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Polymethoxylated flavones metabolites in rat plasma after the consumption of *Fructus aurantii* extract: Analysis by liquid chromatography/electrospray ion trap mass spectrometry

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Abstract

A LC with full scan MS^n method was developed in order to investigate the in vivo absorption and biotransformation of polymethoxylated flavones (PMFs) by analysis of plasma samples from rats after ingestion of *Fructus aurantii* extract. Four parent compounds and six metabolites with intact flavonoid structures were tentatively identified. The metabolites were either glucuronides of parent compounds or glucuronides of demethylated products of parent compounds.

Keywords: LC–MS^n; Polymethoxylated flavones; *Fructus aurantii*; Rat; Metabolism

1. Introduction

Flavonoids are one of the most widespread groups of polyphenolic C_6–C_3–C_6 secondary plant metabolites. Many fruits, vegetables and selected beverages contain substantial quantities. Among the major flavonoids of dietary are polymethoxylated flavones (PMFs) (see Fig. 1), which include a special group of flavonoids present in certain citrus species. PMFs possess important biological properties, including anti-allergic [1], anti-inflammatory [2], anti-oxidant [3], anti-proliferative [4,5], anti-bacterial [6] activities and effects on mammalian metabolism [7].

Since the biochemical properties of PMFs have been studied extensively, information regarding the uptake, metabolism and excretion of this kind of compounds has increasingly received attention. In vitro, the biotransformation of tangeretin (4′,5,6,7,8-pentamethoxyflavone) in rat liver microsomes was investigated and three major metabolites as demethylated and hydroxylated products of the parent compound were tentatively identified [8]. Meanwhile, the biotransformation of nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone) in rat liver S-9 mixture was investigated and one major metabolite as demethylated product of the parent compound was tentatively identified [9]. In vivo, the metabolism of tangeretin in rats was investigated. Results showed that the major metabolites in urine and feces were demethylated or hydroxylated products of the parent compound [10,11] and the major metabolites in serum were glucuronide of demethylated products of the parent compound [11]. Meanwhile, the metabolism of nobiletin in rat was investigated and the major metabolites in urine were demethylated products of the parent compound [12,13]. However, up to now, no previous literature reports could be found which describe the biotransformation of hydroxylated PMFs.

In this paper, a LC coupled to ESI-MS^n method was developed for analysis of rat plasma collected after the ingestion of *Fructus aurantii* extract, which containing high levels of diversified PMFs including hydroxylated PMFs [14].

2. Materials and methods

2.1. Chemicals

The following agents were in LC grade: acetonitrile purchased from Merck (Germany), ethanol, methanol and ethyl

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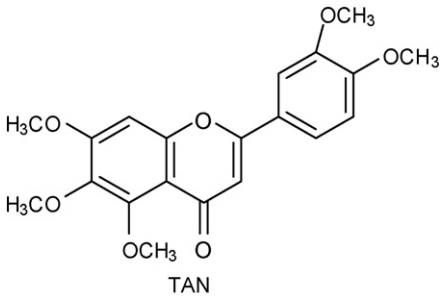

Fig. 1. Structure of a representative PMFs, tangeretin (TAN).

acetate purchased from Yuwang (China). Reverse osmosis Milli-Q water (18.2 MΩ) (Millipore, USA) was used for all solutions and dilutions. Oasis HLB extraction cartridges (1 mL, 100 mg) were purchased from Waters (USA).

2.2. Plant material

F. aurantii were collected from Kai county, Chongqing city, China. The herb was authenticated by Institute of Medication, Xiyuan Hospital of China Academy of Traditional Chinese Medicine. The procedures of extraction were as follow: 100 kg herb was grounded into powder and decocted in 1000 L water at 100 °C for 120 min. Then the residue was collected and re-decocted in 1000 L water at 100 °C for 90 min. The decoction in both times were collected and dried by spray drying. Then 1.5 kg residue was dissolved in 15 L water–ethanol (30:70, v/v). After stirred continuously for 0.5 h, the solution was stored at room temperature for 12 h. The mixture was filtered using Ø7 cm qualitative filter paper and the filtrate was dried with a rotary evaporator at 60 °C. The residue was dissolved in 1 L water and extracted twice by 5.25 and 2.25 L ethyl acetate, respectively. The organic layers in both times were collected and combined, and dried with a rotary evaporator at 60 °C. 10 g residue was dissolved in 100 mL acetonitrile and filtered through 0.45 μm filters. This solution was separated through Purification Factory system (Waters, USA). The fraction of PMFs (FP) (see Fig. 2) was collected and dried with a rotary evaporator at 60 °C. To prepare for the analytical experiment, dry FP was dissolved in acetonitrile to 1 mg/mL and filtered through 0.45 μm filters. For the animal study, dry FP was dissolved in PEG400–water–DMSO (1:1:0.1) to 25 mg/mL and filtered through 0.45 μm filters.

2.3. Animals

The male Sprague–Dawley rats (200 ± 20 g) were obtained from the Laboratory Animal Center at Dalian Medical University. They were housed individually in a constant temperature of 21–24 °C and with a 12 h light–dark cycle and fed standard laboratorial food and water for 1 week. After that, they were divided into two groups of four animals with similar average body weights in each group.

2.4. Drug administration

(a) *Intravenous administration*. The solutions of FP were given to rats via the tail vein at a dose of 50 mg/kg body weight. 0.5 mL blood samples were withdrawn from the suborbital venous plexus of awake rats at 5, 15 min after injection. (b) *Oral administration*. Rats were administered with solution of FP via gastric gavage at a dose of 250 mg/kg body weight. 0.5 mL blood samples were withdrawn from the suborbital venous plexus of awake rats at 30, 60 min post-dosing. Each collected blood sample was transferred to a heparinized micro-centrifuge tube and centrifuged at 8000 × *g* for 10 min. The resulting plasma (100 μL) was then stored at −80 °C for later analysis.

2.5. Sample preparation

Rat plasma was performed using Oasis HLB extraction cartridges. Plasma samples (100 μL) were diluted with 0.9 mL phosphoric acid–water (v:v = 0.4:100). The solutions were vortexed for 1 min and then loaded onto the HLB SPE cartridges

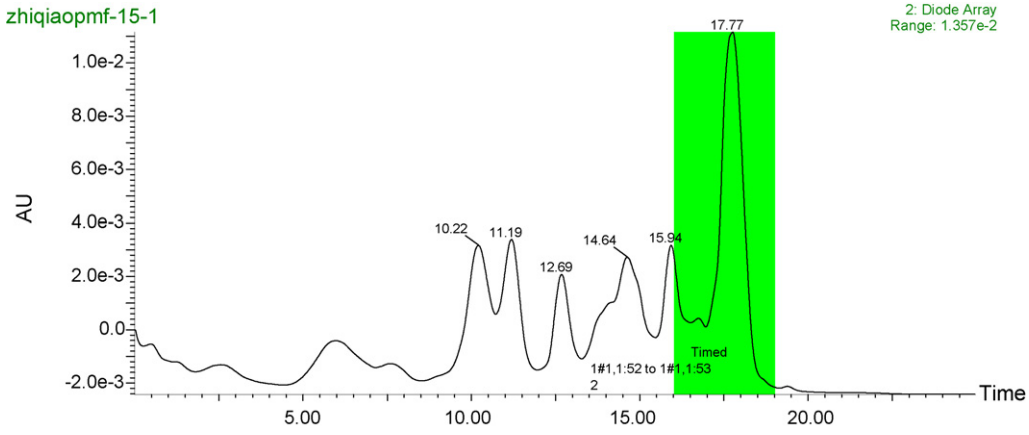

Fig. 2. Chromatogram for the separation of *Fructus aurantii* extract on Purification Factory system. Experimental conditions: column: preparation column, XTerra™ C18 (Waters, USA), 100 mm × 19 mm, 5 μm; eluent: (A) water, (B) acetonitrile. The linear gradient was: 0–5 min, 30% B; 5–15 min, 30–70% B; 15–16 min, 70–95% B; 16–25 min, 95% B. The flow-rate was 150 mL/min and the column temperature was 30 °C. 0.15 mL of sample solution (100 mg/mL) was injected for analysis. The fraction of RT-16 min → RT-19 min was collected as PMFs fraction.

(1 mL, 100 mg), which were preconditioned successively with 2 mL of methanol and 2 mL of water. The cartridges were washed with 2 mL of 10% methanol and then eluted with 2 mL of methanol. The elution was collected and evaporated to dryness at 40 °C with the aid of vacuum centrifugation condense. The residue was dissolved in 0.5 mL acetonitrile and filtered by 0.45 μm filters. The filtrate was kept −20 °C until LC–MS^n analysis.

2.6. Instrumentation

An Agilent 1100 Series LC/MSD Trap XCT (USA) with a photodiode-array detector (DAD) monitoring at 300 nm. Chromatographic conditions were as follow: analytical column, XTerra™ MS C18 (Waters, USA), 150 mm × 2.1 mm, 5 μm; guard column, XTerra™ MS C18 (Waters, USA), 20 mm × 3.9 mm, 5 μm; eluent: (A) water and (B) acetonitrile. The linear gradient was below: 0–20 min, 20–45% B; 20–35 min, 45–80% B. The flow-rate was 0.25 mL/min. 10 μL of sample solution was injected for analysis.

ESI mass spectra were acquired in positive mode by full scan. Nitrogen was used as the nebulizing gas at 35 psi and as drying gas at a flow-rate of 10 L/min and at a temperature of 350 °C. Ions were obtained in the range of *m/z* 100–1000. MS^n spectra were obtained by auto-MS^3 mode (the product-ion of base peak is selected as precursor ion for next-stage MS automatically), the fragmentation amplitude (FA) was set at 1.5 V (SmartFrag: 30–200%) and the MS^n isolation width was 4.0. LC–MS connection was by an I.D. 0.007 in. tube.

3. Result and discussion

3.1. Analysis of FP

Our former study indicated that there were 29 PMFs existing in *F. aurantii* extract [14]. To obtain samples containing high-concentration PMFs for absorption and metabolism experiments, the *F. aurantii* extract was fractionalized by Purification Factory system, and the FP was collected (see Fig. 2). Through the methods developed in our former study to characterized PMFs by LC–MS^n [14], 23 target compounds were tentatively identified in FP (see Table 1 and Fig. 3). The chemical structures of PMFs existing in FP were diversiform, which could represent the whole structural characteristic of such type compound.

3.2. Analysis of rat plasma

The parent compounds and metabolites of PMFs in dosed rat plasma were in trace level which could not form visible peaks in LC–UV chromatogram (see Fig. 4a). Meanwhile, the complex matrix of plasma caused high background in LC–MS total ion chromatogram (TIC) (see Fig. 4b). To detect and characterize those compounds, some analytical methods with high selectivity were needed. In dosed rat plasma, the major metabolites of PMFs are demethylated products of the parent compound and their glucuronides [11]. The molecular weights (MWs) of PMFs in FP have been confirmed (see Table 1), so the MWs of all possible metabolites of those PMFs could be calculated. In this situation, extracted ion chromatogram (EIC) method was used to screen out those metabolites form the complex MS information of dosed

Table 1
The molecular weight (MW), structural identification, and relative concentration (which was represented by the relative peak area (%) "relative peak area = peak area of each compound/peak area of compound 18") of main PMFs detected in FP

No.	RT (min)	Polymethoxylated flavones	The number of methoxyl groups (OCH_3)	The number of hydroxyl groups (OH)	MW	Relative peak area (%)
1	23.5	Tetramethoxyflavone	4	0	342	12.37
2	26.3	Tetramethoxyflavone	4	0	342	7.28
3	20.5	Monohydryoxy-tetramethoxyflavone	4	1	358	4.03
4	26.5	Dihydryoxy-tetramethoxyflavone	4	2	374	3.35
5	20.3	Pentamethoxyflavone	5	0	372	9.19
6	22.9	Pentamethoxyflavone	5	0	372	13.63
7	28.6	Pentamethoxyflavone	5	0	372	52.95
8	19.9	Monohydryoxy-pentamethoxyflavone	5	1	388	1.75
9	21.1	Monohydryoxy-pentamethoxyflavone	5	1	388	11.10
10	23.3	Monohydryoxy-pentamethoxyflavone	5	1	388	5.15
11	26.5	Monohydryoxy-pentamethoxyflavone	5	1	388	2.66
12	30.7	Monohydryoxy-pentamethoxyflavone	5	1	388	3.37
13	31.6	Monohydryoxy-pentamethoxyflavone	5	1	388	2.73
14	22.3	Dihydryoxy-pentamethoxyflavone	5	2	404	2.31
15	23.1	Dihydryoxy-pentamethoxyflavone	5	2	404	10.05
16	30.3	Dihydryoxy-pentamethoxyflavone	5	2	404	1.42
17	22.0	Hexamethoxyflavone	6	0	402	1.88
18	25.7	Hexamethoxyflavone	6	0	402	100.00
19	30.5	Hexamethoxyflavone	6	0	402	1.42
20	22.4	Monohydroxy-hexamethoxyflavone	6	1	418	5.59
21	28.4	Monohydroxy-hexamethoxyflavone	6	1	418	56.54
22	32.2	Monohydroxy-hexamethoxyflavone	6	1	418	3.32
23	27.4	Heptamethoxyflavone	7	0	432	65.81

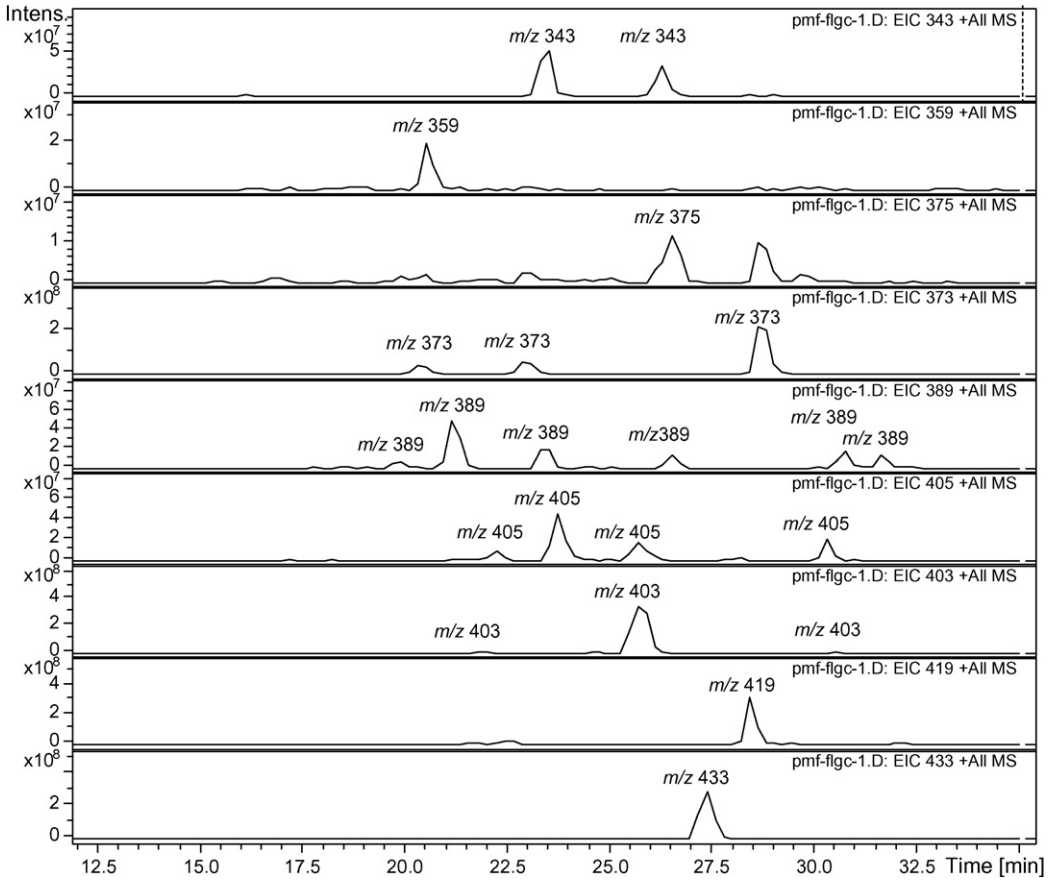

Fig. 3. LC–MS EIC trace of PMFs existing in FP.

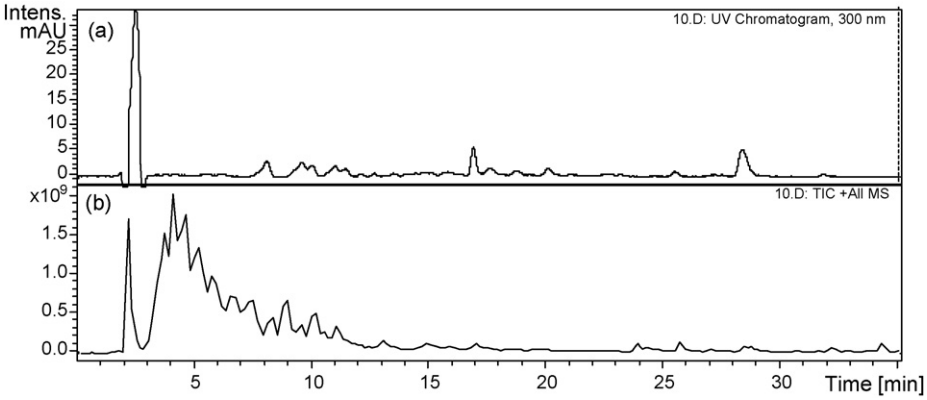

Fig. 4. LC–UV chromatogram (a) and LC–MS TIC (b) of rat plasma (intravenous administration; samples were withdrawn at 5 min after injection).

rat plasma by their MWs. Our former study indicated that the protonated flavones of PMFs dissociated predominantly via loss of one or two methyl radical and then formed the fragments of $[M+H-15]^{\bullet+}$ or $[M+H-30]^{\bullet+}$, which were the diagnostic characteristic for PMFs and could be used to characterize such type compounds [14]. So the further identification of those metabolites needs their MS^n data.

Take the compound 18 as an example to demonstrate the procedure of identification of the parent compound and metabolites of PMFs in dosed rat plasma. Table 2 shows MWs of parent compound and all possible metabolites of the compound. By

Table 2
The structures and MWs of parent compound and all possible metabolites of compound 18 (MW 402)

No.	Flavone derivative	MW/aglycone	MW/glucuronide
1	Parent compound	402	–
2	Monohydryoxypentamethoxy	388	564
3	Dihydryoxytetramethoxy	374	550

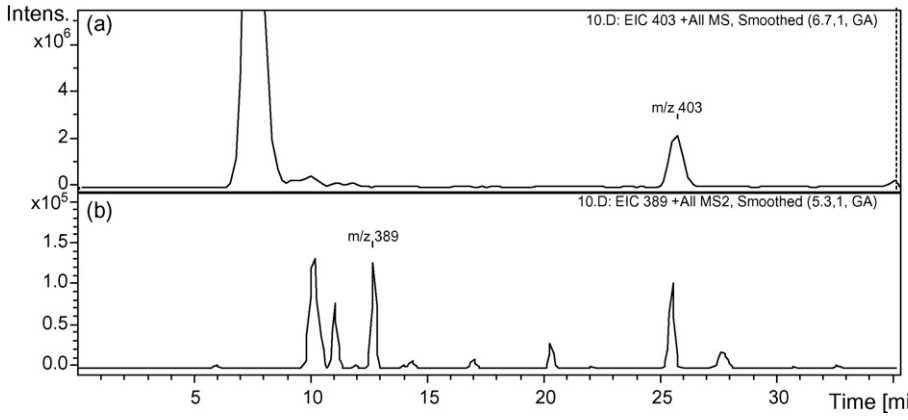

Fig. 5. LC–MS EIC of rat plasma (intravenous administration; samples were withdrawn at 5 min after injection). (a) EIC trace of *m/z* 403 in first-stage MS; (b) EIC trace of *m/z* 389 in second-stage MS.

Table 3
LC–MS^n identification of PMFs parent structures and metabolites detected in plasma after the administration of *Fructus aurantii* extract by rats

No.	RT (min)	Compounds identification	$[M+H]^+$ (*m/z*)	MS^2 fragments (*m/z*)	MS^3 fragments (*m/z*)	Parent compounds (no.)	Location[a]			
							a	b	c	d
1	23.4	Tetramethoxyflavone	343	328,313	–	1	+	+	+	+
2	12.2	Monohydryoxy-tetramethoxyflavone glucuronide	535	359	344,329	3	+	+	–	–
3	28.6	Pentamethoxyflavone	373	358,343	–	7	+	+	+	+
4	12.9	Monohydryoxy-pentamethoxyflavone glucuronide	565	389	374,359	9	+	+	–	–
5	14.2	Monohydryoxy-pentamethoxyflavone glucuronide	565	389	374,359	18	–	+	–	–
6	22.3	Dihydryoxy-pentamethoxyflavone glucuronide	581	405	390,375	15	+	+	–	–
7	25.7	Hexamethoxyflavone	403	388,373	–	18	+	+	+	+
8	13.9	Monohydroxy-hexamethoxyflavone glucuronide	595	419	404,389	21	–	+	–	–
9	17.1	Monohydroxy-hexamethoxyflavone glucuronide	595	419	404,389	23	+	+	–	–
10	27.4	Heptamethoxyflavone	433	418,403	–	23	+	+	+	+

[a] a: Intravenous administration, samples were withdrawn at 5 min after injection; b: intravenous administration, samples were withdrawn at 15 min after injection; c: oral administration, samples were withdrawn at 30 min after gavage; d: oral administration, samples were withdrawn at 30 min after gavage.

those MWs, the parent compound and potential metabolites of compound 18 in dosed rat plasma were screened out through EIC method. The EIC trace of *m/z* 403 in first-stage MS shows a visible peak (see Fig. 5a), which exhibits that parent compound of compound 18 exists probably in plasma. Its retention time (RT) (see Tables 1 and 3) and MS^2 information (see Fig. 6) confirmed its structure further. The EIC trace of *m/z* 389 and *m/z* 375 in first-stage MS do not show visible peak, which means the unconjugatedly demethylated products of the parent compound do not exist in dosed rat plasma. The EIC trace of *m/z* 565 and *m/z* 551 in first-stage MS both show complex interferential signals, which cannot meet our need. Literature reported that the base fragments of glucuronides of flavones in second-stage MS were their aglycones, corresponding to the cleavage of glucuronide unit ($[M+H-176]^+$) [11,15]. So, the EIC trace of *m/z* 389 and *m/z* 375 in second-stage MS were screened for the glucuronides of demethylated products of the parent compound. Only EIC trace of *m/z* 389 shows a visible peak (see Fig. 5b). The precursor ion of *m/z* 389 is *m/z* 565 (Fig. 7a), meanwhile the next-stage MS information of which accords with the characteristic of PMFs (see Fig. 7b). These data indicate the compound is glucuronide of monohydroxy-pentamethoxyflavone.

Follow up the analytical procedure above, four parent compounds and six metabolites existing in dosed rat plasma were detected and tentatively identified (see Table 3). No matters by oral or by intravenous administration, the four parent

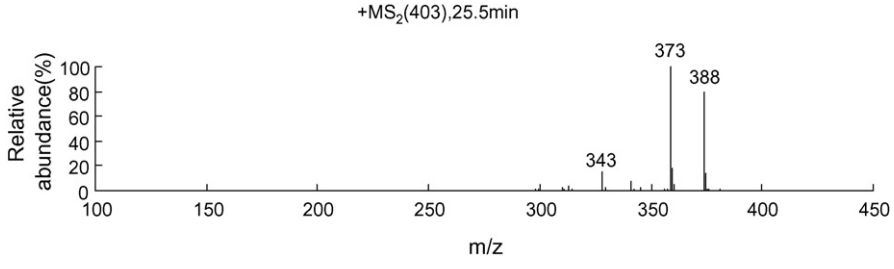

Fig. 6. MS^2 spectrum of the compound with *m/z* 403 in rat plasma.

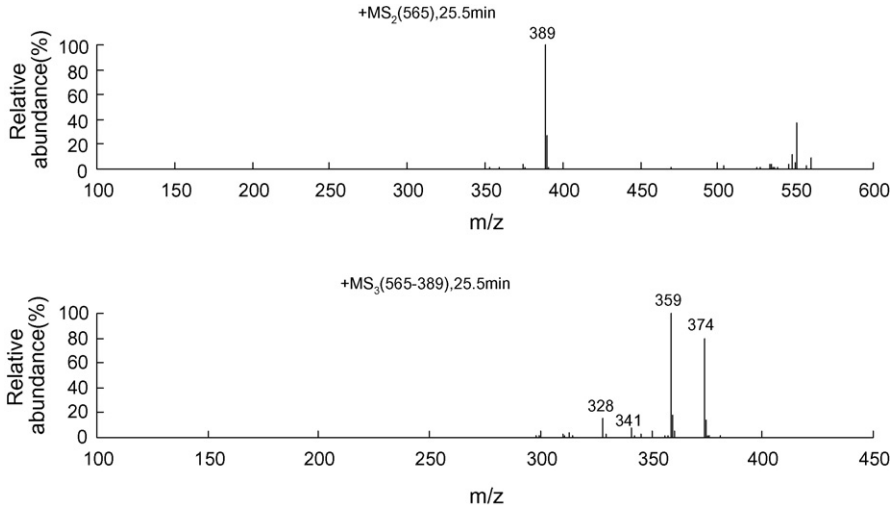

Fig. 7. MS^n spectra of compound with *m/z* 565: (a) MS^2 spectrum (precursor-ion was *m/z* 565 ($[M+H]^+$)); (b) MS^3 spectrum (precursor-ion was *m/z* 389 ($[M-176+H]^+$)).

compounds were detected in all dosed rats plasma samples. Investigating further, we found the common characteristic of the four compounds was without hydroxyl group on their structure. For having no position to conjugate glucuronic acid or sulfate group, they can be absorbed intact through alimentary tract and reach systemic circulation. By comparing the RT (see Tables 1 and 3), the identity of those parent compounds existed in plasma could be confirmed. They had high relative concentration in PF and the absolute amounts of ingestion of them were big enough to do further pharmacokinetic analysis.

The six metabolites are all glucuronides of hydroxylated PMFs and the parent compounds of them require further investigation. For example, monohydroxy-hexamethoxyflavone glucuronide (*m/z* 595) detected in plasma was either from heptamethoxyflavone as its glucuronide of demethylated product or from monohydroxy-hexamethoxyflavone as it glucuronide. While investigating further, we found the time of their appearance in blood was different (see Fig. 8 and Table 3). From the difference we could conclude that metabolite 8 (RT = 13.9 min) was from heptamethoxyflavone. The occurrence of demethylation took a little time, causing its later appearance. Therefore metabolite 9 (RT = 17.1 min) was concluded as metabolite of monohydroxy-hexamethoxyflavone. The appearance in blood of metabolites 4 and 5 (*m/z* 565) showed the same phenomenon, which approved our conclusion further. Since the appearance in blood of demethylated product of parent compound needs more than 5 min, the metabolite 2 (*m/z* 535) and metabolite 6 (*m/z* 581) were characterized as glucuronides of parent compounds. The parent compounds of those metabolites must have high level in the exact dosed, which ensured the detection of metabolites in dosed plasma. Followed such principle, the identity of parent compounds for all metabolites characterized were confirmed (see Table 3).

Though 23 PMFs existed in FP dosed, only four parent compounds and six metabolites existing in dosed rat plasma were detected. The result was caused by the definite sensitivity of the analytical method. More other parent compounds and metabolites existed probably in the dosed rat plasma, while the amounts of them were below the detectable range of the analytical method. That is to say, only the compounds with high relative concentration in the extracts could be selected as target compounds for further pharmacokinetic studies.

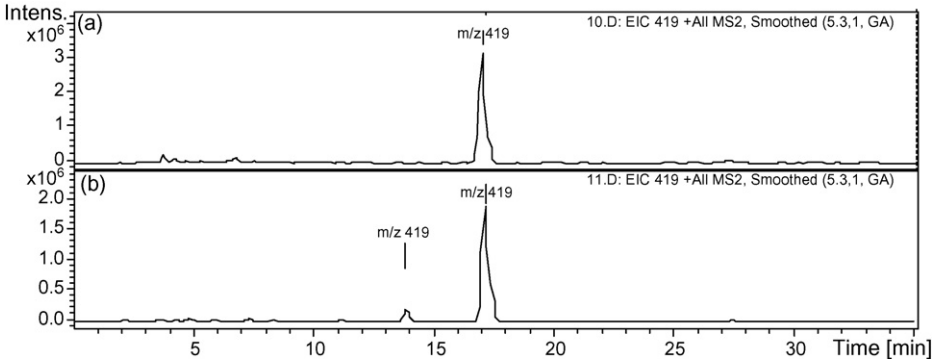

Fig. 8. LC–MS^2 EIC (EIC trace of *m/z* 419) of rat plasma (a) intravenous administration, samples were withdrawn at 5 min after injection; (b) intravenous administration, samples were withdrawn at 15 min after injection.

4. Conclusion

The present study is the first to report on the use of LC with full scan MS^n to analyse metabolites of PMFs appearing in rat plasma. It provides a much more detailed analysis than that of earlier studies which were carried out after the ingestion of tangeretin [11]. Although 23 PMFs were readily detected in FP, the amounts of most parent compounds and metabolites in plasma were below the detectable range of the analytical method used in this study. Only four parent compounds and six metabolites existing in dosed rat plasma were tentatively identified. The data presented in this paper demonstrate the value of LC coupled full scan MS^n for the analysis of trace levels of natural products in impure extracts, especially when reference compounds were unavailable. Further information on the position and orientation of substituent groups would require the use of NMR. However, this would involve not only extensive sample purification but also a requirement for several orders of magnitude more analyte than the low nanogram quantities required for LC–MS^n. With many of the plasma PMFs metabolites detected in trace levels in the present study, this would not be a practical proposition.

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