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Fingerprint Analysis of *Ginkgo biloba* Extract and Ginkgo semen in Preparations by LC-Q-TOF/MS

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ABSTRACT

An ultra-high performance liquid chromatography with tandem quadrupole/flight mass spectrometry (LC-Q-TOF/MS) method was developed for the chromatographic fingerprint analyses of *Ginkgo biloba* extract and Ginkgo semen. All samples were extracted with 70% methanol and part of each extract was hydrolyzed by acids. The chromatographic analyses of these materials were performed on a C18 analytical column (3.0 × 150 mm, 2.7 μm) with gradient elution using methanol and water with 0.01% formic acid at a flow rate of 0.5 mL/min. Mass spectrometry was performed in the negative-ion mode ESI to determine the compounds in *G. biloba* extract and G. semen by comparison with the accurate molecular weight of the reference compounds and literature reported. In addition, Ginkgolide J and Bilobalide were identified from G. semen by this method for the first time. The method was successfully used to analyze compounds in *G. biloba* extract and G. semen with significantly reduced analytical time, and also applied in the analysis of more complex components in *G. biloba* products.

Key words: LC-Q-TOF/MS, fingerprint, *Ginkgo biloba* extract, Ginkgo semen, Ginkgolide J, Bilobalide

INTRODUCTION

Many clinical experience and researches indicated that Chinese medicines have significant function in the treatment of human diseases, improvement of the quality of life and prolongation of life. Owing to the chemical complexity of Chinese herbal medicines, the authenticity and quality of raw materials are key factors to medical effectiveness. Therefore, it is important to establish an analytical method to identify the authenticity of raw materials and their related products[1].

For complex botanicals, such as *Ginkgo biloba* extracts, the quantitation of single or selected targeted compounds does not provide the whole picture of the botanicals. For this reason, the chromatographic fingerprint technology was accepted by the World Health Organization in 1991 as a strategy for the identification and evaluation of herbal medicines[2]. Fingerprint profiling has been widely accepted as a tool for quality control by regulatory authorities worldwide[3].

Ginkgo biloba (Ginkgoaceae) is one of the oldest known trees on earth with fossil records dating back more than 200 million years. G. semen has a long history of use in China as a traditional medicine for various ailments. Scientific studies of *G. biloba* since the 1950s have indicated that the leaf of *G. biloba* is a useful remedy for a wide range of disorders[4]. Active compounds in the leaves of *G. biloba* could improve blood circulation, discourage clot formation, reinforce the walls of the capillaries and protect nerve cells from harm when deprived of oxygen[5]. Its seeds was reported in the treatment of cough, asthma, enuresis, alcohol misuse, pyogenic skin infections and worm infections in the intestinal tract[6].

Flavonoids and terpene trilactones are believed to be associated with most of the pharmacological properties of the leaf extracts of *G. biloba.* Althoough flavonoids can be found in many other plants, ginkgolides and bilobalide are unique components of *G. biloba*[7]. So far, eight diterpenoids have been reported in *G. biloba*[8,9].

Most of the studies involving chromatographic fingerprint analysis of *G. biloba* extracts were carried out by high performance liquid chromatography with ultraviolet (HPLC/UV) [10-14]. However, HPLC/UV is not suitable for detecting terpene trilactones because of their poor UV absorption property.

The advantages of using mass spectrometry (MS) include the abilities to determine the molecular weight and obtain fragmental structural information[15]. Coupling HPLC with MS is a powerful technique that can provide both compositional and structural information and make online qualitative and quantitative analyses possible for plant

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extracts[16,17]. HPLC/MS also avoids the time-consuming process of isolating all the compounds for identification. Besides, time-of-flight mass spectrometry (TOF-MS) can provide excellent accurate mass measurement over a wide dynamic range and distinguish the isotope pattern, providing important additional information for the determination of the elemental composition. Therefore, TOF-MS is a powerful tool for identifying target compounds in a highly complex mixture[18]. However, there were few reports on the fingerprint and quantitative analyses of *G. biloba* extracts and related health foods using LC-Q-TOF/MS.

According to Taiwanese regulations, *G. biloba* extract products are regarded as medicines, while those containing G. semen belong to the dietary supplement category. Therefore, a clear and fast method to differentiate between herbal preparations containing *G. biloba* and G. semen should be established.

In this study, ultra-high performance liquid chromatography with tandem quadrupole/time-of-flight mass spectrometry was applied to analyze the differences in compositions between the extracts of *G. biloba* leaves and G. semen. In addition, a new fingerprint detection method was established to make a distinction among these related products.

MATERIALS AND METHODS

I. *Standard Solution Preparation*

The reference compounds (Ginkgolides A-C, and J, Bilobalide, Quercetin, Kaempferol and Isorhamnetin) were accurately weighed and dissolved in methanol to obtain solutions of suitable concentration. The standard solutions were stored under refrigeration at 4°C. The reference substances (> 98% purity) were purchased from Nacalai.

II. *Sample Collection and Preparation*

Four samples of the *G. biloba* extracts were obtained from the European and Chinese markets. Twenty samples of G. semen were obtained from the Taiwanese market.

Each powdered sample of *G. biloba* extract (5 mg) and G. semen (0.5 g) was extracted with 70% methanol (10 mL) by ultrasonication at room temperature for 30 min. The extract were filtered with a 0.22 μm Millipore filter membrane. The filtrates were used for LC-Q-TOF/MS analysis.

The products were claimed that products containing *G. biloba*, each equivalent to 0.5 g of G. semen, were extracted with 70% methanol (10 mL) by ultrasonication at room temperature for 30 min. The extracts were filtered with a 0.22 μm Millipore filter membrane. The filtrates were then used for LC-Q-TOF/MS analysis and repeated three times.

III. *LC-Q-TOF/MS Analysis*

All analyses were performed on an Agilent 1290 Series UHPLC system (Agilent, USA), including binary solvent manager, sampler manager, column compartment, and TOF-MS detector controlled with Agilent MassHunter software. Chromatographic separation was performed on a C18 analytical column (3.0 × 150 mm, 2.7 μm) (Advanced Materials Technology, USA). The column temperature was maintained at 30°C and the injection volume was set at 0.1 μL. The reference substances and samples were eluted by a gradient mobile phase system consisting of H_2O with 0.01% formic acid (A) and methanol with 0.01% formic acid (B), as follows: 20 - 30% A at 0 - 5 min, 30 - 40% A at 5 - 10 min, 40 - 40% A at 10 - 15 min, 40 - 45% A at 15 - 20 min, 45 - 45% A at 20 - 22 min, 45 - 50% A at 22 - 30 min, 50 - 60% (A) at 30 - 35 min and 60 - 75% (A) at 35 - 40 min. The flow rate was set at 0.5 mL/min. The electrospray source of the MS was operated in both positive and negative modes and the operating parameters were: drying gas (N_2) flow rate: 10.0 L/min; drying gas temperature: 320°C; nebulizer: 35 psi; capillary: 3000 V; octopole RF: 750 V; and fragmentor voltage: 120V. Mass spectra were obtained over the mass range of m/z 50 - 3000, and accurate mass measurement of all mass peaks was also recorded.

The accurate mass data for the molecular ions were processed using the software MassHunter Workstation B 5.0 software (Agilent Technologies, Palo Alto, CA, USA), which can provide a list of possible elemental formulae.

IV. *Fingerprint Analysis of Data Processing*

(I) *Fingerprint of a Computational Analysis*

The Agilent difference analysis of the Mass Profiler software was applied to analyze the chromatographic data based on retention time (RT) and accurate mass (Mass) of the common peaks of the sample output. The persent RSD values obtained were less than 0.80.

(II) *Fingerprint Differences in the Degree of Calculation and Analysis*

The Agilent Mass Profiler Professional Metabolomics software was used for data analysis. The chromatographic data of *G. biloba* extract and G. semen were calculated according to their relative retention times (RT), exact mass (Mass) and peak abundance (Abundance). The *p* value for the difference in composition obtained was less than 0.05.

RESULTS AND DISCUSSION

I. *Optimization of HPLC-ESI-Q-TOF-MS Conditions*

In previous studies[14], the use of a mobile phase system consisting of acetonitrile and water in the analysis of *G. biloba* extract could not separate ginkgolides A and B. In the present study, a mobile phase system consisting of methanol (A) and water (B), both containing 0.01% (v/v) formic acid, was chosen for the analysis following an elution

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gradient program: 0 - 5 min, 20 - 30% A; 5 - 10 min, 30 - 40% A; 10 - 15 min, 40 - 40% A; 15 - 20 min, 40 - 45% A; 20 - 22 min, 45 - 45% A; 22 - 30 min, 45 - 50% A; 30 - 35 min, 50 - 60% A; 35 - 40 min, 60 - 75% A.

To achieve better detection, the MS measurement was optimized and a statistical orthogonal design was employed for optimization. The scans of *G. biloba* extract and G. semen in negative ion mode provided more information than those in the positive ion mode (Figure 1). Therefore, the fingerprint analyses of *G. biloba* extract and G. semen were carried out in the negative ion mode.

II. *Identification of Proprietary Components Using Mass Fragmentation*

The constituents in *G. biloba* extract and G. semen were identified by comparing with reference compounds and literatures reported on the basis of their HPLC retention times and mass spectra. Tables 1 - 4 are lists of the retention time, mass fragmentation and substance names for the numbered peaks in the chromatograms of *G. biloba* extract and G. semen. The fragmentation pathways of flavonoids and their glycosides by tandem mass spectrometry were consistent with literature reports(19-21) .

The use of mass spectral analysis to elucidate the structures of flavonoids is important and indispensable(22-26). In recent years the rapid development of mass spectrometric techniques provides a variety of options for the analysis of flavonoids. In general, the fragment ions from the rupturing of two C-C covalent bonds in the C ring of flavonoid aglycones

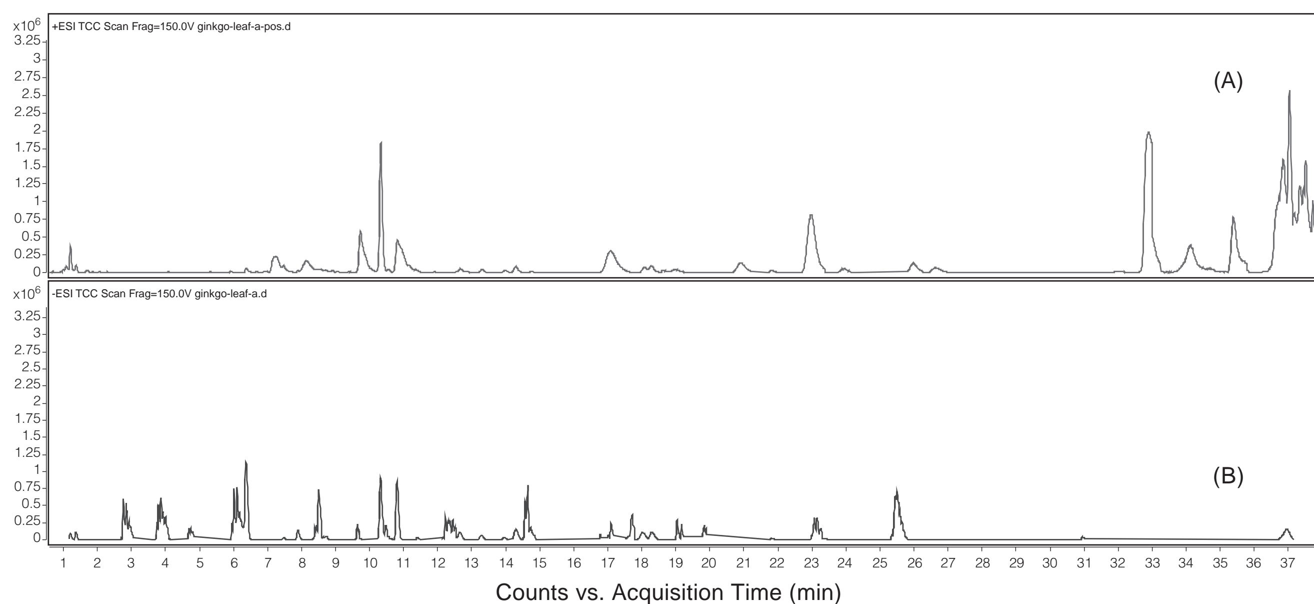

Figure 1. Mass scan in different modes for *Ginkgo biloba* extract. (A) positive ion mode and (B) negative ion mode.

Table 1. Compounds identified in *Ginkgo biloba* extract (before acid hydrolysis)

Name	Formula	RT	Mass	Diff(ppm)
Bilobalide	$C_{15}H_{18}O_8$	6.36	326.1001	-0.40
Ginkgolide A	$C_{20}H_{24}O_9$	10.33	408.1425	1.17
Ginkgolide B	$C_{20}H_{24}O_{10}$	10.82	424.1374	1.09
Ginkgolide C	$C_{20}H_{24}O_{11}$	7.88	440.1317	-0.27
Ginkgolide J	$C_{20}H_{24}O_{10}$	7.46	424.1362	-1.77
kaempferol-3-[6’’’-*p*-coumaroylglucosyl-β-(1→4)-rhamnoside]	$C_{36}H_{36}O_{17}$	18.00	740.1957	0.61
kaempferol 3-(2G-rhamnosylrutinoside)	$C_{33}H_{40}O_{19}$	9.62	740.2183	2.58
kaempferol 3-*O*-glucoside	$C_{21}H_{20}O_{11}$	12.56	448.1010	1.07
luteolin 3’-*O*-glucoside	$C_{21}H_{20}O_{11}$	14.50	448.1019	2.89
isorhamnetin 3-*O*-rutinoside	$C_{28}H_{32}O_{16}$	13.29	624.1687	-0.46
kaempferol 3-*O*-rutinoside	$C_{27}H_{30}O_{15}$	12.65	594.1583	-0.31
kaempferol-3-*O*-β-D-glucorhamnoside	$C_{27}H_{30}O_{15}$	14.74	594.1584	-0.14

Table 2. Compounds identified in *Ginkgo biloba* extract (after acid hydrolysis)

Name	Formula	RT	Mass	Diff (ppm)
target-compound1	$C_{15}H_{12}O_6$	10.20	288.0625	-2.97
Quercetin	$C_{15}H_{10}O_7$	17.24	302.0426	-0.05
Isorhamnetin	$C_{16}H_{12}O_7$	25.36	316.0581	-0.57
Bilobalide	$C_{15}H_{18}O_8$	6.37	326.1005	1.08
Ginkgolide A	$C_{20}H_{24}O_9$	10.31	408.1421	0.14
Ginkgolide B	$C_{20}H_{24}O_{10}$	10.79	424.136	-2.24
Ginkgolide J	$C_{20}H_{24}O_{10}$	7.48	424.1362	-1.73
Ginkgolide C	$C_{20}H_{24}O_{11}$	7.88	440.1319	0.08
Rutin	$C_{27}H_{30}O_{16}$	10.54	610.1531	-0.46
Kaempferol	$C_{15}H_{10}O_6$	23.20	286.0474	-1.21

Table 3. Compounds identified in Ginkgo semen (before acid hydrolysis)

Name	Formula	RT	Mass	Diff(ppm)
Bilobalide	$C_{15}H_{18}O_8$	6.36	326.1001	-0.58
Ginkgolide A	$C_{20}H_{24}O_9$	10.33	408.1425	1.24
Ginkgolide B	$C_{20}H_{24}O_{10}$	10.82	424.1374	1.95
Ginkgolide C	$C_{20}H_{24}O_{11}$	7.88	440.1317	2.27
Ginkgolide J	$C_{20}H_{24}O_{10}$	7.46	424.1362	0.47
target-compound1	$C_{26}H_{34}O_{16}$	5.81	602.1849	0.38
target-compound2	$C_{24}H_{22}O_{12}$	5.75	502.1111	-3.06
target-compound3	$C_{35}H_{20}O_3$	5.25	488.1412	0.36
target-compound4	$C_{11}H_{12}O_5$	9.39	224.0684	-2.8

Table 4. Compounds identified in Ginkgo semen (after acid hydrolysis)

Name	Formula	RT	Mass	Diff (ppm)
target-compound2	$C_7H_{10}O_7$	2.47	206.0428	0.54
target-compound1	$C_8H_{12}O_7$	3.84	220.0579	-1.85
target-compound8	$C_{11}H_{12}O_6$	5.84	240.0628	-2.3
target-compound4	$C_{12}H_{14}O_6$	7.82	254.0801	3.69
Bilobalide	$C_{15}H_{18}O_8$	6.37	326.1007	1.67
Ginkgolide A	$C_{20}H_{24}O_9$	10.32	408.1427	1.7
Ginkgolide B	$C_{20}H_{24}O_{10}$	10.81	424.1393	5.57
Ginkgolide J	$C_{20}H_{24}O_{10}$	7.47	424.1371	0.37
Ginkgolide C	$C_{20}H_{24}O_{11}$	7.87	440.1330	2.5
target-compound6		6.97	466.1602	0.4
target-compound5	$C_{26}H_{26}O_{12}$	7.81	530.1424	-0.06
target-compound3		9.41	540.1971	0.6
target-compound7		8.38	594.1681	0.46

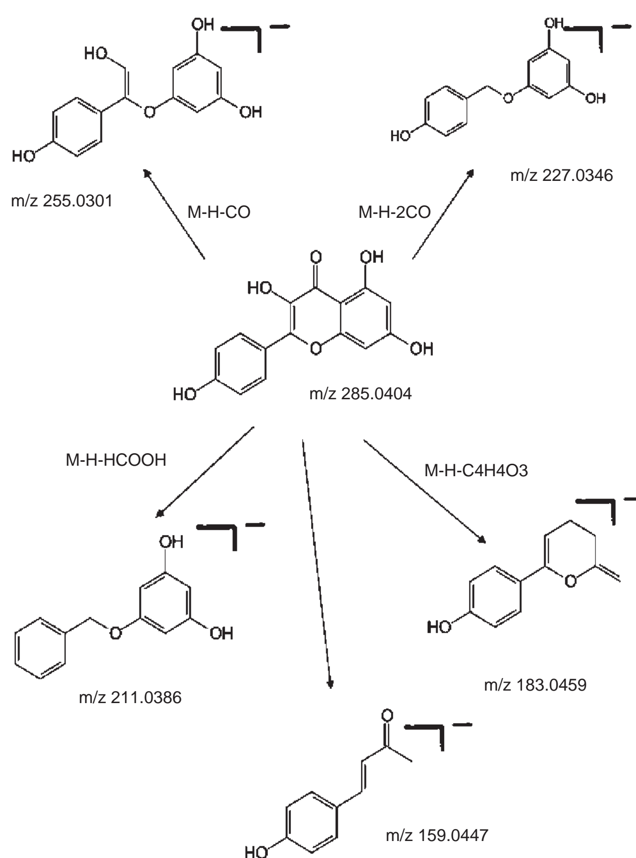

Figure 2. Fragmentation pathways of Kaempferol.

reflect the number and form of the A and B rings substituents, which are the main information used to distinguish the aglycone structure. This theoretical identification of flavonoid glycosides was applied, herein, to identify the components in *G. biloba* extract (before acid hydrolysis). The results indicated that seven unknown components of flavonoid glycosides **1** - **7** were inferred by tandem mass spectrometric fragmentation, and the difference of the mass spectral characteristics of each flavonoids (Figures 2 and 3) provided a possible structure, which were then compared with literature reported to confirm the correct compound.

From the fragments with *m/z* 255.0301, 227.0346, and 227.0346 obtained from the cleavage of the C ring of Kaempferol, and the fragment with *m/z* 159.0447 by Retro-Diels-Alder (RDA) cleavage, it could be speculated that the targets **1**-**3**, **6**, and **7** contained Kaempferol aglycone. On comparison with literature data[(27-36)], target **1** was found to be kaempferol-3-[6'''-*p*-coumaroylglucosyl-β-(1→4)-rhamnoside], target **2** was kaempferol 3-(2G-rhamnosylrutinoside), target 3 was kaempferol 3-*O*-glucoside, target **6** was kaempferol 3-*O*-rutinoside, and target **7** was kaempferol-3-*O*-β-D-glucorhamnoside (Figures 4 - 8).

From the fragments of isorhamnetin with *m/z* 300.0292, 285.0389 and 271.0257, those with *m/z* 255.0313 and 243.0296 by cleavage of the C ring, and those with *m/z* 151.0054 and 107.0140 by RDA cleavage, it could be speculated that the target **5** contained isorhamnetin aglycone

and rutin glycosylation. On comparison with the literaturel data[37], target **5** was confirmed as isorhamnetin 3-*O*-rutinoside (Figure 9).

Target **3** and **4** had the same molecular weight but different retention times, suggesting that they were isomers. The mass spectral data in Figure 10 showed that target **3** was kaempferol 3-*O*-glucoside and target **4** was luteolin 3'-*O*-glucoside[38,39].

III. *Structural Confirmation of the Compounds Using Molecular Structure Correlator Software*

For the structural confirmation of compounds with no authentic standard available, the Molecular Structure Correlator software was used to facilitate automatic structural assignment. For example, the proposed structure of isorhamnetin 3-*O*-rutinoside was loaded into the analysis software windows and the possible ion fragments were assigned with predicted score values. As shown in Figures 4 - 10, we could propose fragmentation pathways of compounds based on their MS^2 spectra.

IV. *Mass Spectrometric Fngerprinting of G. biloba Extract and G. semen*

The HPLC fingerprint profiles of four samples of *G. biloba* extracts and twenty samples of G. semen before and after acid hydrolysis were comparied (Figure 11 - 12),

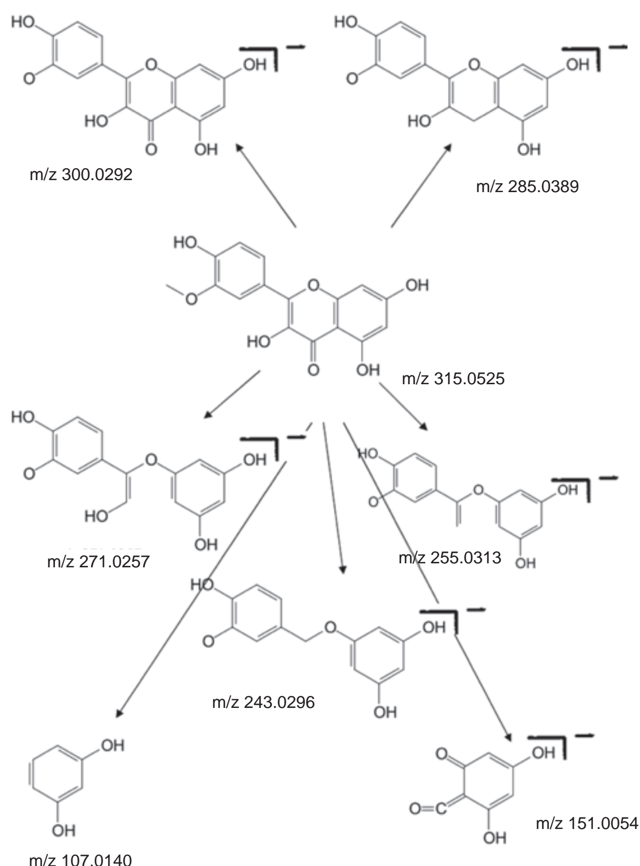

Figure 3. Fragmentation pathways of Isorhamnetin.

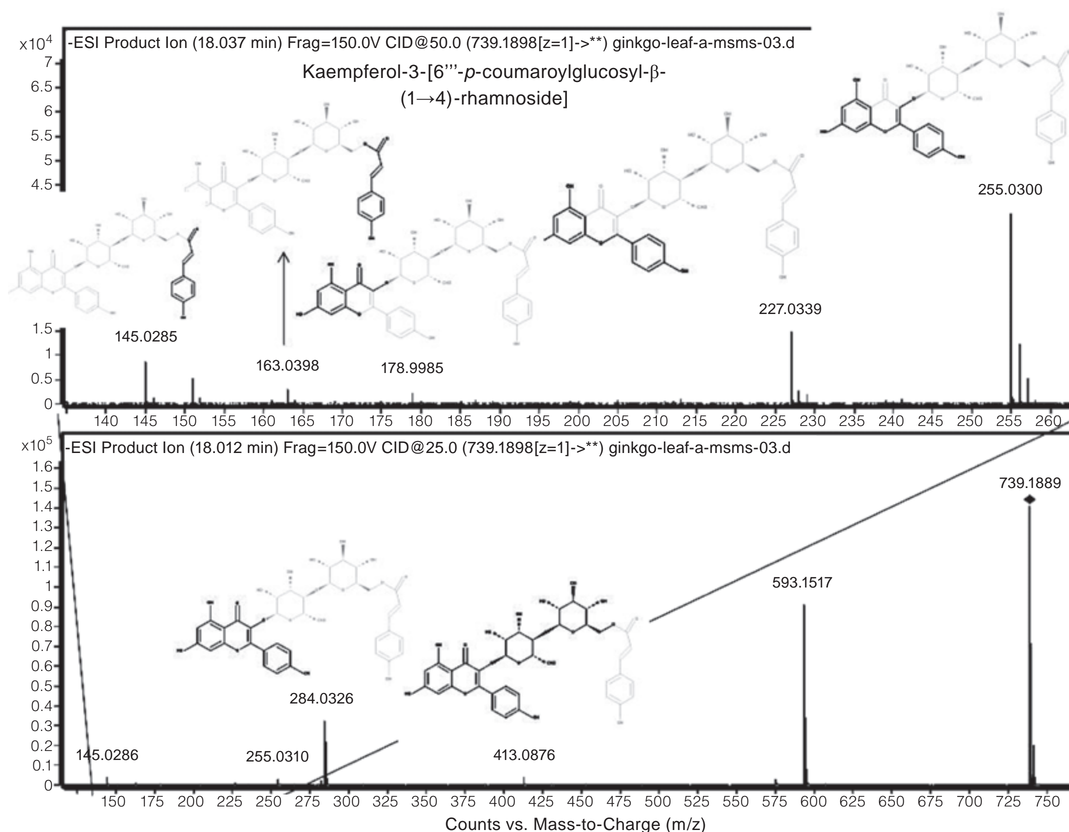

Figure 4. Fragmentation pathway of kaempferol-3-[6'''-*p*-coumaroylglucosyl-β-(1→4)-rhamnoside].

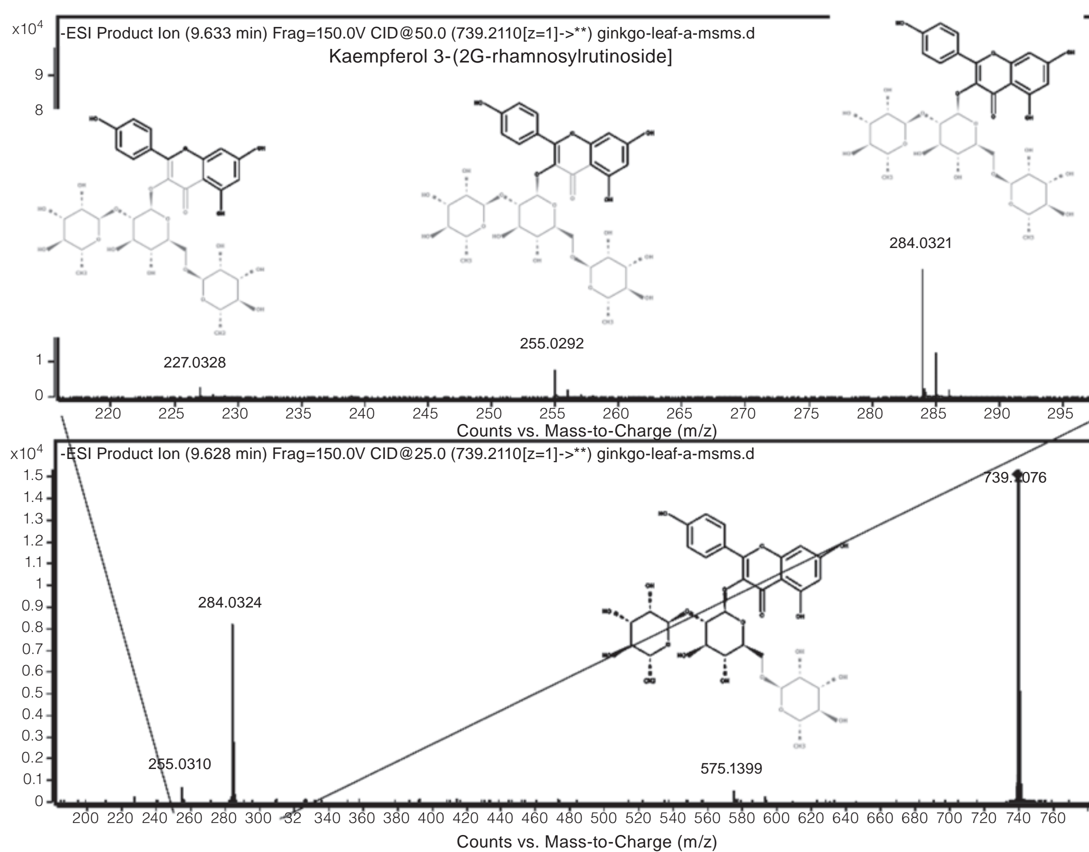

Figure 5. Fragmentation pathway of kaempferol 3-(2G-rhamnosylrutinoside).

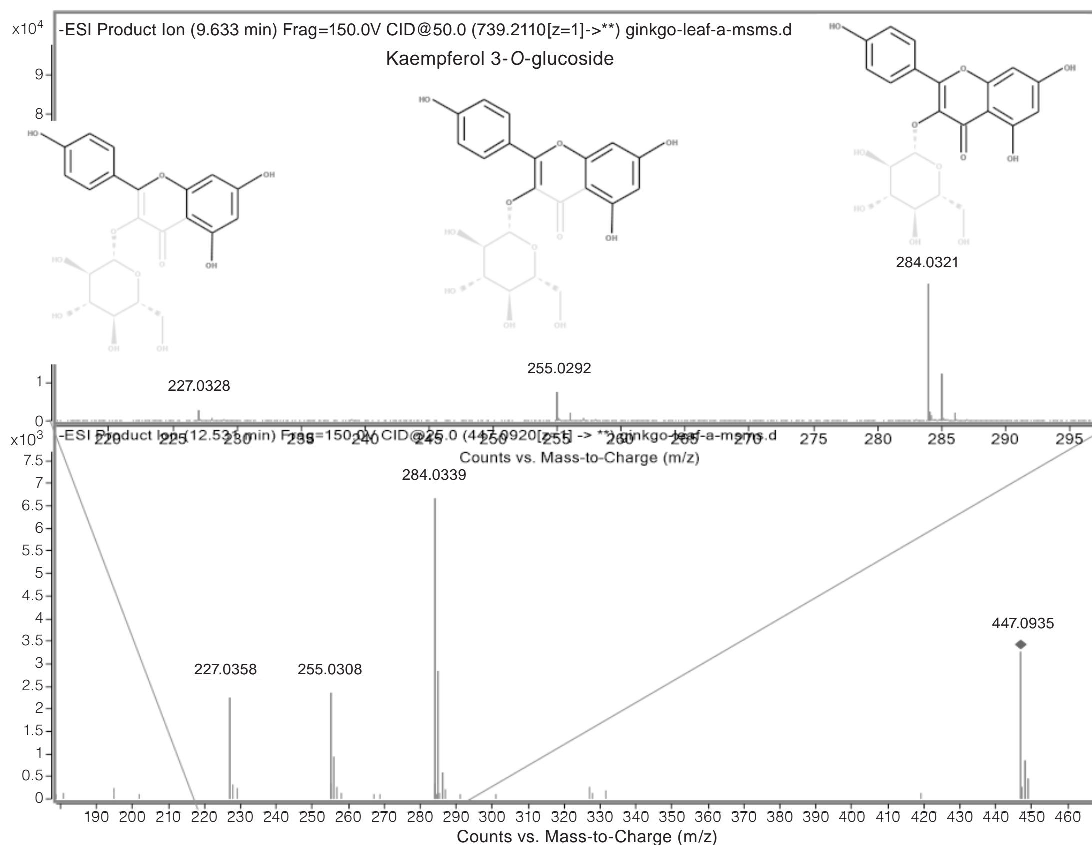

Figure 6. Fragmentation pathway of kaempferol 3-*O*-glucoside.

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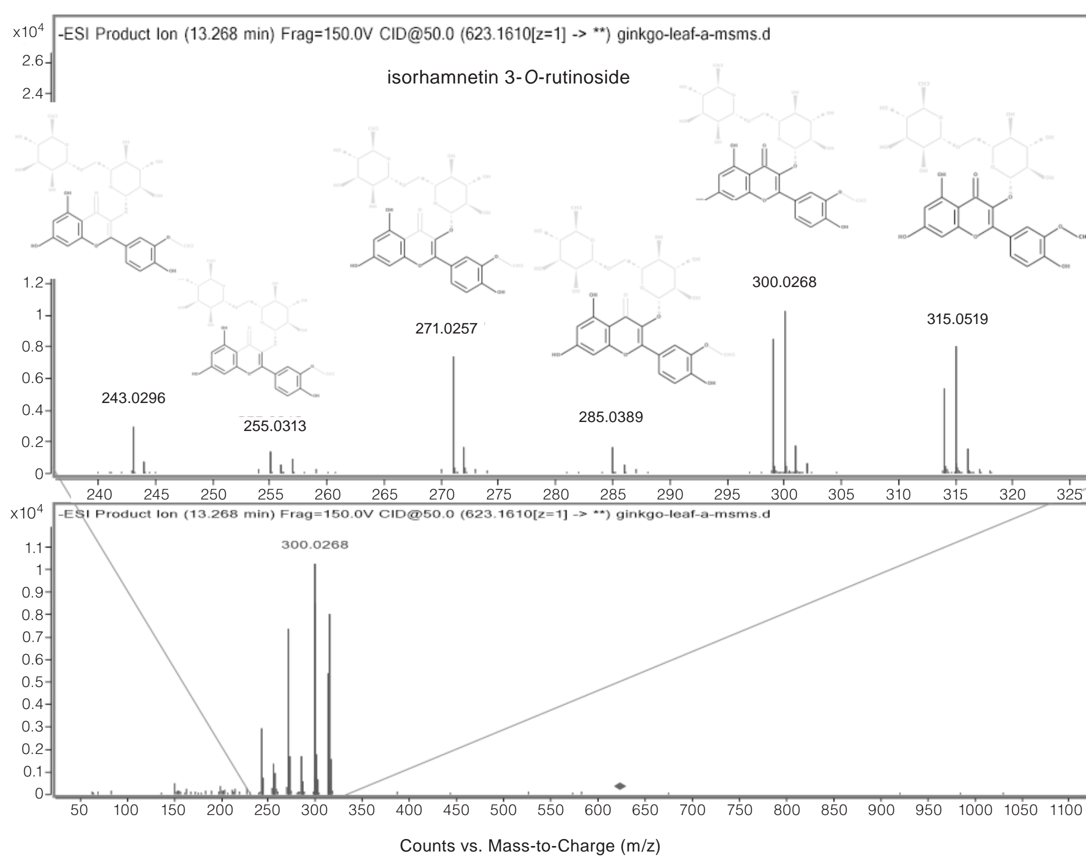

Figure 7. Fragmentation pathway of kaempferol 3-*O*-rutinoside.

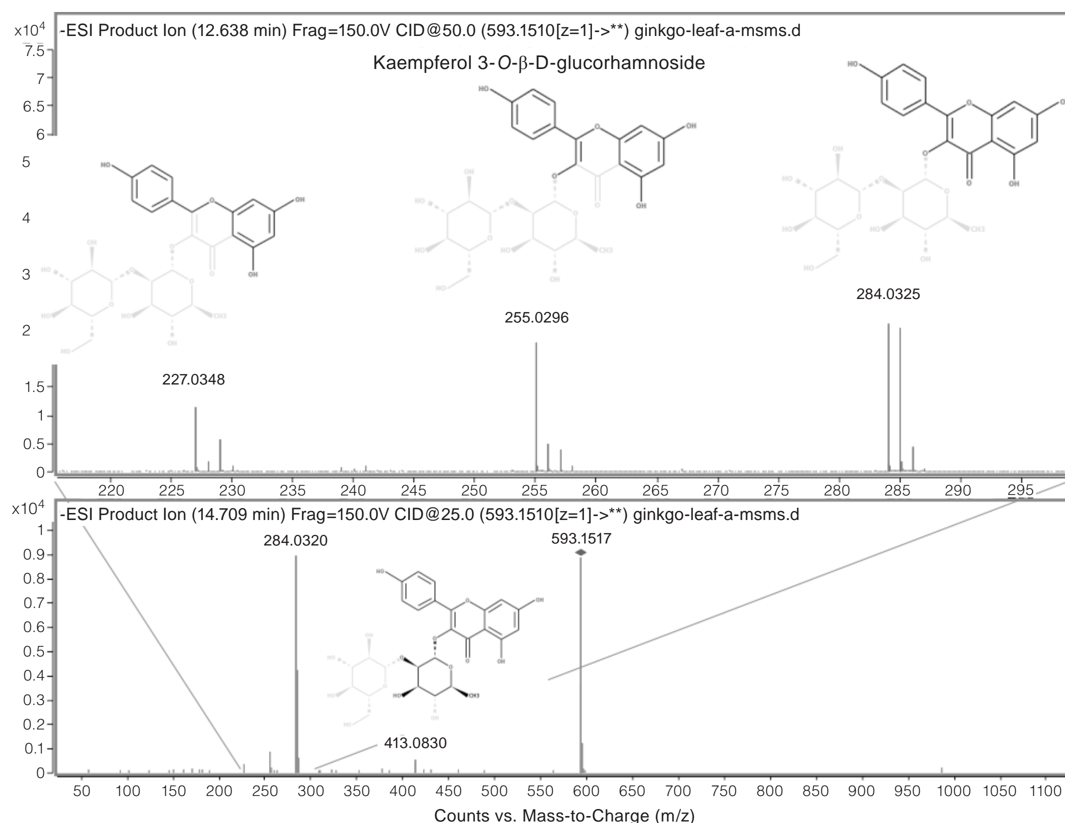

Figure 8. Fragmentation pathway of kaempferol-3-*O*-β-D-glucorhamnoside.

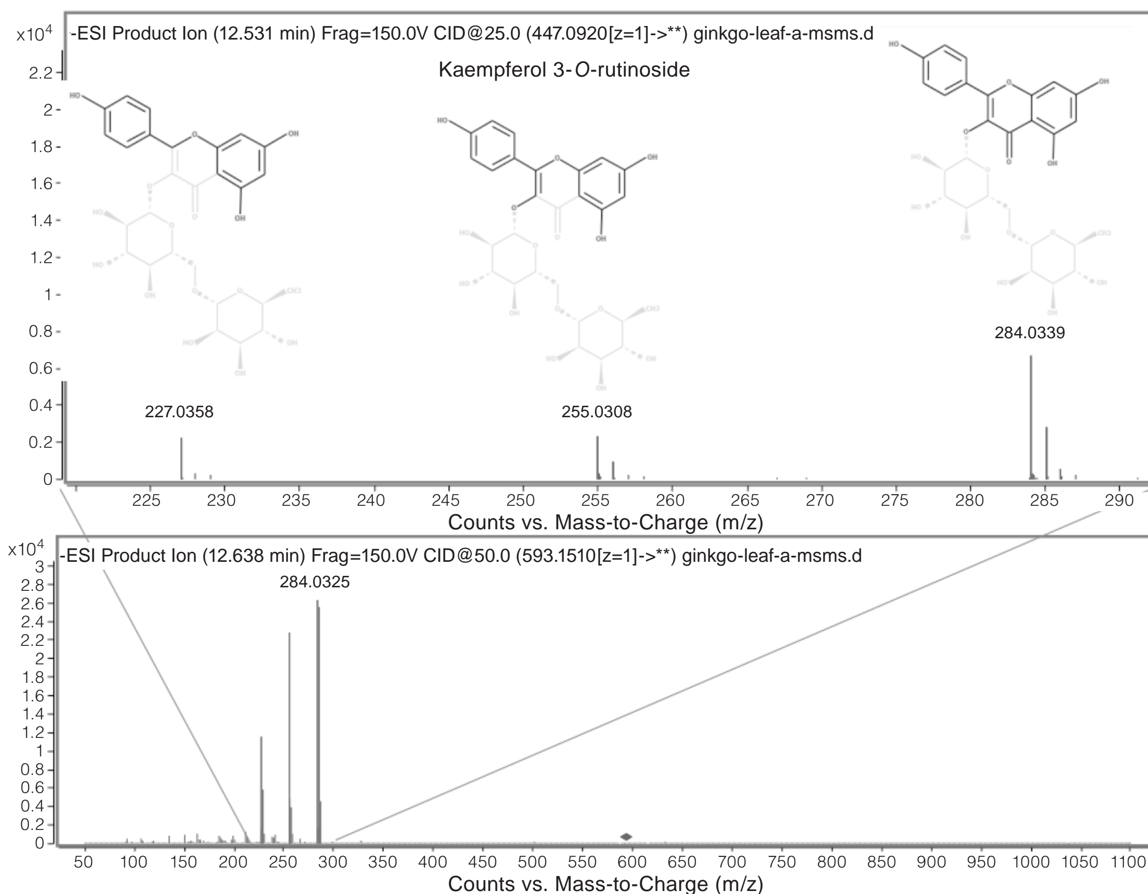

Figure 9. Fragmentation pathway of isorhamnetin 3-*O*-rutinoside.

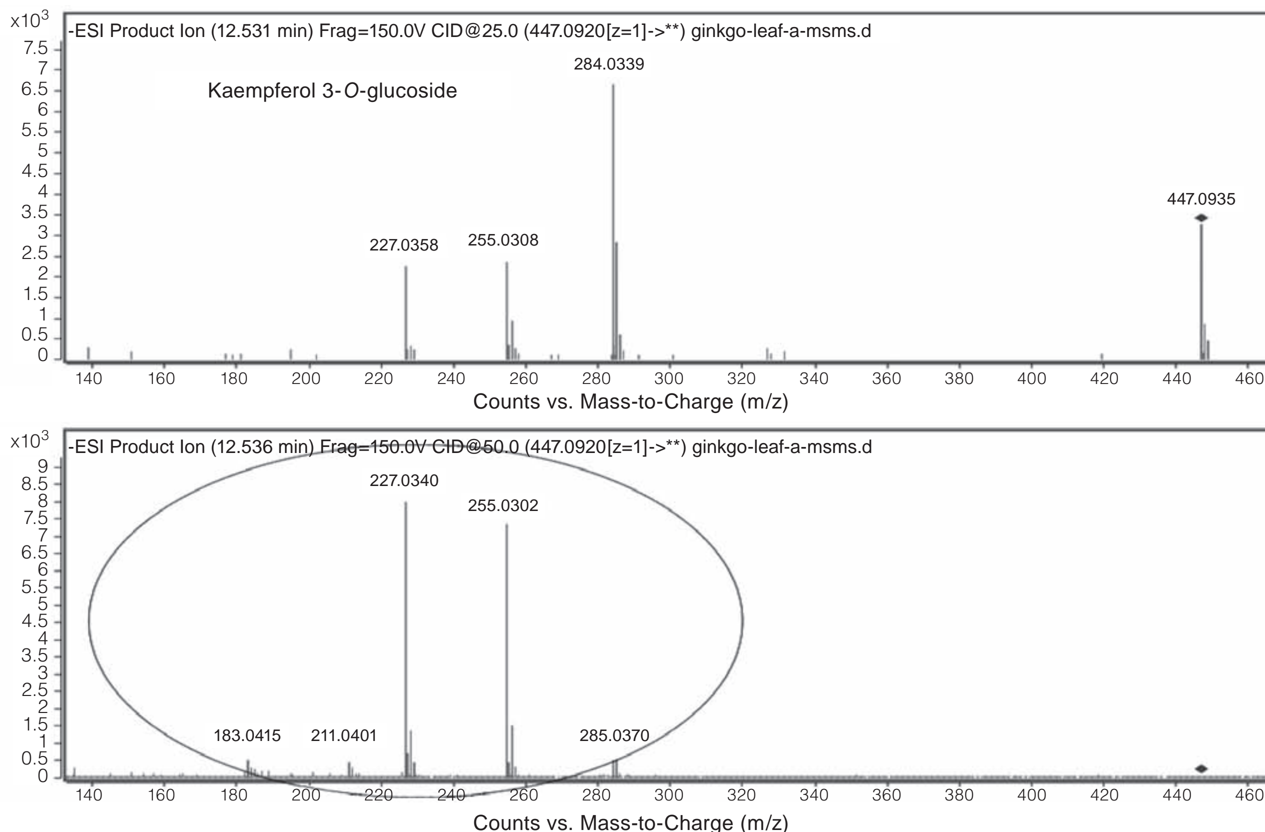

Figure 10. Fragmentation pathways of the isomer.

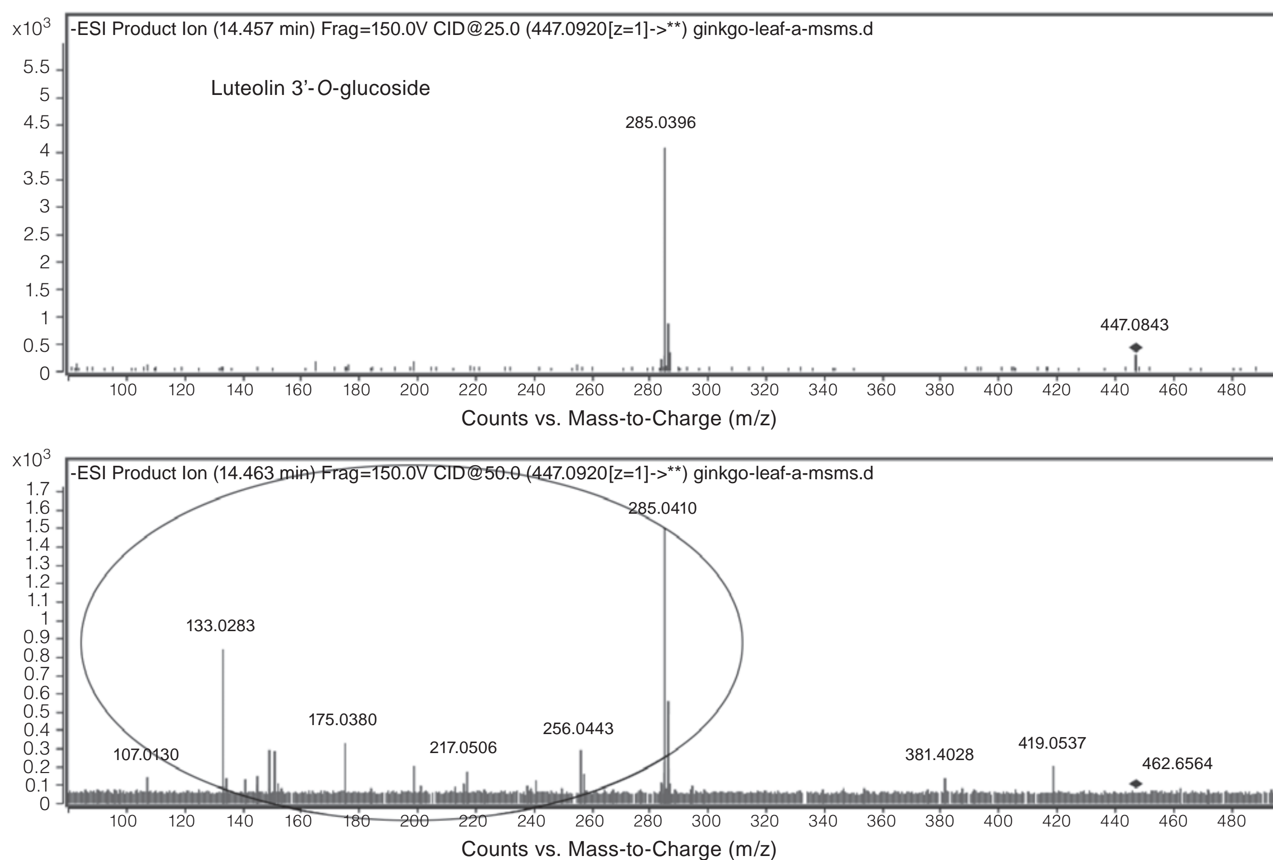

Figure 10. Continued.

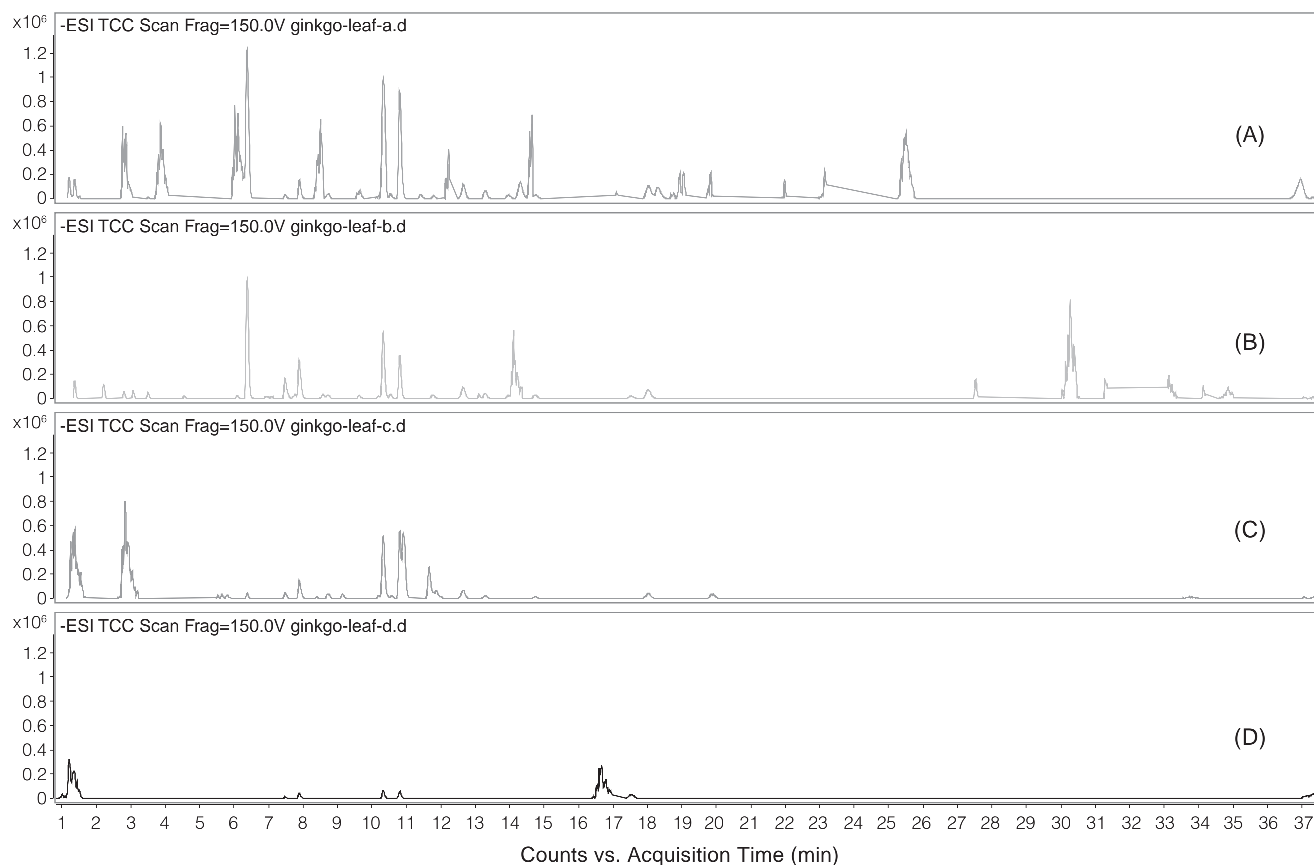

Figure 11. Mass scan in different sample of *Ginkgo biloba* extract. (A)(B) obtained from the European markets and (C)(D)were Chinese.

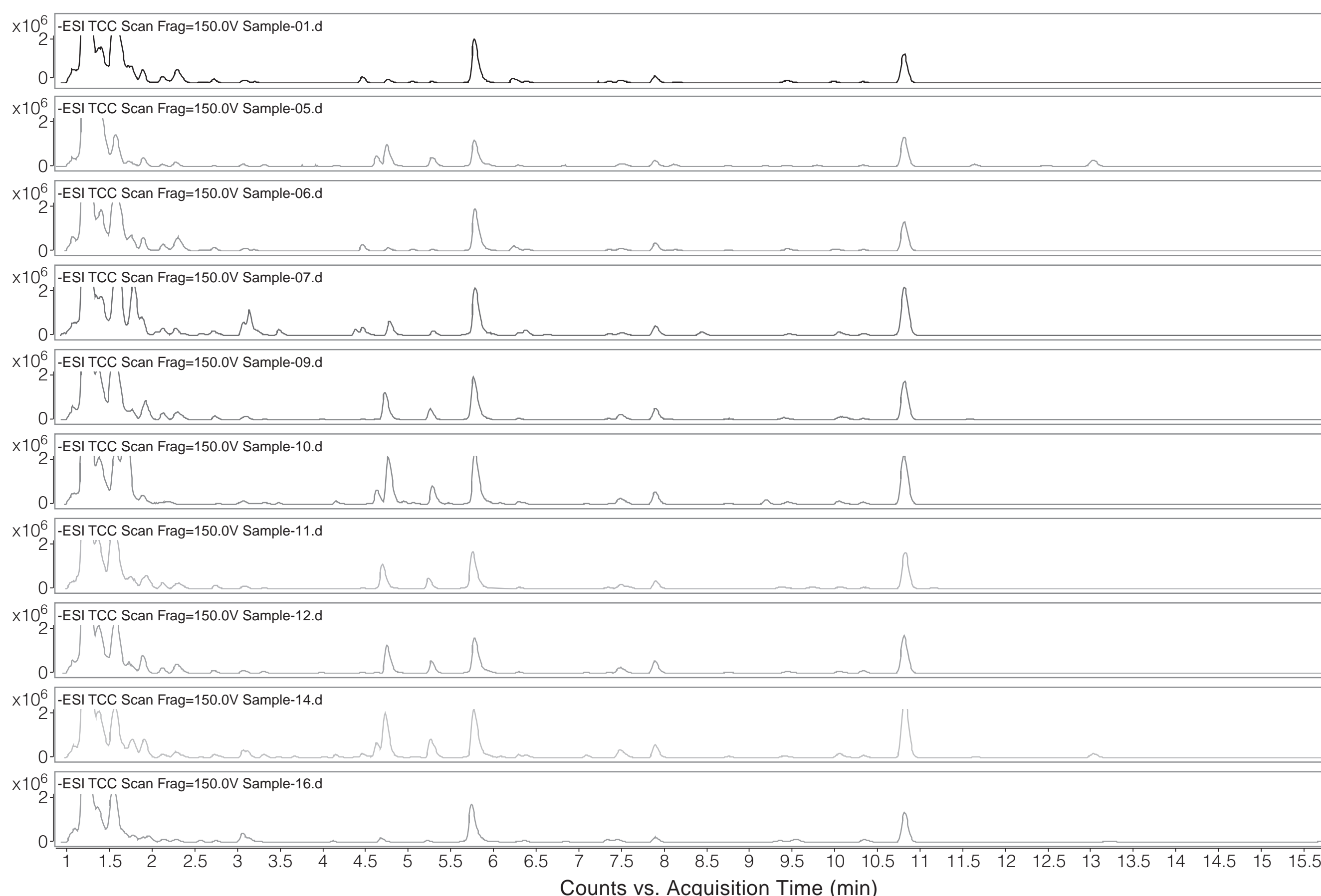

Figure 12. Mass scan in twenty sample of G. semen.

respectively. The limit setting the persent RSD values of the tested compounds was less than 0.80, and the test *p*-value to sort out individual compositions of *G. biloba* extract and G. semen was less than 0.05. The TOF-MS data is used to comfirm the chemical components in each material including (1) *G. biloba* extract (before acid hydrolysis), (2) *G. biloba* extract (after acid hydrolysis), (3) G. semen (before acid hydrolysis), (4) G. semen (after acid hydrolysis), retention time of each compound, the precise molecular weight and the formula of these speculated ingredients were shown in Tables 1 - 4. As shown in Figures 13 - 14, the method could be successfully used to analyze compounds in *G. biloba* extract and G. semen qualitatively and quantitatively and greatly decrease the analytical time. It could also be applied to the analysis of *G. biloba* product with more complex components. In addition, Bilobalide and Ginkgolide J were found in G. semen for the first time.

CONCLUSIONS

In this paper, ginkgolide J and bilobalide were identified in G. semen using HPLC-ESI-Q-TOF-MS in the negative ion mode for the first time. This newly established method was successfully applied to simultaneously identify the chemical constituents in *G. biloba* extract and G. semen. Thus, the method described is useful for the rapid analysis of multiple constituents in *G. biloba* extract and G. semen.

TOF-MS can detect the molecular weight-charge ratio of the substances quantitatively with high sensitivily. On comparison with other detectors, the mass accuracy of TOF-MS was constant over a wide range of injection volumes and multiple frequencies of injection, where a complicated and heavy matrix used in the ionic trap MS or quadrupole ionic resource was common for TCM analytes. Thus, TOF-MS is a useful tool for the quality control of TCM.

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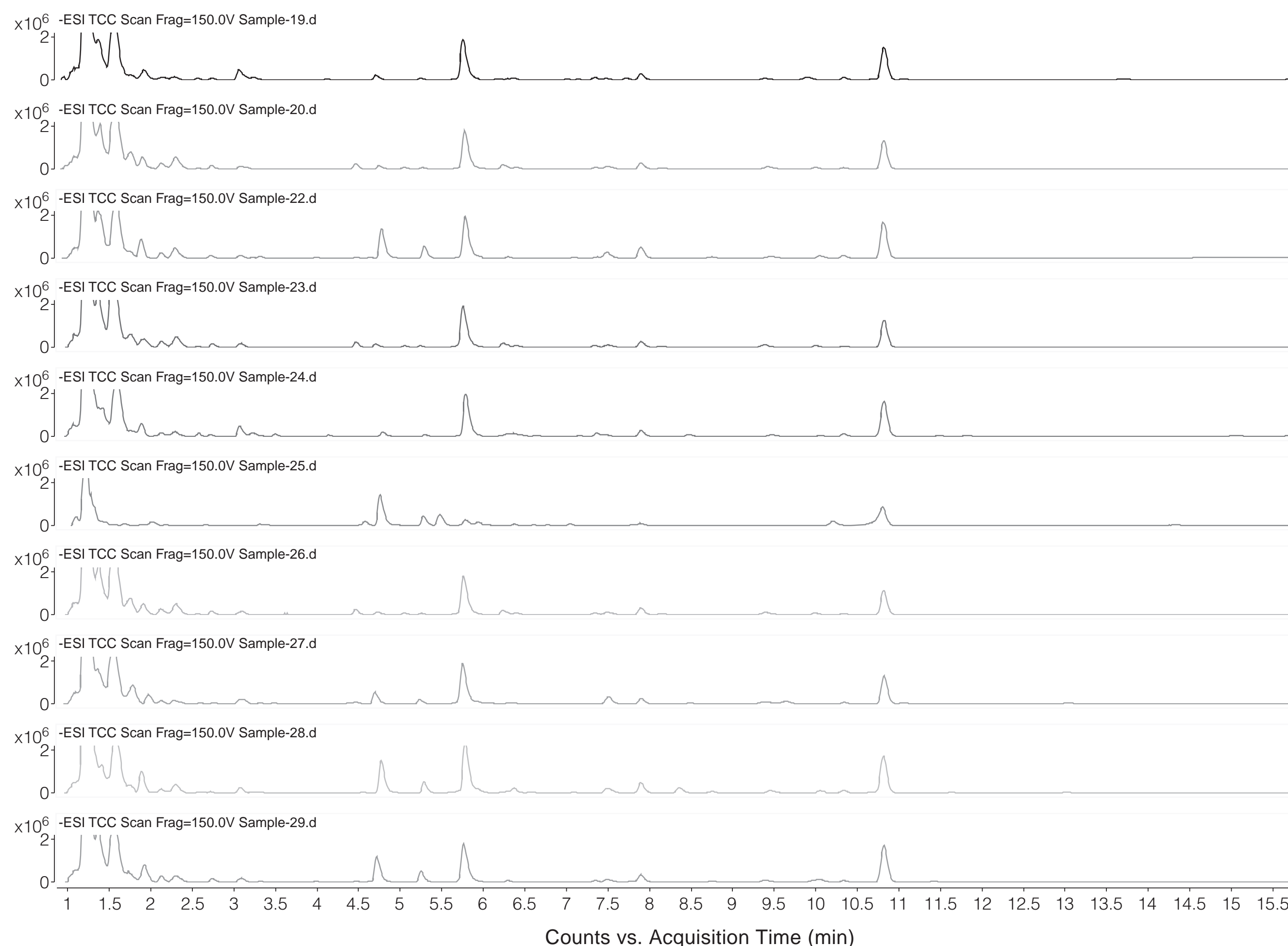

Figure 12. Continued.

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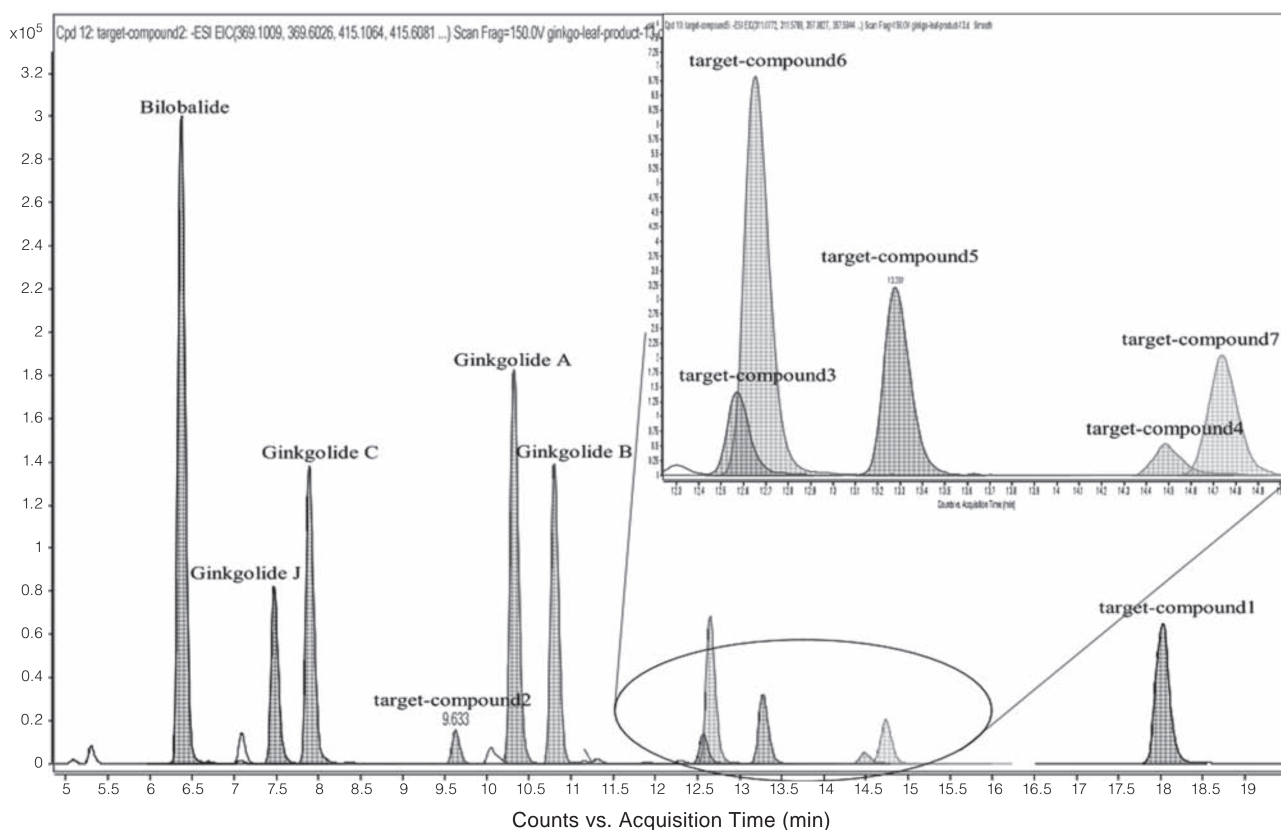

Figure 13. The target compounds in the product containing *Ginkgo biloba* extract (before acid hydrolysis).

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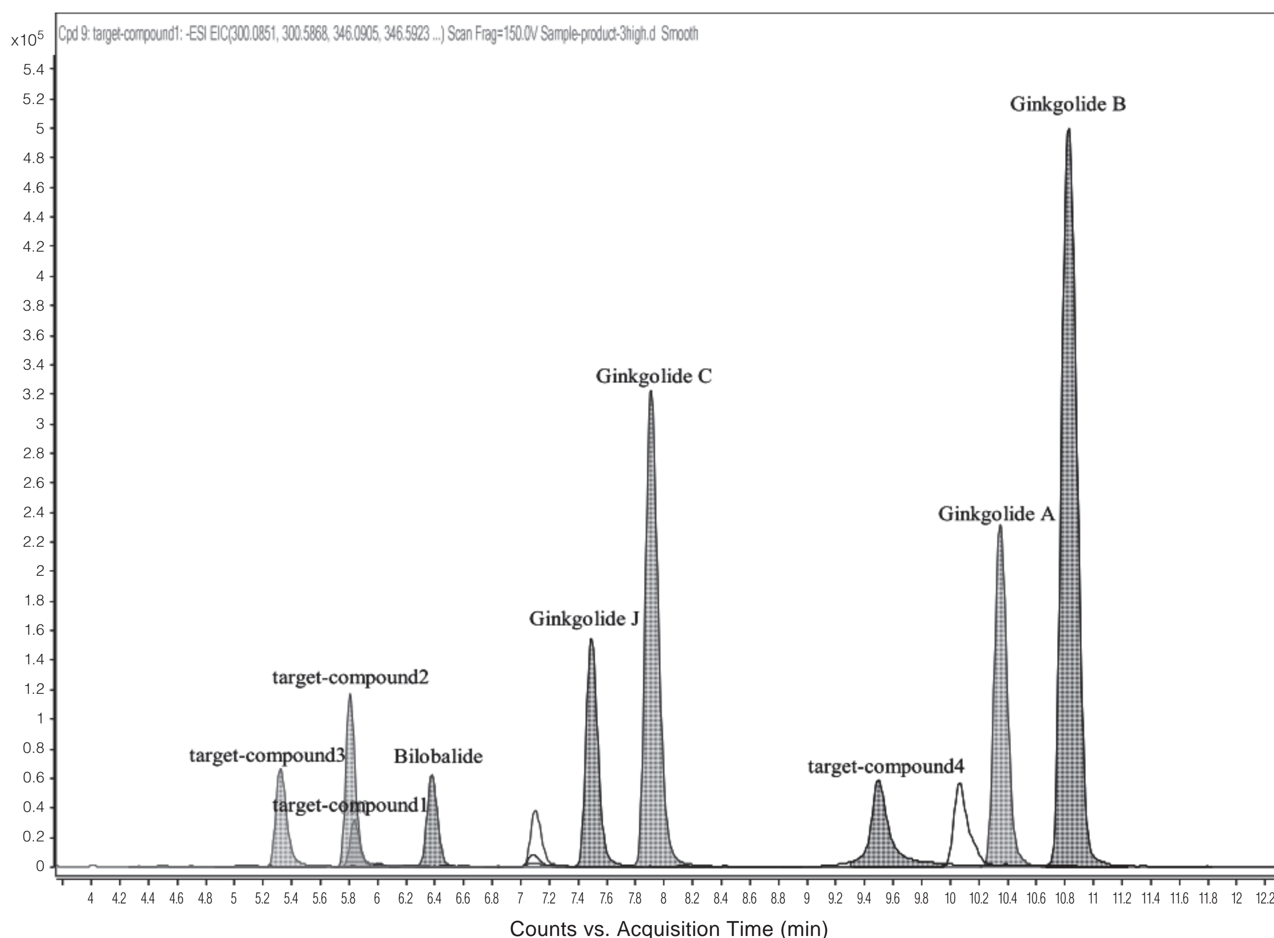

Figure 14. The target compounds in the product containing Ginkgo semen (before acid hydrolysis).

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