

# Analysis of Flavonoids from *Ginkgo Biloba* Extracts Using Ultra High Performance Liquid Chromatography—High Resolution Time-of-Flight Mass Spectrometry

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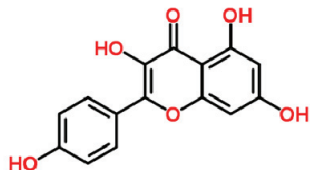
Key Words: *Ginkgo Biloba* Extracts, Accurate Mass, Fragmentation, High Resolution Time-of-Flight, Metabolites Databases, MS, MSc<sup>2</sup>, UHPLC Separation

## 1. Introduction

*Ginkgo biloba* extracts (GBEs) have been used as herbal medicines for many centuries in China.<sup>[1]</sup> GBEs are considered as alternative medicines for prevention and treatments of a number of illnesses such as heart disease and Alzheimer's disease, as well as cardiovascular and bronchial pathologies. GBEs are becoming one of the most widely used herbal supplement products in the world, especially in the US and in Europe.<sup>[2]</sup>



Figure 1. *Ginkgo biloba*.



Flavonoids are secondary plant metabolites. They have diverse structural features and high medical significance as antioxidants and free radical scavengers. *Ginkgo biloba* extracts (GBEs) contain a variety of pharmacological active flavonoids. Global regulatory agencies are working to establish analytical methodologies for the quality of control flavonoid containing natural products. LC-MS/MS is becoming the prominent technique used to address the needs due to its high selectivity, sensitivity, speed, and comprehensive coverage.<sup>[3]</sup>

In this study we have developed a reliable and robust LC-MS/MS method to compare and characterize some major flavonoids and their glycoside derivatives presented in four commercial GBEs matrices using a fast UHPLC separation technology coupled to high resolution TOFMS on the Citius™ LC-HRT. Accurate mass information on molecular species as well as comprehensive CID (MSc<sup>2</sup>) was used to determine structures of flavonoid compounds from the complex GBE matrices. Public access metabolite databases were utilized to confirm the identifications of compounds.

## 2. Results and Discussions

The UHPLC-TOFMS profiles (m/z 100-1000) for the 4 GBE samples are provided in Figure 1.

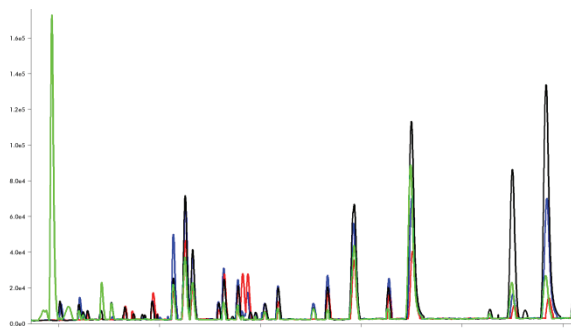


Figure 1. UHPLC-ESI TOFMS Profiles of 4 GBE Samples (m/z = 100-1000). The green trace is of a multivitamin supplemented with GBE.

Selected ion chromatograms of m/z of 714.2248 in the four GBEs are shown in Figure 2. There was a major component at a retention time near 380 seconds, present in all four commercial GBEs. However, the minor component at 270 seconds was present in only two of the four samples.

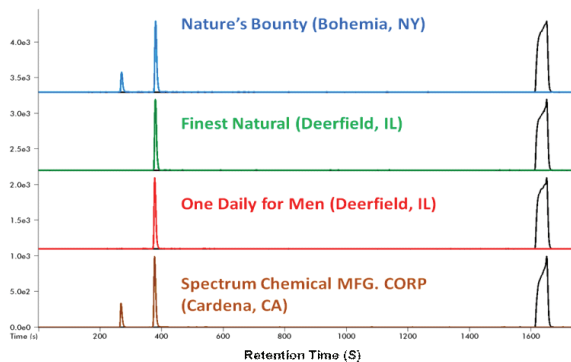


Figure 2. UHPLC-ESI TOFMS Profiles of m/z 714.2248 and mass calibrant m/z 622.029 of 4 GBEs.

The MS and MS<sup>2</sup> spectra of the major peak from Figure 2 are shown in Figure 3.

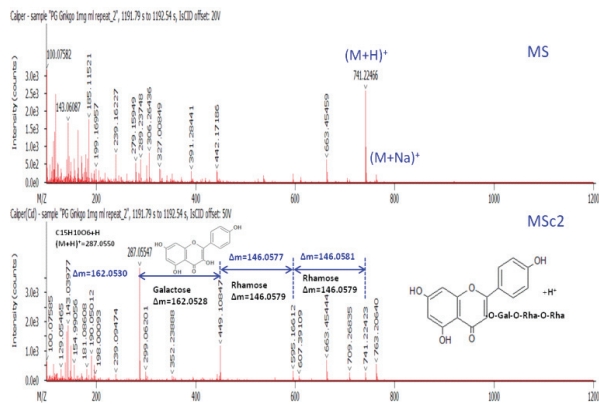


Figure 3. MS and MS<sup>2</sup> of m/z 741.2245 at retention time of 380 seconds.

The precursor MS spectrum showed an ion at m/z 741.2248 with its apparent sodium adduct at m/z 763.2062. At an elevated energy offset of 50 V, significant fragmentation was observed with sequential loss of monosaccharides including deoxyhexose (e.g. rhamnose) and hexose (e.g. galactose).

Table 1 shows the formula calculations of the precursor ions (protonated and sodiated) and their fragment ions. High resolution and high mass accuracy in both MS and MS<sup>2</sup> provide confidence in assigning the formula for both precursor and fragment ions.

Table 1. Mass calculation of MS and MS<sup>2</sup> ions from precursor ion of 741.2245.

Ions	Species	Formula	Mass	Δ PPM
741.2245	M+H	C33H41O19	741.2237	1.0793
763.2062	M+Na	C33H41O18Na	763.2056	0.7862
595.166	M+H-Rhamnose	C27H31O15	595.1663	-0.5041
449.1084	M+H-Rhamnose	C21H21O11	449.1078	1.3360
287.0554	M+H-Galactose	C15H11O6	287.055	1.3935

Database searching based on a precursor ion at m/z 741.2248 resulted in many candidates. Based on the MS<sup>2</sup> fragmentation pattern, the compound is tentatively identified as Kaempferol 3-rhamno-(1-4)-rhamno-(1-6)-glucoside. It possesses two rhamnose residues on its non-reducing end and is expected to provide the loss of two rhamnose moieties during the CID process. Its structure is shown in Figure 4.

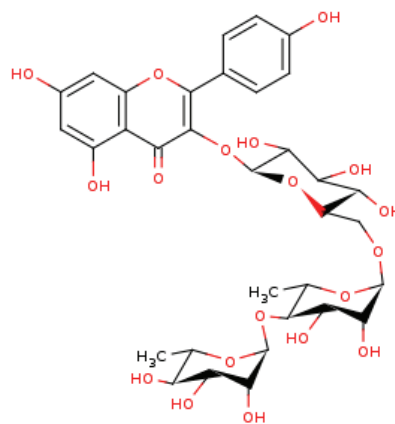


Figure 4. Kaempferol 3-rhamno-(1-4)-rhamno-(1-6)-glucoside.

The MS and MS<sup>2</sup> spectra of the minor peak were shown in Figure 5. The MS spectrum indicates a mixture of at least two major compounds at m/z at 741.2247 Da and 757.2194 da. MS<sup>2</sup> at an elevated energy offset of 80 V resulted in the production of rich fragment ions. Here the loss of two galactose moieties, or sequential loss rhamnose and galactose moieties, were observed. No additional loss of monosaccharide residues was observed. Proposed structures are shown in Figures 6 and 7.

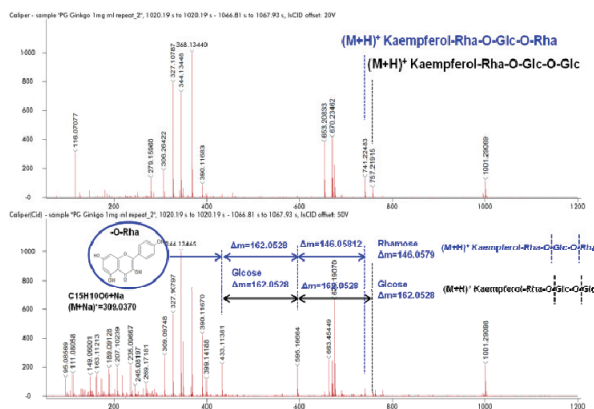


Figure 5. MS and MS<sup>2</sup> of m/z 741.224.

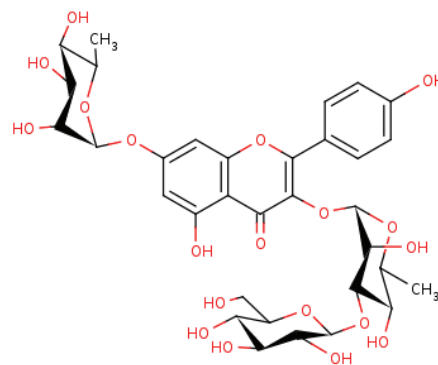


Figure 6. Kaempferol 3-glu-(1-3)-rhamnoside-7-rhamnoside.

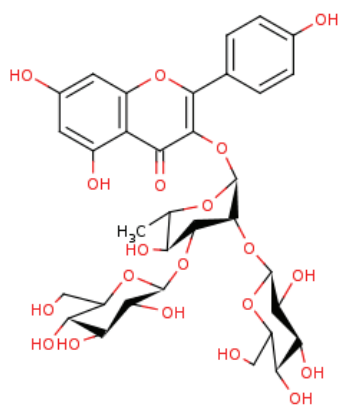


Figure 7. Kaempferol 3-glu-(1-2)-[glu-1-3]] rhamnoside.

Selected ion chromatograms for 611.1622 in the four GBEs are shown in Figure 8. Several potentially isomeric species are detected.

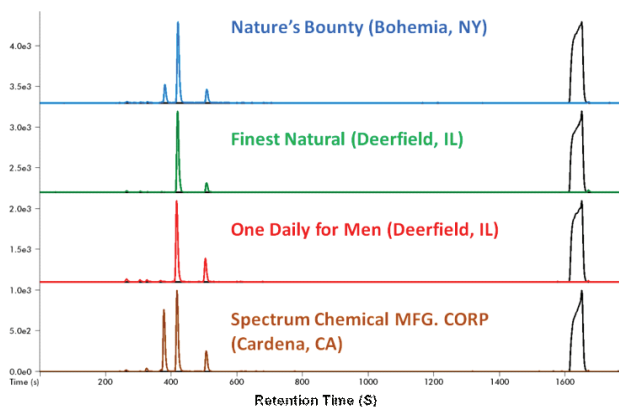


Figure 8. UHPLC-ESI TOFMS Profiles of  $m/z$  611.1612 and IS of  $m/z$  622.029 of 4 GBE Samples.

Each GBE showed unique profile. There was a major peak at ca. 420 seconds in all four commercial GBEs. However, the minor peak of 380 seconds was only present in two of the four samples.

Precursor ion MS spectrum showed an ion at  $m/z$  611.1613 with its supposed sodium adduct at  $m/z$  633.14350. At an elevated energy offset at 80 V, sequential loss of rhamnose and galactose occurred. In addition, an ion at  $m/z$  147.04424, diagnostic of rhamnose, is shown in Figure 9.

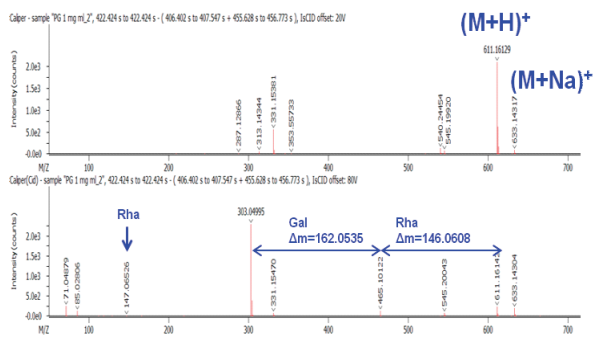


Figure 9. MS and  $MS^2$  of  $m/z$  611.1613 at retention time of 420 seconds.

The structure of the proposed analyte, quercetin 7-rutinoside, is provided in Figure 10.

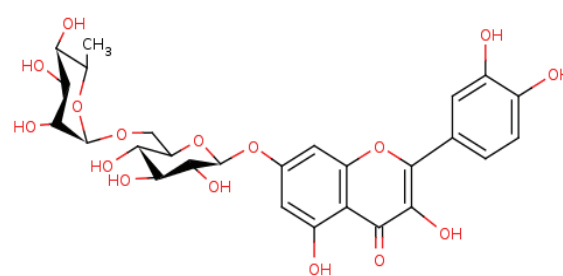


Figure 10. Quercetin 7-rutinoside.

The MS and  $MS^2$  spectra of the minor peak at 380 seconds is shown in Figure 11. The MS spectrum indicated that there were mixtures of at least two major compounds at  $m/z$  at 741.2247 Da and 611.1611 da.  $MS^2$  at an elevated energy of offset of 80 V resulted in the sequential loss rhamnose and galactose from the ion of 611.1611. A proposed structure for the compound at  $m/z$  611.1611 of quercetin 3-gal-(1-4)-rhamnoside is presented in Figure 12.

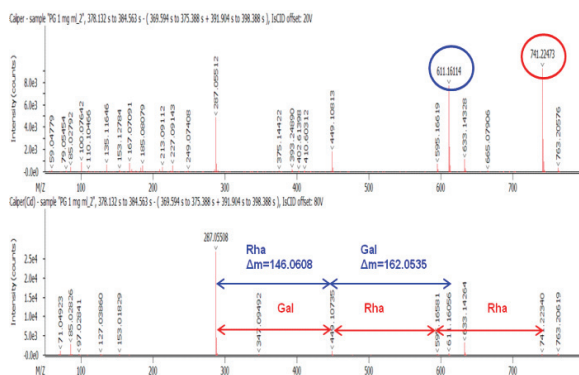


Figure 11. MS and  $MS^2$  of  $m/z$  611.1611 and 741.2247 at 380 seconds.

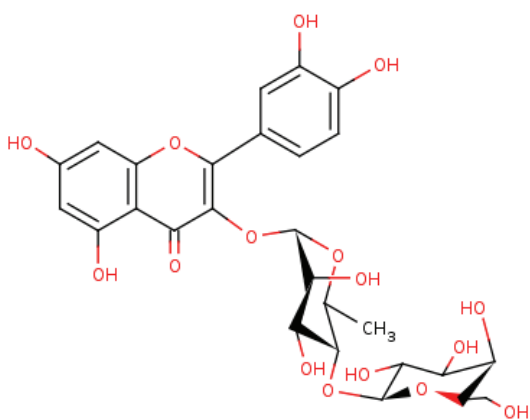


Figure 12. Quercetin 3-gal-(1-4)-rhamnoside.

### 3. Conclusions

It was demonstrated that UHPLC, in combination with Citius™ LC-HRT High Resolution TOFMS provides a powerful tool for the structural elucidation of major flavonoid glycosides in commercial GBEs products. Accurate precursor ions in MS and fragment ions in MS<sup>2</sup> showed less than 2 ppm mass accuracies which facilitated characterization of these unique class compounds using public databases. The results clearly demonstrated the accurate mass capabilities of the Citius in the analysis of flavonoid glycosides in complex matrices.

### 4. References

- <sup>1)</sup> van Beek TA. *Ginkgo biloba*. Harwood Academic Publishers; Australia: 2000.
- <sup>2)</sup> Luo Y. *J Alzheimers Dis*. 2001;3:401–407.
- <sup>3)</sup> Yongkai Sun, Wenkui Li, John F. Fitzloff, and Richard B. van Breemen. *J Mass Spectrom*. 2005; 40(3): 373–379.

### 5. Sample Preparations

Four commercially-available *Ginkgo biloba* extracts were used in this study. Three were dietary supplements and the fourth was food-grade. The powder from the samples was weighed and suspended in 70% ethanol 30% water solvents at 10 mg/mL. The stock solution was mixed well and centrifuged at 10,000 RPM for 10 min. A portion of the clear stock solution was diluted to 0.5 mg/ml in the ethanol/water for analysis.

### 6. Experimental Conditions

A LECO Citius LC-HRT high resolution mass spectrometer equipped with an Agilent 1290 UHPLC was used in this study.

#### Mass Spectrometry Conditions

Data were acquired in both positive and negative ion modes using the electrospray interface on the LECO Citius LC-HRT in high-resolution (HR) mode (R(FWHM) = 50,000). Mass calibration was achieved using Agilent Tune Mix (Agilent, CA). Key acquisition parameters are summarized below.

Nozzle Temperature (°C)	100
Desolvation Heater Temp (°C)	900
Desolvation Flow (s/m)	7.5
Spectral Acquisition Rate	2.5 spectra/second
CID Mode	Pulsed (MS & MS <sup>2</sup> )
Fragmentation Energy (V)	60
Spectral Range (m/z)	50 to 1000
Fragmentation Energy (V)	60

#### UHPLC Conditions

Agilent 1290 Infinity UHPLC with a Waters 1.8 μm HSS T3 C18 column (2.1x100 mm) was used in this work. The UHPLC column heater chamber was maintained at 40°C during the experiment. The LC-MS grade solvents (Honeywell Burdick & Jackson) were 0.1% formic acid both in water (A) and acetonitrile (B). The gradient was 0-2 min, 2%B; 2-22 min, linear increase to 100%; 26-26.5 min, 2%B with a final hold for 2 min. During the time frame of 26 min to 26.5 min, reference pump in the Citius LC-HRT TOFMS delivered at 30 μL/min of Agilent ESI Tuning Mix for post data acquisition calibrations.



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