High Resolution GC/MS Analysis of Ginkgolic Acids in *Ginkgo biloba* Plants, Extracts, and Dietary Supplements Using the Agilent 5975C Series GC/MSD

Application Note

Food Testing & Agriculture

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Abstract

A high-resolution GC/MS method using the Agilent 7890 GC and the Agilent 5975C Series GC/MSD has been developed and validated for the analysis of potentially harmful ginkgolic acid (GA) species that is superior to current HPLC methods. Ozonolysis was used to identify the position of double bonds in four different unsaturated GA isomers. The 15:1 isomer was dominant in all the plant samples, and the ratio of Δ10 to Δ8 GA15:1 can be used to determine the *Ginkgo biloba* plant part (seed or leaf) source of the GAs present in commercial dietary supplements and other ginkgo products.

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Introduction

Products and extracts derived from *Ginkgo biloba* L. (Ginkgoaceae) make up one of the most widely used categories of phytopharmaceuticals, dietary supplements, or herbal medicines. Purported therapeutic benefits most commonly associated with these products include improved blood circulation, slowing of the aging process, treatment of cardiovascular and neurodegenerative disorders, and improved cognitive functions [1].

However, as is often seen with herbal medicines and dietary supplements, *G. biloba* extracts and commercial products have also been associated with harmful health effects, including carcinogenic, cytotoxic, neurotoxic, tumor promoting, or mutagenic properties [1]. While the validity of many of these unfavorable health claims is not well established, it is generally accepted that most of the adverse effects are due to the bioactive alkylsalicylic acids present in these products, such as ginkgolic acids (GAs).

A simple, sensitive, and validated analytical method for the detection and quantitation of these alkylsalicylic acids in commercial products containing *G. biloba* is needed. While liquid chromatography is the currently accepted analytical method, it provides inadequate resolution of GAs that differ by carbon number and unsaturation (C13:0/C15:1 or C15:0/C17:1), as well as double bond positional isomers.

This application note describes a previously published GC/MS method [1] that produces high-resolution separation of the individual isomers of C13, C15, and C17 GAs in ginkgo leaves, extracts, and commercial products. It was also used to determine the double bond position and concentration of each isomer. This method was developed using the Agilent 7890 GC and the Agilent 5975C Series GC/MSD, and it provided resolution that could not be attained with the usual HPLC techniques.

Experimental

Sample preparation

A total of 19 plant samples and reference standards including leaves, seeds, and leaf extracts, as well as 21 dietary supplements, were prepared as described [1].

Reagents and standards

Four ginkgolic acid standards (C13:0, C15:0, C15:1, and C17:1) and the internal standard anthracene were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and methylene chloride were purchased from Fisher Scientific (Pittsburgh, PA). GC grade *n*-hexane, formic acid, and the derivatization reagent trimethylsulfonium hydroxide (TMSH) solution (~0.25 M in methanol, for GC derivatization) were obtained from Sigma-Aldrich.

Instruments

The GC/MS method was performed using an Agilent 7890GC configured with an Agilent 7693A Automatic Liquid Sampler and coupled to an Agilent 5975C Series GC/MSD.
The instrument conditions used are shown in Table 1.

Table 1. GC and Mass Spectrometer Conditions

GC conditions	
Analytical column	Agilent J&W HP-88, 30 m × 0.25 mm, 0.25 µm cross-linked (88% cyanopropyl) arylpolysiloxane (p/n 112-8837)
Injection temperature	260 °C
Injection mode	Split ratio 25:1
Oven program	2 minutes at 150 °C 8 °C/min to 220 °C 3 °C/min to 250 °C 2 minutes at 250 °C
Column flow	1 mL/min constant flow
Carrier gas	Helium
Transfer line temperature	280 °C
GC run time	25 minutes
MS conditions	
Ionization mode	Electron ionization at 70 eV
Ion source temperature	230 °C
Solvent delay time	4 minutes
Acquisition mode	Scan (40–500 amu) and SIM (*m/z* 161 and 178 IS)

Data analysis

Comparison of the spectra with the Wiley and NIST databases using a probability-based matching algorithm was performed to achieve compound identification, along with comparison of relative retention indices (RI) to literature and standard reference values.

Results and Discussion

Method performance and validation

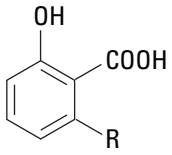
The primary challenge of GA analysis is full resolution of the individual acids and their isomers, specifically for the component pairs C13:0/C15:1 and C15:0/C17:1 (Figure 1). Figure 2 shows that this GC/MS method clearly resolved these difficult component pairs, using the *m/z* 161 (methoxy indenone) cation common to all GAs. This ion is generally the base peak in the GA spectrum, and is a very sensitive and specific probe for all GAs [1]. Figure 2A shows the separation of GA standards, and 2B demonstrates the high-resolution separation of GAs extracted from *Ginkgo biloba* leaves.

Several method parameters were validated pursuant to the International Conference on Harmonisation (ICH) guidelines, including intra- and interday precision, selectivity, linearity, accuracy, limit of detection (LOD), and limit of quantitation (LOQ). Triplicate intraday relative standard deviations (RSDs) ranged from 1.3–2.1 %, while the interday RSD for nine analyses was 3.8 %.

Using the selected ion monitor mode at *m/z* 161, no other components or contaminants were detected in the retention time window available for the GAs, indicating high selectivity. Correlation coefficients (R^2) for the calibration plots were > 0.99 over a concentration range of 5–250 µg/mL, demonstrating excellent linearity.

To determine the accuracy of the method, a leaf sample was spiked with a known amount of GA standards. A 0.5 g sample was first exhaustively extracted six times as described [1] and then dried. After the addition of 4–5 µg of C13:0, C15.0, C15:1, and C17:1 GAs, the samples were extracted, derivatized with trimethylsulfonium hydroxide (TMSH), and analyzed using the optimized GC/MS method. The percentage recoveries were 87.7 % (C13:0), 89.5 % (C15:0), 95.4 % (C15:1), and 89.2 % (C17:1).

The LOD, determined from the signal-to-noise ratio of 3:1, was 0.5 µg/mL, and the LOQ was 1.5 µg/mL for all of the GAs, demonstrating good sensitivity.



Ginkgolic acids

R = $C_{13}H_{27}$ (C13:0)
R = $C_{15}H_{31}$ (C15:0)
R = $C_{15}H_{29}$ (C15:1)
R = $C_{17}H_{33}$ (C17:1)
R = $C_{17}H_{31}$ (C17:2)

Figure 1. Chemical structures of the ginkgolic acids in Ginkgo biloba.

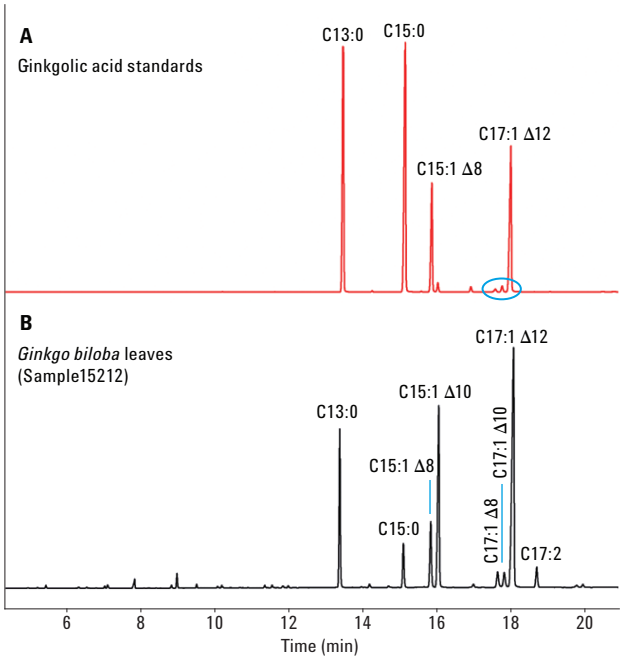


Figure 2. SIM GC/MS chromatograms at m/z 161 of GA standards (A) and sample 15212 (Ginkgo biloba leaves) (B).The circled region in 1A highlights the position of the C17:1 Δ8 and Δ10 isomers present in the standard, 90 % of which was shown to be the Δ12 isomer.

Double bond localization

Because the precise roles of the various isomeric GAs in the pharmacological effects of *G. biloba* have never been demonstrated, this method was used to characterize GA isomeric composition. The double bond positions were determined using ozonolysis of both the C15:1 and C17:1 standards [1]. The C15:1 GA standard was confirmed to contain only the Δ8 double bond isomer, as the manufacturer stated. Subsequent analysis of *G. biloba* plant parts and products revealed the presence of the C15:1 Δ8 isomer as well. While the C17:1 standard contained three isomers (Δ8, Δ10, and Δ12) whose relative abundances are shown in Figure 2, the Δ12 isomer represented more than 90 % of this GA. This was in contrast to the manufacturer's claim that this standard contained only the Δ10 isomer.

Analysis of plant and dietary supplement samples

The method was then used to determine the GA content of 19 *G. biloba* samples, including isomer composition. The sample set included seeds, sarcotesta (fleshy seed coat), leaves, and dried alcoholic extracts of leaves. Wide variations in GA content were observed (Table 2), with the seeds having the lowest concentration range (4–39 ppm). While dried extracts gave slightly higher GA values (3–47 ppm), leaf samples exhibited the widest concentration range (42–534 ppm), with no systematic pattern.

Table 2. Ginkgolic Acid Content of Plant Parts and Dietary Supplements

Sample type	Total GA concentration range (ppm)	C15:1 Δ10/Δ8 ratio range
Leaves	42–534	1–3
Leaf extract	3–47	1–3
Seeds	4–39	0.1–0.5
Sarcotesta	143 (one sample)	0.2
Dietary supplements	1–56	1–3

Ozonolysis revealed that the major GA constituent present in every type of plant sample was either C15:1 Δ8 or Δ10. In addition, the C15:1 isomer Δ10/Δ8 ratio was indicative of the source of the plant sample (Table 2). Within the limited data set, this ratio was in the range of 1–3 for all leaf and leaf extract samples, while seed and sarcotesta samples gave a Δ10/Δ8 ratio of 0.1 to 0.5. This ratio, therefore, enables the determination of the plant part source of the *G. biloba* GAs present in dietary supplements. In fact, all of the dietary supplements that contained detectable amounts of the C15:1 isomers gave ratios between 1 and 3 (Table 2), indicating that these commercial products were all derived from leaves, rather than seeds or sarcotesta. The resolution and quantitation of the C15:1 Δ8 and Δ10 isomers that enables such product characterization is only possible using gas chromatographic methods.

Conclusions

A GC/MS method is now available for the high resolution analysis of ginkgolic acid (GA) species. Unlike HPLC methods, which cannot provide full resolution of GAs differing in carbon number and unsaturation, this GC/MS method is capable of separating and quantitating the GA isomers. Ozonolysis was used to determine the double bond positions in four unsaturated GAs. The C15:1 Δ10/Δ8 ratio can be used to determine the plant part source (for example, leaves versus seeds) of the GAs present in a limited set of commercial dietary supplements. Such product characterization is currently only possible using GC methods.

Reference

1. M. Wang, *et al.* "High-resolution gas chromatography/mass spectrometry method for characterization and quantitative analysis of ginkgolic acids in Ginkgo biloba plants, extracts, and dietary supplements" *J. Agric. Food Chem.* **62**, 12103-12111 (2014).

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Printed in the USA
March 12, 2015
5991-5634EN

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