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High-Performance Liquid Chromatography–Electrospray Ionization-Mass Spectrometry Study of Ginkgolic Acid in the leaves and Fruits of the Ginkgo Tree (*Ginkgo biloba*)

Xian-guo He*, **Matthew W. Bernart** †, **Gary S. Nolan**, **Long-ze Lin,** and **Michael P. Lindenmaier**
Research Laboratory of Natural Products Chemistry, A.M. Todd Botanicals, 4091 W. 11th Avenue, Eugene, OR 97402

Abstract

A method is developed for qualitative analysis of ginkgolic acids in the leaves and fruits of *Ginkgo biloba* by high-performance liquid chromatography (HPLC)–electrospray ionization-mass spectrometry technique. Negative ionization mode is successful in obtaining a very abundant deprotonated molecule $[M - H]^-$. The mass detection sensitivity is higher than ultraviolet detection but relies heavily on the concentration of acetic acid in the HPLC eluent, which consists of acetonitrile–water–acetic acid. The method is also very specific for the analysis of ginkgolic acid with no interferences from the sample matrix.

Introduction

Ginkgolic acid is a mixture of several 2-hydroxy-6-alkylbenzoic acids in which the alkyl chain may contain Δ^8An 15:1 (I), Δ^{10}An 17:1 (II), or An 13:0 (III). The abbreviations An 15:1, An 17:1, and An 13:0 are used to specify *n*-pentadecenyl, *n*-heptadecenyl, and *n*-tridecyl, respectively. Their structures are shown in Figure 1.

Compounds I and II are the major ginkgolic acids in ginkgo leaves (I, 52%; II, 40%; and III, 8%) (1). The structures of ginkgolic acids are similar to anacardic acid, a principal constituent of cashew nuts (2). Anacardic acid is also a mixture of 2-hydroxy-6-alkylbenzoic acids in which the alkyl chain (C^{11} or higher) is fully saturated or is a monoene, diene, or triene. Because anacardic acids are structurally similar to urushiol of poison ivy, German authorities maintain that ginkgolic acids must be thoroughly removed from ginkgo extracts. These products are widely used as herbal medicines for sharpening memory, increasing blood flow, and reducing the risk of heart attacks.

Indian and Japanese scientists have dispelled the Western misbelief. Recent Indian research has shown that ginkgolic acids possess antioxidant, radical-scavenging, and anti-inflammatory activities (3). Japanese scientists reported that ginkgolic acids showed antitumor activities (4). Irie et al. (5) reported that ginkgolic acids definitely inhibit GPDH, a key enzyme in the synthesis of triacylglycerol, which is related to obesity. Evidence obtained now suggests that as long as the carboxy group in ginkgolic acids is intact, there is no tendency to cause allergic reactions (6). No matter how this debate proceeds, the analysis of ginkgolic acids in ginkgo extract is becoming increasingly important.

Relatively few reports have been published on this subject. Thin-layer chromatography (TLC) was used early on for qualitative analysis (7). Individual ginkgolic acids from ginkgo leaves were determined quantitatively through gas chromatography–mass spectrometry (MS) as trimethylsilyl derivatives (8). Various HPLC analyses of anacardic or ginkgolic acids using reversed-phase columns have been published (5,9). Both studies employ an isocratic elution with methanol–water–acetic acid in different compositions. There is still a need for developing a rapid and sensitive method for the analysis of ginkgolic acids. Furthermore, there is no report on high-performance liquid chromatography (HPLC)–MS research of ginkgolic acids to date.

During our continuing study on HPLC–electrospray ionization (ESI)-MS for the analysis of natural products from botanical extracts (10,11), it was found that ginkgolic acids are very easily ionized in electrospray MS in a negative-ion mode. Thus, this technique was applied to analyze ginkgolic acids in the leaves and fruits of the ginkgo tree using a simple sample preparation procedure.

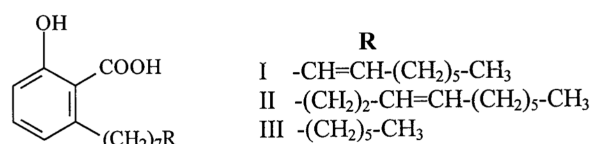

Figure 1. Structures of ginkgolic acids.

* Author to whom correspondence should be addressed.
† Current address: Molecular Probes Inc., 4849 Pitchford Ave., Eugene, OR 97402.

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Experimental

Materials

Equipment

An HPLC 1090 series II instrument (Hewlett-Packard, Palo Alto, CA) with a photodiode-array detector (DAD) set at 308 nm was coupled with an HP5989B quadrupole mass spectrometer. Ultraviolet (UV) spectra were taken in the range of 200–500 nm. Chromatographic conditions were as follows: column, Waters (Milford, MA) Symmetry Shield RP_{18}, 5 µm, 2.1 × 150 mm with sentry guard column; isocratic elution with acetonitrile–H_2O–acetic acid (92:7:1) at 15 min; flow rate was 0.5 mL/min; temperature was 45°C; mass range measured, 200–600 u; quadrupole temperature was reduced to 120°C to help prevent oxidation of the mass filter; EM Volts, 2173. The spectra were acquired in the negative-ion mode. The electrospray interface was an HP 59987A. Drying N_2 (40 mL/min) and additional O_2 at 20 mL/min was mixed in preventing corona discharge. Temperature was 320°C. Nebulizing N_2 was 5.5×10^5 Pa (80 psi). The HPLC was connected directly to the MS without stream splitting.

Chemicals

HPLC-grade water, acetonitrile, and ethanol (VWR, Seattle, WA) were used for HPLC analysis. Reagent-grade acetic acid, methanol, hexane, and ethyl acetate (VWR) were used for sample preparation.

Methods and solutions

Plant material and sample preparation

Ginkgo leaves and fruits were purchased from Asia Natural (San Francisco, CA).

Ginkgo fruit extract

Ginkgo fruits were husked and then frozen. A 2-g sample of frozen sarcocarp was ground in a Diamonite mortar and refluxed with 20 mL ethanol for 1 h. The sample solution was filtered through a 0.45-µm nylon Acrodisk 13-mm filter (Gelman, Ann Arbor, MI); a 20-µL sample was injected into the HPLC for analysis.

Ginkgo leaf extract

A 200-g amount of dried ginkgo leaf powder was refluxed with 500 mL hexane–ethyl acetate (1:1) for 1 h. The sample was filtered and reduced in a vacuum. Approximately 2 g of extract was

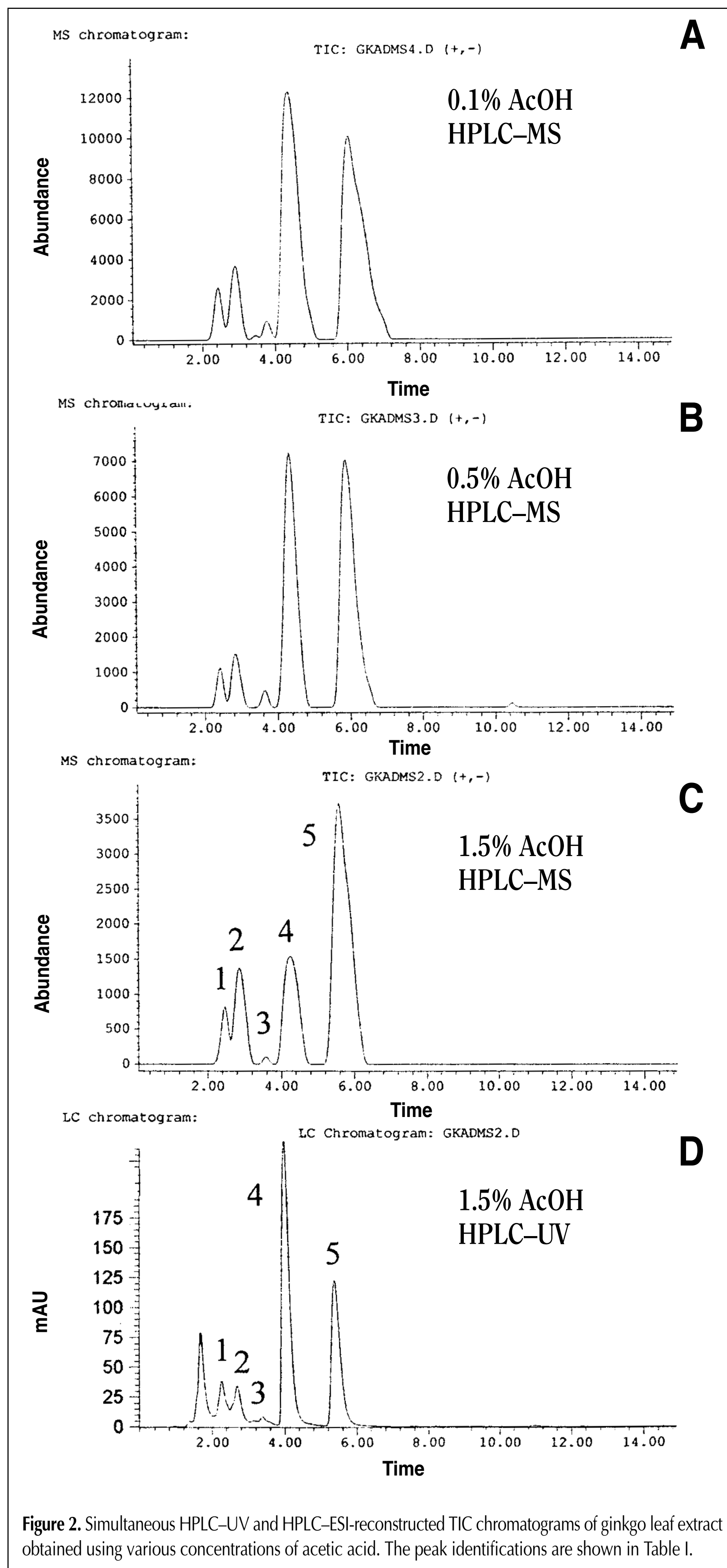

Figure 2. Simultaneous HPLC–UV and HPLC–ESI-reconstructed TIC chromatograms of ginkgo leaf extract obtained using various concentrations of acetic acid. The peak identifications are shown in Table I.

applied to a preparative C_{18} column (45 × 35 mm), Bakerbond octadecyl C_{18}, 40 µm (J.T. Baker, Phillipsburg, NJ), which was eluted with 150 mL acetonitrile–H_2O (95:5, v/v). Ginkgolic acid can be monitored by TLC, because it fluoresces brightly under long-wave UV light.

The eluate was concentrated under a vacuum. Residue was dissolved in 20 mL ethanol. After filtering through a Acrodisk 13-mm filter, a 4-µL sample was injected into the HPLC for analysis.

Results and Discussion

Identification of ginkgolic acid I and II by HPLC–DAD-ESI-MS analysis

Previously, most reversed-phase HPLC separations of anacardic or ginkgolic acids used different compositions of methanol–water–acetic acid as the mobile phase. In positive ESI mode, the addition of acetic acid reduces the pH to improve ESI positive-ion detection sensitivity, but we found that ginkgolic acid did not ionize in the positive ESI mode; therefore, we employed the negative ESI mode. Negative-ion ESI operation requires special solvent considerations, usually at high pH levels. Therefore, for highly sensitive negative ion analysis, it is important to use a basic eluent. The common method is ammonium hydroxide postcolumn addition to increase the pH, thus improving ESI negative-ion detection (12).

In the present experiment, ginkgolic acids were found to still have strong ionization at pH 3. Figure 2D shows an HPLC–UV (308 nm) chromatogram of ginkgo leaf extract at 1.5% acetic acid in acetonitrile–H_2O (92:6.5, v/v). Figure 2C shows a simultaneous HPLC–reconstructed total ion current (TIC) chromatogram, but with MeOH–H_2O as eluent, ginkgolic acids exhibited poorer ionization.

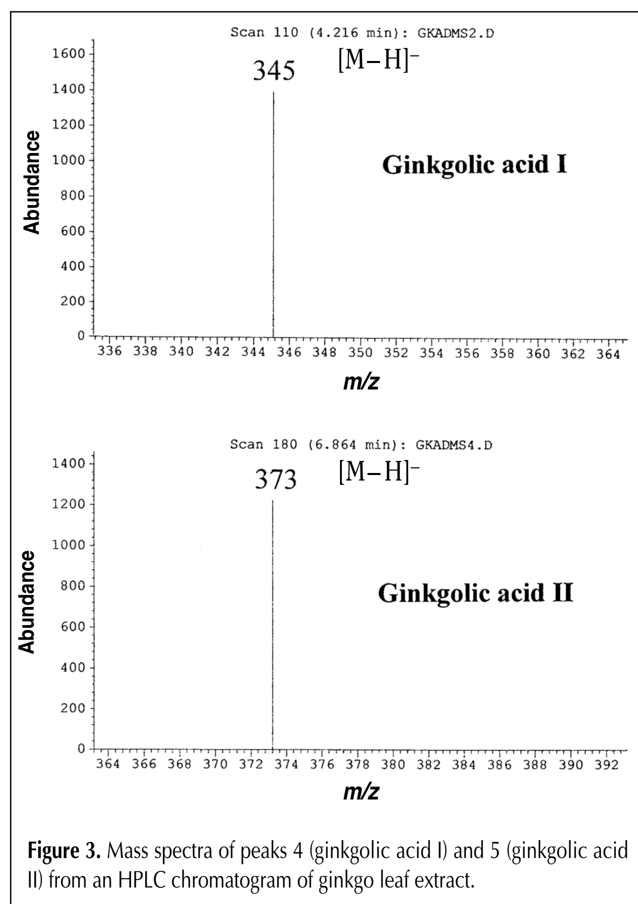

Figure 3. Mass spectra of peaks 4 (ginkgolic acid I) and 5 (ginkgolic acid II) from an HPLC chromatogram of ginkgo leaf extract.

The mass spectra of peaks 4 and 5 are shown in Figure 3. A singly deprotonated molecule $[M-H]^-$ at *m/z* 345 for ginkgolic acid I and *m/z* 373 for ginkgolic acid II were observed. Both peaks gave very high $[M-H]^-$ ion abundance. The UV spectra of peaks 4 and 5 are shown in Figure 4. This is a typical UV spectrum for ginkgolic acid.

This data is identical with the data of ginkgolic acid I and II published in the literature (4). Furthermore, the nuclear magnetic resonance (NMR) data of purified peaks 4 and 5 also confirmed our identification (4). Peaks 1–3 have not been identified, but could be assigned as ginkgolic acid derivatives based on their UV and characteristic MS data (Table I).

The effects of acetic acid concentration on MS detection sensitivity

The present results showed that increasing the concentration of acetic acid in the eluent increases HPLC peak sharpness in UV detection but decreases the MS sensitivity.

Figure 5 shows the relationship between TIC values and concentration of acetic acid in acetonitrile–water as eluent. Interestingly, ginkgolic acid I (peak 4) was more affected by the concentration of acetic acid than ginkgolic acid II (peak 5, Figure 2A–C). As a practical consideration, we have chosen 1% acetic acid in the eluent. It not only provided a good HPLC separation but also demonstrated abundant detectable pseudomolecular ion $[M-H]^-$.

Analysis of the ginkgo fruit extract

Figure 6 shows the simultaneous HPLC–UV and HPLC–reconstructed TIC chromatogram of ginkgo fruit extract. Ginkgo fruit contains three times as much ginkgolic acids as ginkgo leaves (5). Due to the lower concentration of our fruit sample (a 2 g/20 mL extract from fruits, compared to an extract from 200 g/20 mL leaves), only small peaks 4 and 5 (ginkgolic acid I and II) are shown in UV detection, yet these are strong peaks in TIC detection. Obviously, the mass detection sensitivity was higher than UV detection.

HPLC–MS has been widely used in phytochemical analysis of

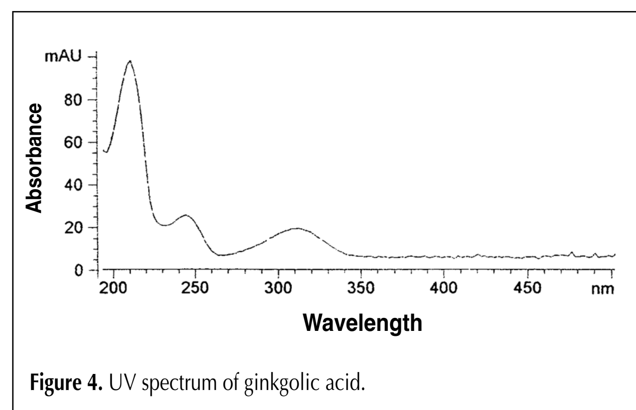

Figure 4. UV spectrum of ginkgolic acid.

herbal extracts. So far, the flavonoids and saponins have been successfully examined by this technique because of their easy ionization (13,14). However, relatively few phenolic acids have been studied.

Galletti et al. (15) reported that ferulic acid, coumaric acid, and vanillic acid can produce protonated molecules $[M + H]^+$ in thermospray interface and discharge ionization using loop injections. However, sensitivity was low. They postulated that negative-ion HPLC–thermospray-MS may yield increased sensitivity in comparison with the positive ion data.

Gioacchini et al. (16) analyzed the same phenolic acids using HPLC–ESI-MS in a Nova-pak phenyl column (MeOH–H_2O–HOAc as eluent, with 0.3mM Et_4NI at pH 5.7). Only their deprotonated molecules $[M - H]^-$ are generated with no adducts or other ions.

Hofte et al. (17) analyzed hop acids with HPLC–ESI-MS. The sensitivity is superior in the negative-ionization mode at pH 3 (Zorbax SB C_8 column, MeOH–H_2O, 90:10, v/v, with 1% HOAc as eluent). The mass spectra are dominated by a deprotonated molecule.

Our results provide another example to show that ESI negative-ionization mode is suitable for the analysis of phenolic acids. Further quantitative analysis is planned when very pure standard ginkgolic acids can be isolated.

Table I. Peak Assignments for the Analysis of Ginkgo Leaf Extract

Peak number	t_R* (min)	$[M - H]^-$ (m/z)	UV λ_{max} (nm)	Identification
1	2.3	361	245, 310	n.i.†
2	2.7	389	245, 310	n.i.†
3	3.4	369	n.d.‡	n.i.†
4	4.0	345	245, 310	ginkgolic acid I
5	5.4	373	245, 310	ginkgolic acid II

* t_R, retention time.
† n.i., not identified.
‡ n.d., not detected.

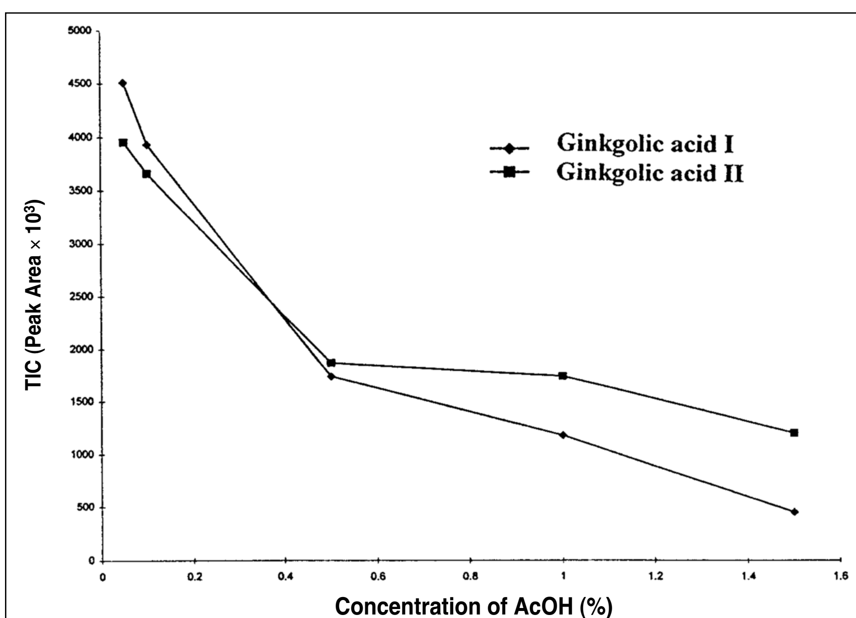

Figure 5. Effect of the concentrations of acetic acid (%) on TIC values (peak areas) in HPLC–ESI-MS analysis.

Conclusion

The present results showed that HPLC–ESI-MS in a negative-ion mode can be used for detecting minor ginkgolic acids in ginkgo leaves and fruits. MS sensitivity was greater than UV detection at 308 or 245 nm. The method is very specific for the analysis of ginkgolic acids, because the other components in ginkgo samples either were not extracted in the sample preparation or did not show any mass spectra in the current HPLC–MS condition.

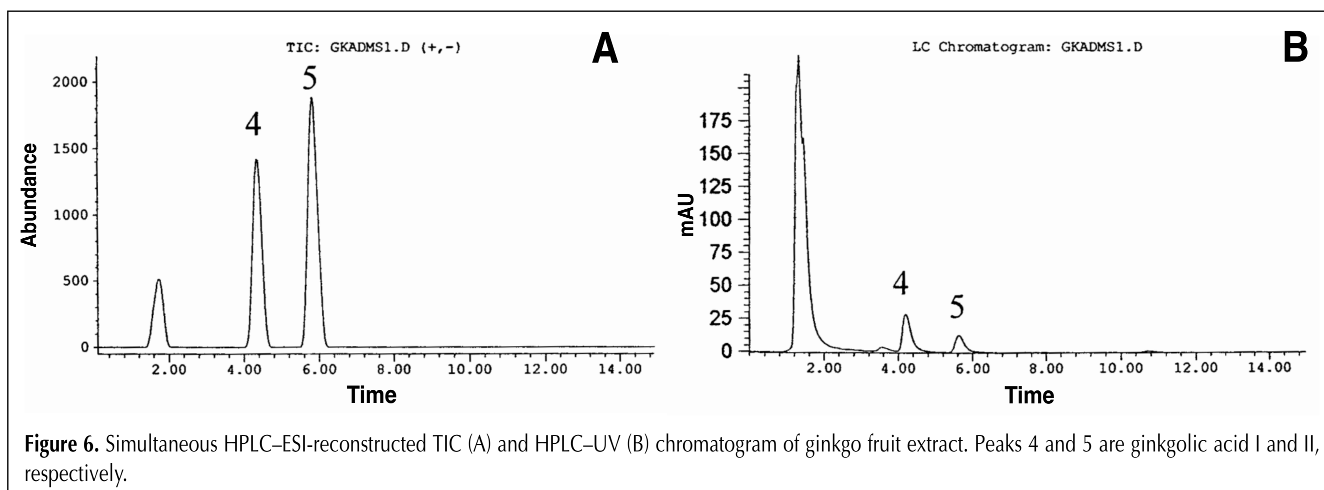

Figure 6. Simultaneous HPLC–ESI-reconstructed TIC (A) and HPLC–UV (B) chromatogram of ginkgo fruit extract. Peaks 4 and 5 are ginkgolic acid I and II, respectively.

Acknowledgments

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