

Detection of fortification of ginkgo products using nanoelectrospray ionization mass spectrometry†

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We report here a negative ionization nanoelectrospray ionization mass spectrometry (nanoESI-MS) technique that simultaneously detects active components, terpenes and intact flavonol glycosides, and toxic ginkgolic acids in ginkgo products. Unlike the conventional methods that hydrolyze flavonol glycosides to flavonoids for analysis, this technique directly detects intact flavonol glycosides, enabling differentiation of these natural glycosides from the synthetic flavonoids. Thus, it allows the detection of fortification of ginkgo products, alleviating a common problem encountered by the conventional methods. Analysis of 14 commercial ginkgo products using this technique demonstrates large variations and deviation from the well-accepted standardized ginkgo extract. Four products showed evidence of fortification with synthetic surrogates. Two products were found to have toxic ginkgolic acids that exceed the $5 \mu\text{g g}^{-1}$ limit by as much as 60000 fold. These results emphasize the importance of appropriate monitoring of ginkgo product quality.

Introduction

Simultaneous characterization of multiple classes of compounds in complex mixtures, such as herbal medicine, is an analytical challenge, because they often possess different chemical and physical properties, requiring tedious sample treatment processes and different analytical techniques. Electrospray mass spectrometry has shown promise for ionizing and detecting different compounds. However, the capability of electrospray MS for simultaneous and quantitative analysis of complex herbal mixtures has not been fully explored. The present study is to develop and demonstrate a negative ionization nanospray tandem quadrupole time-of-flight technique for quantitative fingerprinting of complex herbal products. A widely used herbal plant, *Ginkgo biloba*, is used to demonstrate the capability of this technique because of the complexity and difficulty involved in the determination of the wide range of compounds in ginkgo.^{1–7}

The major active components of *Ginkgo biloba* are various terpene trilactones (including ginkgolides and bilobalide) and flavonol glycosides (see ESI Fig. 1 for their structures†). A number of studies have shown that both classes of compounds of ginkgo extracts present at appropriate concentrations are required to achieve beneficial effects.^{1,3,7,8} The accepted composition of “standardized ginkgo extracts” is 6% of terpenes and 24% of flavonol glycosides.^{1,7} In addition to these active components, ginkgo plants also contain ginkgolic acids that are toxic allergens. It is recommended that the concentration of ginkgolic acids should be less than $5 \mu\text{g g}^{-1}$ in the extracts.^{1,9–11} Therefore,

proper quality control is required to ensure the appropriate composition of all active components and minimum amounts of toxic ginkgolic acids in the products.^{1,12–14}

Currently, no single technique is able to quantitatively determine various terpenes, flavonol glycosides, and ginkgolic acids in a single analysis. Therefore, the processes of quality control of ginkgo products are complex and time consuming. In addition, there is no quantitative technique available for direct analysis of intact flavonol glycosides. Conventionally, the flavonol glycosides are hydrolyzed with acid to flavonoids quercetin (Q), kaempferol (K), and isorhamnetin (I) (see ESI Fig. 2†), which are then separated by reverse phase liquid chromatography followed by absorbance or fluorescence detection.^{15,16} The contents of flavonol glycosides are inferred from the measurement of the three flavonoids (Q, K and I). As a result of the hydrolysis procedure, this method cannot differentiate between the fortified synthetic flavonoids and the intact flavonol glycosides that originate from the ginkgo plants. Differentiation between them is important because bioavailability, pharmacodynamics and pharmacokinetics of intact flavonol glycosides and the hydrolysis products (flavonoids) are different.^{16–19}

The objective of this study is to develop an analytical technique that can simultaneously detect all the three groups of components in ginkgo products and can differentiate the intact flavonol glycosides from the fortified flavonoids. Here we report the development of a negative ionization nanoelectrospray mass spectrometry (nanoESI-MS) technique, and demonstrate its application to rapid analysis of ginkgo products. This method is useful for quality control and for detection of product adulteration.

† Electronic supplementary information (ESI) available: Supplementary Figs. 1–6. See <http://www.rsc.org/suppdata/an/b4/b415501g>/
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Experimental

Reagents and materials

Standards of ginkgolides A, B, C, and bilobalide and ginkgolic acids I and II were obtained from LKT Laboratories (Montreal, Quebec, Canada). Quercetin dehydrate (Q), kaempferol (K), isorhamnetin (I) and rutin (RGQ) were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). Three standard ginkgo leaf extracts (g1, g2 and g3) were received as gifts from Kinetana Group Inc. (Edmonton, Canada), which were provided by Ningbo Traditional Chinese Factory (China), China Nutraceutical Ltd. (China). Another ginkgo extract (GK1000) was provided by Herb-Pharma (Oakville, Oregon, USA). Fourteen commercial ginkgo products were obtained from local food stores, pharmacies, and natural product specialty stores (Edmonton, AB, Canada). Barbaloin (Sigma-Aldrich, St. Louis, MO, USA) was used as the internal standard for quantification of ginkgolic acids I and II in commercial samples. HPLC-grade ammonium acetate, methanol and water (Fisher Scientific, Fair Lawn, New Jersey, USA) were used throughout the experiments.

Samples

Stock standard solutions (1 mg mL^{-1}) of ginkgolides A, B, C, bilobalide, ginkgolic acids I and II, quercetin dehydrate (Q), kaempferol (K), and isorhamnetin (I) and ginkgo extracts were separately prepared by dissolving appropriate quantities of these standards in methanol. Working standard solutions were diluted to $10\text{--}100 \text{ }\mu\text{g mL}^{-1}$ in (50 : 50) methanol : water and 5 mM ammonium acetate.

Extraction of ginkgo products was optimized varying the percentage of methanol (20% to 100%) in water (see ESI Fig. 3†). With increasing the percentage of methanol from 20% to 100%, the amount of ginkgolic acids increased, whereas other components did not change significantly. Therefore, all the commercial samples (1 mg) were extracted using 1 mL of 100% methanol then diluted with aqueous ammonium acetate buffer to be $100 \text{ }\mu\text{g mL}^{-1}$ in 50% MeOH and 5 mM ammonium acetate for the subsequent nanoESI-MS analysis.

Instrumentation

A QSTAR Pulsar I mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) was equipped with a nano-electrospray source (Protana, Denmark). The conductive capillary tips for nano-electrospray were purchased from Proxeon Biosystem (Denmark). The system was operated in both positive and negative mode, as specified in the text, with the vacuum of $\sim 10^{-7}$ Torr, and the curtain gas at a flow rate of 1.31 L min^{-1} .

In the single MS scan mode, the mass measurements ($200\text{--}900 \text{ amu}$) were performed using the time-of-flight (TOF) section of the instrument with a resolution of 10,000 (FWHM), while Q1 and Q2 were operating in RF-only mode. To obtain quality mass spectra various instrumental parameters (such as declustering potential, focusing potential, nanospray voltage, and positive or negative ion scan), in addition to the solution composition (solvent and buffer), were systematically

optimized. The optimized conditions for acquiring mass spectra of ginkgo extracts included a nanospray voltage of -900 V , a first declustering potential (DP1) of -5 V , a second declustering potential (DP2) of -10 V , and a focusing potential (FP) of -5 V .

In order to obtain reproducible mass spectrum of standardized ginkgo extracts that can be used for fingerprinting, fragmentation of analytes is minimized or eliminated in order to acquire a mass spectrum that is closely representing what is in solution. The ionization and fragmentation of the commercially available standards of biloba, ginkgolides A, B, C, ginkgolic acids I and II, quercetin dehydrate, kaempferol, and rutin (RGQ) was individually studied under different conditions. After optimizing the instrument parameters with individual standards, we then optimized conditions for ginkgo extracts (see ESI Fig. 4†).

In the MS/MS mode, the parent ion was selected by Q1 with a mass window of 1 Da at low resolution, and fragmented in Q2 by collision-induced dissociation with collision energy of -10 to -40 eV and collision gas setting of 5. The resulting product ions were analyzed by the TOF analyzer with a four-anode detector. Analyst QS software (Applied Biosystems, Foster City, CA, USA) was used for the spectrum acquisition and data analysis. Igor Pro software (WaveMetrics, Lake Oswego, OR, USA) was used to plot the spectra.

Quantification of ginkgolic acids

A calibration curve was obtained for quantification of ginkgolic acids in the commercial samples using barbaloin as the internal standard. A linear relationship between the ratio of the intensity of standard ginkgolic acid I or II ($5\text{--}15 \text{ }\mu\text{g mL}^{-1}$) to that of the internal standard ($0.6 \text{ }\mu\text{g mL}^{-1}$) was obtained with r^2 0.9870. This was used to quantify the concentrations of ginkgolic acids I and II in the commercial samples, when the samples ($100 \text{ }\mu\text{g mL}^{-1}$) were spiked with a fixed concentration of barbaloin ($0.6 \text{ }\mu\text{g mL}^{-1}$). No standard is available for ginkgolic acid III, so it was estimated using ginkgolic acid I as the standard.

Results

Our primary focus is to develop a method that is able to determine the intact flavonol glycosides, terpenes, and ginkgolic acids in a single analysis. We chose nano-electrospray mass spectrometry (nanoESI-MS) as the platform to develop a technique that can alleviate the two main problems, the need for hydrolysis of flavonol glycosides to flavonoids: quercetin, kaempferol, and isorhamnetin, and the lack of chromospheres in terpenes. We initially examined the suitability of both positive and negative ionization techniques for the analysis of the three classes of ginkgo compounds: terpenes (biloba and ginkgolides A, B, C), intact flavonol glycosides, and ginkgolic acids. Although positive ionization can simultaneously detect the three classes of components in ginkgo, the positive ion spectrum is complicated by the formation of multiple adduct-ions of each compound, for example, $(\text{M}+\text{H})^+$, $(\text{M}+\text{Na})^+$, $(\text{M}+\text{K})^+$, and $(\text{M}+\text{NH}_4)^+$. In comparison, the negative ionization produces a typical ion of

(M-H)⁻ for each of the target compounds under the optimized conditions.

Fig. 1 shows a typical negative ion mass spectrum of a standardized ginkgo leaf extract using nanoESI-MS, demonstrating that ginkgo terpene trilactones and intact flavonol glycosides are simultaneously detected. The standardized ginkgo leaf extract contains mainly these two classes of active compounds (Fig. 1A). To confirm that the nanoESI-MS negative ionization technique can also determine ginkgolic acids, standards of ginkgolic acids I and II (10 µg mL⁻¹) were spiked into the same ginkgo extract. Fig. 1B shows all three classes of important ginkgo components, terpenes, intact flavonol glycosides, and ginkgolic acids, can be determined simultaneously with a single analysis.

In addition to the simultaneous determination of the three groups of target compounds, another major advantage of the technique is the ability to differentiate the natural intact flavonol glycosides from the fortified flavonoids. Fig. 2 shows three mass spectra from the analysis of (A) a standard ginkgo leaf extract (50 µg mL⁻¹), (B) the same extract fortified with 5 µg mL⁻¹ of (Q), and (C) a commercial ginkgo product that is evidently fortified with synthetic flavonoids (K, Q, and I). RGK, RGQ, RGI, RGMMeM, R₂GK, and R₂GQ (see ESI Fig. 1 for their structures[†]) are the primary intact flavonol glycosides detected in the standardized ginkgo extract

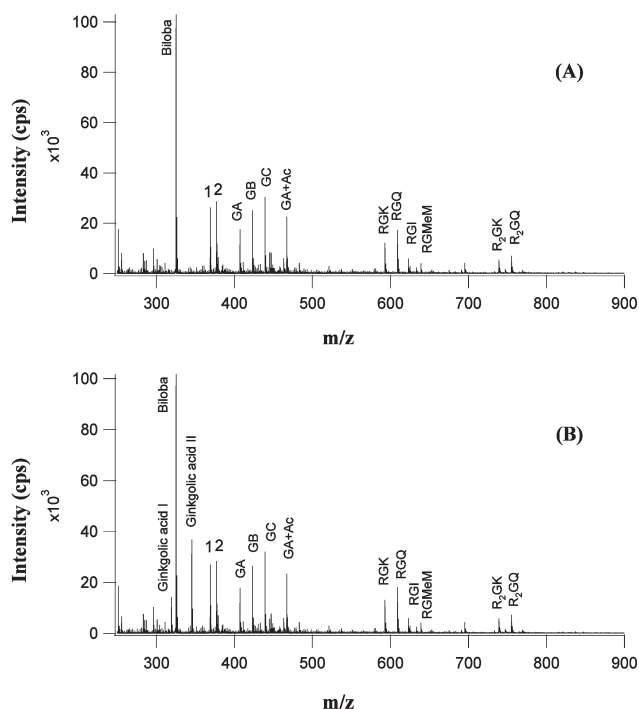


Fig. 1 Typical negative ion mass spectra of (A) standard ginkgo extract (50 µg mL⁻¹), and (B) the same standard ginkgo extract spiked with 10 µg mL⁻¹ standards of ginkgolic acids I and II. GA, GB, and GC are ginkgolides A, B, and C, respectively. GA+Ac corresponds to an acetylated form of GA. RGK, RGQ, RGI, RGMMeM, R₂GK, R₂GQ are intact flavonol glycosides. The structures and molecular weights of the ginkgolides and flavonol glycosides are shown in the ESI Fig. 1. † Peak 1 is an unidentified peak. Peak 2 represents GA-2CH₃ (*m/z* 377.0904, mass accuracy 0.18 ppm). Tandem mass spectrometry (MS/MS) of GA confirmed the peak 2.

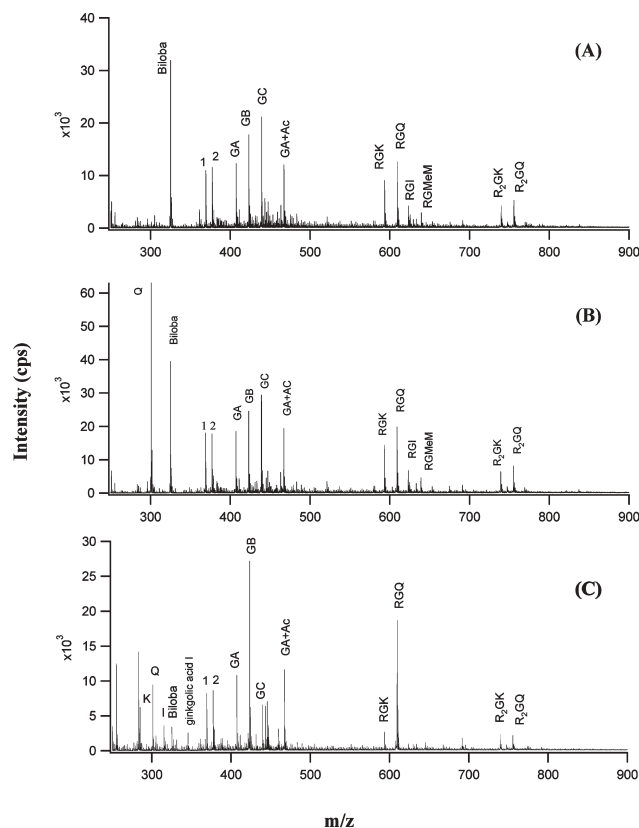


Fig. 2 Mass spectra of (A) a standard ginkgo extract (50 µg mL⁻¹), (B) the same extract spiked with 5 µg mL⁻¹ of standard quercetin, and (C) a commercial ginkgo product showing differentiation between intact flavonol glycosides and synthetic flavonoids (Q, K, and I). The peak identities are the same as in Fig. 1.

(Fig. 2A). Their surrogate products of fortification, such as quercetin (Q), can be differentiated from the authentic flavonol glycosides (Fig. 2B). These flavonoids (K, Q, and I) are not detectable in the standardized ginkgo extract (Fig. 2A). However, they are substantial (approximately 2 to 4% of Q and K in the product) in a commercial product that is most likely fortified with these flavonoids (Fig. 2C). This fortification (product adulteration) would not be recognized by the conventional methods that involve hydrolysis of intact flavonol glycosides followed by the analysis of flavonoids as the hydrolysis products. The nanoESI-MS technique described here is able to clearly distinguish the intact flavonol glycosides that are present in the ginkgo from the flavonoids that are readily synthesized. Therefore, this technique is useful for monitoring ginkgo product adulteration.

We further demonstrate the application of this technique for rapid determination of both active and toxic components in various ginkgo products, which is essential to the screening of product quality. We reveal that the market products have large variations in the composition and concentrations of active and toxic components of ginkgo products. Out of 14 ginkgo products available in local stores, only three have the composition and contents of the major active components closely resemble those in the standard extracts (Fig. 3A). Five products do not meet the requirement of 6% terpenes and 24%

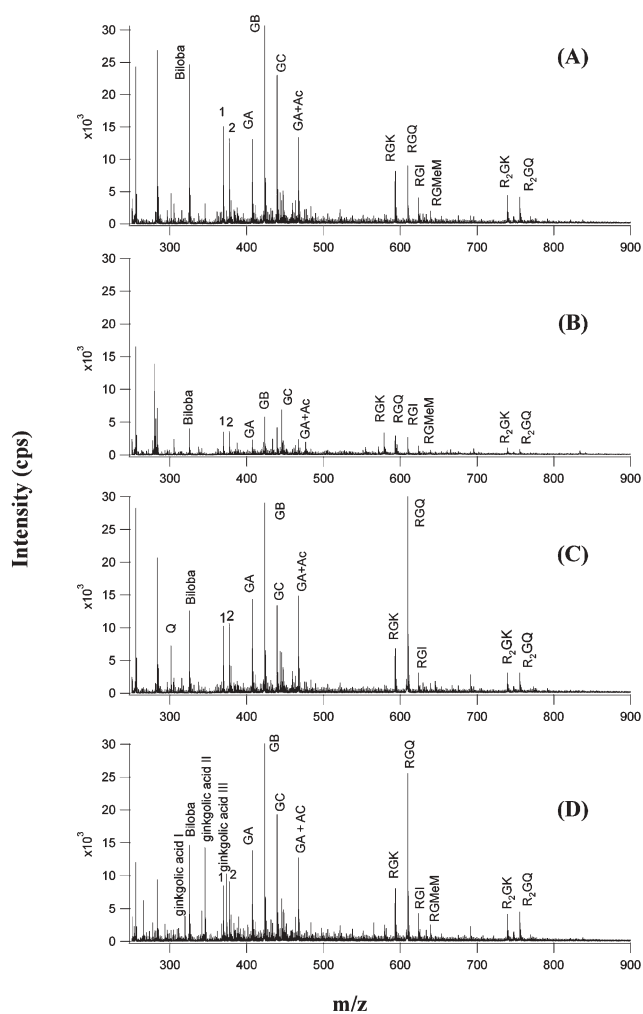


Fig. 3 Representative mass spectra of selected commercial ginkgo products ($100 \mu\text{g mL}^{-1}$), showing large variations in the composition and concentrations of active and toxic components and fortification. Sample (A) met the requirement of the standard ginkgo extract. Sample (B) contains low levels of active components. Sample (C) showed elevated levels of Q and RGQ, evidence of possible fortification. Sample (D) contains unacceptable high levels of ginkgolic acids (approximately 10% each). The peak identities are the same as in Fig. 1.

flavonol glycosides (Fig. 3B): two products contain very low levels of active ginkgo compounds ($<1/4$ of the standard extract), two samples contain less than 50% of the active components, and one sample contains some unknown components that were detected together with low levels of active components. Similar mass spectral profiles of these products suggest that there is no product fortification although the contents of active compounds vary in these products. Four products show potential fortification with synthetic flavonoids (Q and/or K, I) and rutin (RGQ), such as those shown in Fig. 2C and Fig. 3C. The product shown in Fig. 3C contains unusually high levels of Q and RGQ. The ratio of RGQ/RGK in this product is 4.7, more than four times higher than that in standard extracts (Fig. 1A) and most other products (Fig. 3A). The unusually high levels of Q and RGQ in this product suggest the potential addition of Q and

RGQ. Pure compounds of Q and RGQ are commercially available.

Toxic ginkgolic acids are detected in two of the 14 products. Fig. 3D shows an example of a commercial ginkgo product obtained from a local food store that contains ginkgolic acids I, II, and III at a concentration of 9–11 μg per 100 μg product (approximately 10% each in the product), respectively. The ginkgolic acids were quantified using the external calibration curves with barbaloin as the internal standard, as described in the Experimental section. Although the active components are present at reasonable levels with the exception of an unusually high level of RGQ compared to the standard extract (Fig. 1A), the total concentration of ginkgolic acids in the product is about 30%, which is 60000 times over the $5 \mu\text{g g}^{-1}$ (0.0005% in the extract) limit. Both MS/MS spectra and accurate mass confirmed the identity of ginkgolic acids. In addition, we analyzed three samples from three different lots produced by the same manufacturer. All three samples showed high concentrations of ginkgolic acids (3–11 μg per 100 μg each in the product). This suggests that the ginkgolic acids were not properly removed from the ginkgo extract used to produce this product. The detection of ginkgolic acids is important because of concerns over their potential allergenic effects.

Discussion

The well-recognized composition of ginkgo products is based on the pioneering work of Dr Schwabe, who systematically standardized and patented the ginkgo extract, named as “EGb 761”.^{1,7,20,21} After over three decades of a series of optimization and pharmacological and toxicological investigation, ginkgo extract consisting of 6% terpenes, 24% flavonol glycoside, and $<5 \mu\text{g g}^{-1}$ ginkgolic acids has been accepted as the “standardized ginkgo extract”.^{20,21} Clinical trials using ginkgo products of this standardized extract composition have shown beneficial effect for treating various diseases, such as cerebrovascular and peripheral circulatory disorders, asthma, coughs, bladder inflammation, blenorrhagia, and alcohol abuse.^{1–7} Some recent studies suggested that the standardized ginkgo extracts may delay the onset of neurodegenerative disorders, such as Alzheimer’s, Parkinson’s, and Huntington’s disease, and may be used as potential preventive agent for cancer because active components in ginkgo act as radical scavengers and provide antioxidant activity.^{3,4,22–26} However, many commercial ginkgo products do not meet this standard, as demonstrated from the analysis of 14 commercial products. It is alarming that 2 of the 14 products contain toxic ginkgolic acids at a level of as much as 60000 fold higher than the recommended limit of $5 \mu\text{g g}^{-1}$. Previous studies have also shown large variations in ginkgo products manufactured in different countries.^{27–30} Therefore, it is very important to monitor product quality in order to protect public health.

Two main analytical challenges are simultaneous determination of intact flavonol glycosides, terpenes and ginkgolic acids, and the ability to detect product fortification. The negative ionization nanoESI-MS technique enables rapid analysis of the three classes of target compounds without the need for complicated sample pre-treatment processes. It takes less than 120 s to obtain a quality mass spectrum with accurate mass

and MS/MS information for identification. It also offers the day-to-day reproducibility of a MS spectrum as demonstrated by the analysis of four of the standardized ginkgo extracts (ESI Fig. 5†) and repeated analysis of a standard ginkgo extract over 18 days (ESI Fig. 6†). Thus this technique is useful for fingerprinting and quality control of complicated mixtures such as ginkgo products.

Product fortification is a common problem associated with natural products. Detection of fortification presents an analytical challenge. The conventional methods for ginkgo analysis involve hydrolysis followed by the analysis of flavonoids. Because of the hydrolysis procedure, the previous methods are unable to distinguish the fortified flavonoids from the natural flavonol glycosides. The method described here can easily detect the intact flavonol glycosides without complicated acid digestion and extraction processes, and is capable of differentiation between synthetic flavonoids (K, Q, and I) and intact flavonol glycosides (mono-, di-, tri- glycoside). This further provides a means of detecting potential adulteration with synthetic flavonoids, alleviating the problems encountered by conventional methods.

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