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Development of Nanoelectrospray Mass Spectrometry Technique for Simultaneous Detection of Active and Toxic Components in Ginkgo Products

by

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Abstract

A negative ionization nanoelectrospray ionization mass spectrometry technique was developed for the simultaneous detection of both active and toxic components in ginkgo products. This technique dramatically simplified the analytical procedures for ginkgo analysis. Unlike the conventional methods that hydrolyze flavonol glycosides to flavonoids for analysis, this technique detects intact flavonol glycosides directly, enabling these natural glycosides to be differentiated from the synthetic flavonoids. Fingerprinting of 14 commercial ginkgo products using this technique revealed large variations between brands. Three products showed evidence of fortification with synthetic surrogates. This method was also used to quantify these compounds in the 14 different commercial ginkgo extracts. Two products were found to contain toxic ginkgolic acids that exceeded the 5 μg/g limit by as much as 50000–90000-fold. These results emphasize the importance of appropriate monitoring of ginkgo product quality. This technique is potentially useful for analysis of other complex mixtures.

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List of Abbreviations

m/z	mass-to-charge ratio
APCI-MS	atmospheric pressure chemical ionization mass spectrometry
BB	bilobalide
CE	collision energy
CI	chemical ionization
DP	declustering potential
EI	electron ionization
ESI	electrospray ionization
EGb 761	standardized ginkgo extract
FAB	fast-atom bombardment
FP	focusing potential
FWHM	full width at half maximum
GA	ginkgolide A
GAs1	ginkgolic acids I
GAs2	ginkgolic acids II
GAs3	ginkgolic acids III
GB	ginkgolide B
GC	ginkgolide C
GI	3-O-(β-D-glucosyl) isorhamnetin
GK	3-O-(β-D-glucosyl) kaempferol
GQ	3-O-(β-D-glucosyl) quercetin

HPLC-UV	high performance liquid chromatography-ultraviolet detection
HPLC-RI	HPLC-refractive index detection
I	isorhamnetin
K	kaempferol
MS/MS	tandem mass spectrometry
nanoESI	nano electrospray ionization
NMR	nuclear magnetic resonance spectroscopy
PAF	platelet activating factor
Q	quercetin
QTOF	quadrupole time-of-flight
RK	3-O-(α-L-rhamnosyl) kaempferol
RI	3-O-(β-D-rhamnosyl) quercetin
RGK	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) kaempferol
RGQ	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) quercetin
RGI	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) isorhamnetin
RGMeM	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl)-3'-methylmyricetin
R_2GK	3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) kaempferol
R_2GQ	3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) quercetin
R_2GI	3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) isorhamnetin
SPE	C_{18} solid phase extraction column
THF	tetrahydrofuran

Chapter 1

Literature Review and Objectives

1.1 *Ginkgo biloba*

Ginkgo biloba is one of the world's oldest trees. It was reported that the ginkgo tree survived over millions of years as the only member of a whole class of plants.[1] *Ginkgo biloba* has a long history as a herbal medicine in traditional Chinese medicine; however, extracts of *Ginkgo biloba* leaves for medical use in western countries were only first developed in 1965 by Dr. Willmar Schwabe in Germany. Later, the extraction procedure was standardized. The purified and concentrated form of the extract was patented as "EGb 761" in 1971.[1,2] The EGb 761 is referred to as the standardized ginkgo extract.

The standardized ginkgo extract EGb 761 is produced by extraction of ginkgo leaves using a patented manufacturing process.[1] The process involves leaf extraction, lipid removal, active ingredient enrichment, removal of polyphenolics, liquid-liquid extraction, precipitation, drying, and milling-mixing. The processed extract consists of a number of classes of compounds, including terpene trilactones (i.e., ginkgolides A, B, C, J, and bilobalide), flavonol glycosides, biflavones, proanthocyanidins, alkylphenols, simple phenolic acids, 6-hydroxykynurenic acid, 4-*O*-methylpyridoxine, polyprenols, and heavy metals. The composition of the standardized extract of *Ginkgo biloba* leaves is summarized in Table 1-1.

Table 1-1. Constituents of the standardized ginkgo extract[21]

Compound	%	Compound	%
Flavonol glycosides	24.0	High molecular mass compounds	4.0
Terpene trilactones	6.0	Inorganic constituents	5.0
Proanthocyanidins	7.0	Water, solvent	3.0
Catechins	2.0	Alkylphenols	$\leq$ 5 ppm

The major active components in the *Ginkgo biloba* leaf extract are terpene trilactones and flavonol glycosides. The extract may also contain some toxic compounds such as ginkgolic acids, ginkgols, and cardols, which should be removed using patented preparation processes. An example of the specifications of the contents of the active and potentially toxic components in the standardized ginkgo extract is presented in Table 1-2. The concentrations of the active components, terpenes and flavonol glycosides, should be $\geq$ 6% and $\geq$ 24%, respectively, whereas those of the toxic components, ginkgolic acids, heavy metals, and arsenic, are recommended be limited to concentrations under 5, 20, and 2 ppm, respectively.

Table 1-2. Some specifications for a standardized ginkgo extract[21]

Description	Brown powder with characteristic smell
Identity	Green-brown colour after adding $FeCl_3$ to a 0.1% solution (g/v) in alcohol-water (1:1)
Heavy metals	$\leq$ 20ppm
Arsenic	$\leq$ 2ppm
Ginkgolic acids	$\leq$ 5ppm
Loss on drying	$\leq$ 5% (80°C)
Total flavonoid content	$\geq$ 24% (HPLC-UV)
Total terpene trilactone content	$\geq$ 6% (HPLC-RI)

The standardized extract of ginkgo leaves, EGb 761, has been used for many years to increase peripheral and cerebral blood flow as well as for treatment of dementia.[3] Clinical

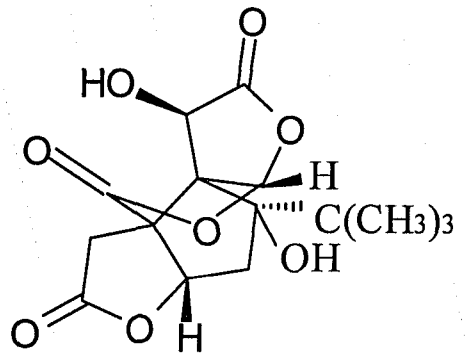
trials have demonstrated that the EGb 761 is effective in mild-to-moderate dementia of Alzheimer's disease patients.[4,5,6] Pharmacological studies have shown that the standardized EGb 761 improves cerebral blood flow in models of focal ischemia[4,7,8] and has a neuroprotective effect during ischemia.[9] Recent studies have suggested that the EGb 761 may have anticancer properties.[10-14] It has been clearly demonstrated that the pharmacological benefits of the EGb 761 result from the combined effects of the radical scavenging properties of flavonoid glycosides and the platelet activating factor (PAF) antagonism of ginkgolides;[15] however, a single compound extracted from ginkgo leaves does not provide the same benefits. Animal studies suggest that the terpenoid and flavonoid compounds of ginkgo extracts provide complementary actions.[16] Therefore, in order to achieve the beneficial effects, the ginkgo extracts require proper quality control to ensure effective compositions of all active components and minimum amounts of potentially harmful components such as ginkgolic acids in the products. Quality control is an essential part of research involving safety, efficacy, and therapeutic reproducibility.

1.2 Major components in ginkgo extracts and their analysis

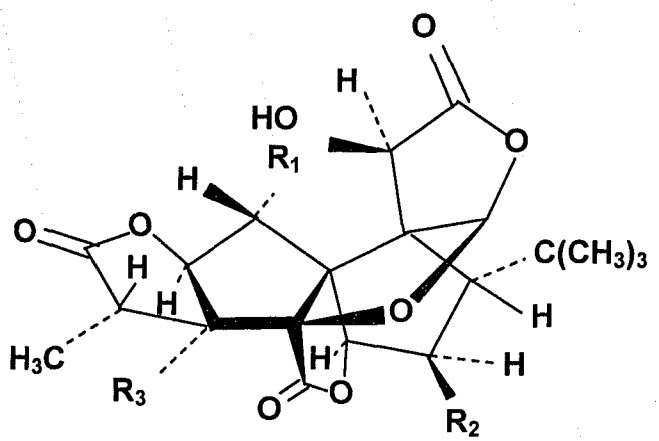
1.2.1 Terpenes

Terpenes are composed of four or six 5-membered rings, three lactones, and a tertiary -butyl group, as shown in Figure 1-1. In 1967, the structures of ginkgolides were first identified by Maruyama et al. using nuclear magnetic resonance spectroscopy (NMR).[17] In 1971, bilobalide was also identified by Nakanishi et al. using an NMR technique.[18] These compounds possess unique physical and chemical properties (three lactones with a tertiary-butyl group) and pharmacological activities. Recent studies indicate that

ginkgolides are involved in the PAF antagonist activity[19] and that bilobalide has neuroprotective properties.[20]



Bilobalide



Ginkgolides

R1:	R2:	R3:	Ginkgolide
H	H	OH	A
OH	H	OH	B
OH	OH	OH	C
H	OH	OH	J
OH	OH	H	M

Figure 1-1. Chemical structures of some terpenes identified in ginkgo extracts

The analysis of terpenes is difficult because these compounds do not have strong chromophores and have poor UV absorption. In addition, they are often present at relatively low concentrations in the extracts. Extraction and sample pre-treatment are also required to avoid the interference of impurities in the analysis of terpenes. One method used methanol-water to extract the active components from ginkgo leaves, a polyamide column to remove flavonoids from the terpenes, and then a C_{18} solid phase extraction (SPE) column to remove other impurities prior to high performance liquid chromatograph (HPLC) analysis.[21] Another method used acetone-water to extract the active components from ginkgo leaves, ethyl acetate with phosphate buffer to extract the terpenes, and a kieselguhr column to purify the sample prior to reverse phase HPLC analysis.

After sample clean-up, different analytical methods can be used to detect terpenes in ginkgo. Reverse phase HPLC (RP-HPLC) with a C_{18} column and UV detection is a widely-used method. The mobile phase usually consists of MeOH-H_2O or MeOH-H_2O-THF (tetrahydrofuran). Several HPLC with UV detection methods[22-24] have been developed for quantification of terpenes. Other detection methods, such as refractive index detection (RI)[25-27] and evaporative light scattering detection,[28-31] have also been used in HPLC analysis. These detection methods have better sensitivity than UV for these coumpunds. The HPLC-RI method has been widely used for analysis of terpenes; however, the HPLC methods require extensive sample extraction and purification procedures, making them time-consuming. These methods are not practical for routine analysis of a large number of samples.

Other methods, such as thin layer chromatography[32-34] and gas chromatography (GC/MS), have been developed and used for analysis of terpenes. These methods also

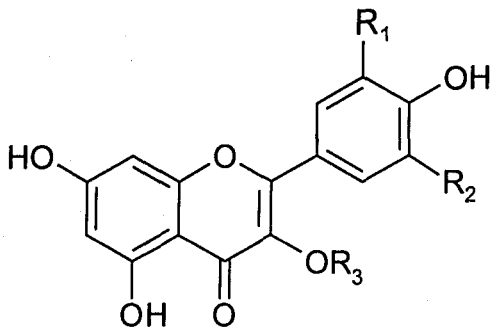
require complicated sample extraction, clean-up, and derivatization procedures. These processes are not only time-consuming, but also introduce chemical background.[35-37] NMR[38] is not often used because it is not best suited for quantification and is rarely available in routine quality control laboratories.

Mass spectrometry techniques can provide structural information for identification and are capable of sensitive detection and quantification. They are most suitable for analysis of compounds that do not have a strong chromophore, such as terpenes. They can provide both qualitative and quantitative information, which is important for quality control. Advances in interfacing HPLC with mass spectrometry detection have made it the most attractive tool for analysis of complex mixtures such as ginkgo extracts. A number of HPLC-MS methods have been developed for analysis of terpenes. These techniques include HPLC separation with thermospray-MS,[39] APCI-MS (atmospheric pressure chemical ionization),[40,41] and ESI-MS (electrospray ionization).[42,43] Thermospray-MS detection is based on the sample solution being heated by electrical resistive heating at the end of the capillary tube, vaporized, and sprayed, forming ions that are directed towards the mass analyzer. Thermospray ionization causes thermal destabilization of terpenes, particularly bilobalide, resulting in poor reproducibility. ESI has been demonstrated to be the most useful technique for coupling with HPLC. ESI is an ionization technique under atmospheric pressure, and it will be described in more detail in Section 1.3.

1.2.2 Flavonol glycosides

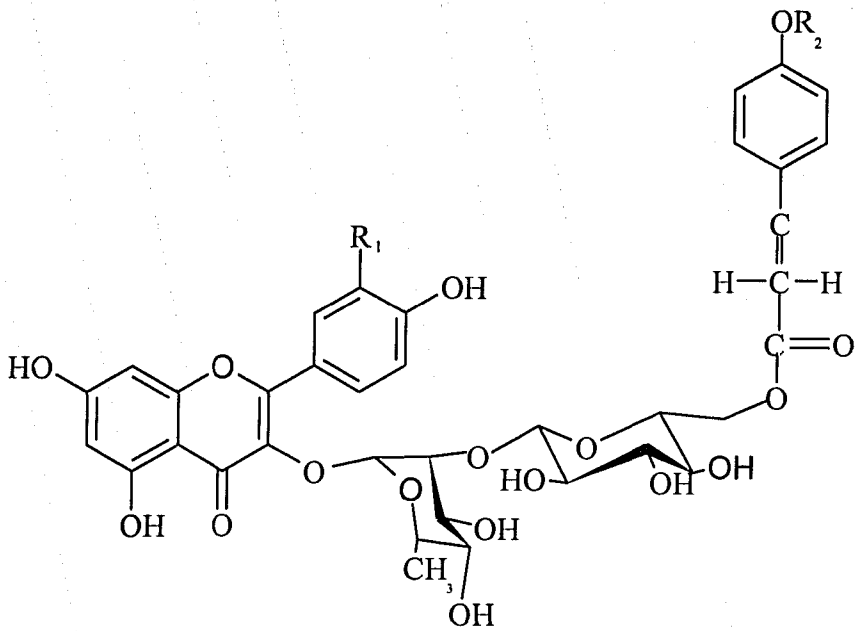
Flavonol glycosides include flavonol aglycones, flavonol mono-, di-, and triglycosides, and acylated flavonol glycosides. Their structures are shown in Figure 1-

2A and 1-2B. The structures are based on 2-phenylbenzopyrone with different hydroxylation and linked with different sugar units. Under acidic conditions, these compounds are hydrolyzed to aglycones. The common aglycones in ginkgo flavonol glycosides have been identified as quercetin (Q), kaempferol (K), and isorhamnetin (I). In sugar unit parts, glucose and rhamnose are the major sugar moieties. In acylated flavonol glycosides, the hydroxyl group of the glucose unit is esterified with coumaric acid.



R1:	R2:	R3:	**flavonol aglycones:**
H	H	H	kaempferol
OH	H	H	quercetin
OCH_3	H	H	isorhamnetin
			flavonol monoglycosides:
H	OH	glu	3-O-(β-D-glucosyl) kaempferol
OH	H	glu	3-O-(β-D-glucosyl) isorhamnetin
H	OH	rha	3-O-(α-L-rhamnosyl) kaempferol
OH	H	rha	3-O-(β-D-rhamnosyl) quercetin
			flavonol diglycosides:
H	H	glc-rha	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) kaempferol
OH	H	glc-rha	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) quercetin
OCH_3	H	glc-rha	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) isorhamnetin
OCH_3	OH	glc-rha	3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl)-3'-methylmyricetin
			flavonol triglycosides:
H	H	glc-$(rha)_2$	3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) kaempferol
OH	H	glc-$(rha)_2$	3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) quercetin
OCH_3	H	glc-$(rha)_2$	3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) isorhamnetin

Figure 1-2A. Chemical structures of flavonol aglycones and mono-, di-, and triglycosides



R_1: H or OH R_2: H or glucosyl

Figure 1-2B. Chemical structures of acylated flavonol glycosides

Flavonol glycosides have different chemical and physical properties than terpenes. These glycosides have strong chromophores and thus are easily detected using UV or fluorescence detection. Analysis of flavonol glycosides is commonly conducted using RP-HPLC-UV methods.[44-49] Hasler and his colleagues developed a HPLC-UV method to detect flavonol glycosides. They used 80% ethanol to extract ginkgo leaves and a C_{18} SPE cartridge to purify the samples.[45] Methanol-tetrahydrofuran (THF)-orthophosphoric acid was used as the HPLC mobile phase with the C_{18} column. This method used a ternary gradient program and separated 33 flavonoids. This method cannot provide quantitative information of flavonoids due to a lack of standards, but it could be used to fingerprint flavonoids based on an increase of the aglycones and a decrease of the glycosides that indicate the degradation process in the ginkgo extracts. Hasler and his

colleagues also developed an indirect HPLC-UV method to quantify the flavonol glycosides after hydrolysis.[45] The ginkgo leaves were first extracted using methanol, then hydrochloric acid was added, and the samples were left to hydrolyze for one hour. The hydrolyzed aglycones (K, Q, I) were purified using a C_{18} SPE cartridge and separated using C_{18} HPLC column separation with a mobile phase of methanol and orthophosphoric acid. The peak areas of K, Q, and I were used for quantification.

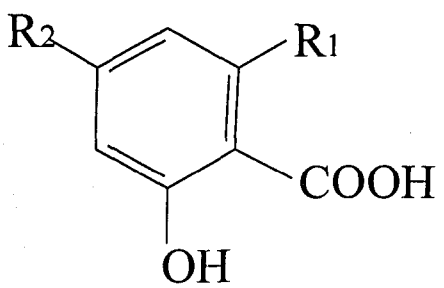
To date, quantitative analysis of flavonol glycosides is usually obtained based on the indirect measurement of three aglycones, quercetin, kaempferol, and isorhamnetin, after the ginkgo extract is hydrolyzed by acid to form the aglycones. The hydrolysis products are subsequently separated by HPLC and detected by UV. The peak areas of the three major aglycones are integrated and calibrated against their standards. The calculation of intact flavonol glycosides was based on the conversion factors of aglycone, which were related to the molecular weight of intact flavonol glycosides (conversion factors: isorhamnetin 2.39, kaempferol 2.64, quercetin 2.51, average 2.51). The quantification of aglycones in ginkgo extracts has been used for quality control of flavonol glycosides in the extracts.[50]

The indirect measurement of aglycones for the analysis of flavonol glycosides cannot differentiate fortified synthetic flavonoids from the intact flavonol glycosides originating in the ginkgo leaves; therefore, this approach cannot detect the adulteration of ginkgo products with synthetic flavonoids such as quercetin, kaempferol, and rutin.[51] Potential adulteration has been detected based on HPLC peak profiles.[51] It is important to develop techniques that can directly identify and quantify the intact flavonol glycosides for quality control.

Other methods have also been reported. For example, Hu et al.[52] reported using the Fourier transform near-infrared spectroscopy to determine the total flavones and total lactones using partial least square mode; however, this method could not provide information about individual flavonol glycosides.

1.2.3 Ginkgolic acids

The structures of ginkgolic acids are shown in Figure 1-3. These compounds consist of alkyl salicylic acids substituted with a saturated or unsaturated alkyl side-chain from 13 to 17 carbons in length (unsaturated alkyl side-chain with one or two double bonds, represented by Cx:y; x represents carbon atom number C_{13}, C_{15} or C_{17}; y represents the number of double bonds). In 1928, Kawamura[53] extracted ginkgo fruit, and obtained and identified one ginkgolic acid ($C_{15:1}$: salicylic acids with alkyl side-chain $C_{15}H_{29}$ and one double bond) using NMR and IR spectroscopy techniques. In 1968, Gellerman and Schlenk[54] extracted ginkgo leaves and obtained several ginkgolic acids. The structures were identified as $C_{13:0}$, $C_{15:0}$, $C_{15:1}$, and $C_{17:1}$ ginkgolic acids using mass and NMR spectra. Some studies reported that these compounds have allergenic,[55] cytotoxic,[56] and neurotoxic properties.[57]



R2: H or OH **R1: Alkyl Chain C13-17**

Figure 1-3. Chemical structures of ginkgolic acids

Figure 1-3. Chemical structures of ginkgolic acids

R_1: $C_{13}H_{27}$ ($C_{13:0}$); $C_{15}H_{31}$ ($C_{15:0}$); $C_{15}H_{29}$ ($C_{15:1}$); $C_{17}H_{33}$ ($C_{17:1}$); $C_{17}H_{31}$ ($C_{17:2}$);

Ginkgolic acids have an aromatic chromophore that makes them suitable for UV detection. The sample extraction and purification processes are also important in the analysis of ginkgolic acids. Ginkgolic acids are often extracted by an organic solvent such as hexane.[58] The solvent is then removed and the dry residue is dissolved in methanol. The extracts are separated by RP (C_{18}) HPLC with UV detection. A similar method used ethyl acetate to extract ginkgo samples.[21] The dry residue was dissolved in methanol. A mixture of acetonitrile and orthophosphoric acid was used as the mobile phase for RP (C_{18}) HPLC separation with UV detection. Some ginkgolic acid peaks cannot be separated well from the impurities in the extracts.

Another method that used hexane to extract ginkgolic acids and a silica gel column to purify the sample with a dual HPLC column system was developed.[60] The first column was a C_{18} column, and the second was an Ag(I)-coated cation exchange column. The Ag(I)-coated column can separate $C_{13:0}$-$C_{15:1}$ and $C_{15:0}$-$C_{17:1}$ (Ag(I) can form complexes with the double bond) while the C_{18} column alone cannot separate these ginkgolic acids peaks. Methanol-formic acid was used as the mobile phase. This method could obtain the baseline separation of five ginkgolic acids, but both the sample preparation and the HPLC separation system was still complex.

Supercritical carbon dioxide has also been used to extract ginkgo leaves.[59] The sample was purified using a silica gel column. Supercritical extraction with carbon dioxide, however, is not commonly available in routine quality control laboratories.

GC-MS has also been used for analysis of ginkgolic acids; however, it requires tedious sample preparation and derivatization.[61] NMR spectrometry (^{1}H-NMR) was also reported for quantification of total ginkgolic acids.[62] However, all these methods require

multiple steps of sample preparation and are time-consuming; therefore, they are not practical for routine analysis.

The concentrations of ginkgolic acids in standardized extracts are generally low. The analysis of ginkgolic acids requires a method that can provide efficient separation and sensitive detection. HPLC with mass spectrometry detection has high sensitivity for the detection of low concentrations of ginkgolic acids. Several LC-ESI-MS methods[63-65] were developed for the analysis of ginkgolic acids in ginkgo. Sample extraction and preparation are still very important parts of these analyses. One method used chloroform to extract the sample.[65] The solvent was then evaporated, and the dry residue was redissolved in methanol. Acetonitrile and acetic acid were used as the mobile phase with RP C_4 HPLC column and electrospray ion trap MS detection in negative ion mode because ginkgolic acids are easily ionized to negative ions. The sensitivity of this method was better than UV detection. Three ginkgolic acids were detected in this study.[65] In another method, hexane with saturated sodium sulfate-sulfuric acid was used to extract ginkgolic acids[63] and the extracts were purified by siliceous earth. After the solvent evaporated, the residue was redissolved in chloroform. Methanol-acetic acid-silver nitrate was used as the mobile phase with a C_{18} column, UV detection, and MS detection in negative ion mode. The silver ion improved the separation of ginkgolic acids. Electrospray mass spectrometry detection was used to identify ginkgolic acids. Four ginkgolic acids were detected in this study.[63] This method also required complex sample preparation.

The current state of the analysis of ginkgo products is that there is no method that can simultaneously determine terpenes, flavonol glycosides, and ginkgolic acids, and no

method that can directly quantify intact flavonol glycosides. These compounds are determined by tedious and complicated procedures and by several different analytical methods and instrumentation. It has been shown that the analysis of flavonoids after acid digestion does not detect the adulteration of ginkgo products. In order to establish meaningful quality control programs for herbal products, it is necessary to develop simple, fast, and reliable analytical methods.

Recently, mass spectrometry techniques have become increasingly more attractive for the analysis of complex samples and have shown superior potential for simple and rapid analysis of multicomponents.

1.3 Mass spectrometry for the analysis of ginkgo products

Mass spectrometers measure the mass-to-charge ratio (m/z) of gas phase ions of molecules. A mass spectrometer consists of a vacuum system, a sample introduction device, an ionization source, a mass analyzer, and an ion detector, as shown in Figure 1-4.

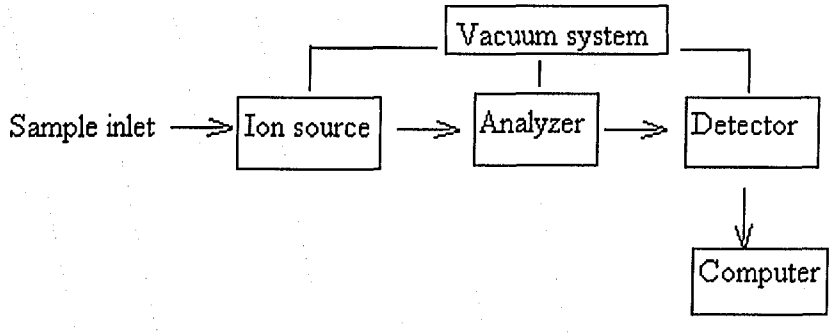


Figure 1-4. Block diagram of a mass spectrometer

Mass spectrometry techniques have become very important for a large variety of applications in the analysis of inorganic, organic, and bio-organic chemicals, including drug testing and drug discovery in pharmaceutical industries. The advantages of mass spectrometry in different analyses rely extensively on the ionization method and analyzer.

1.3.1 ESI and nanoES ionization processes

Ionization is one of the most important processes in mass spectrometry detection. Analytes in a solution must first be transferred into gas phase ions for detection. There are a number of ionization techniques such as electron ionization (EI), chemical ionization (CI), fast atom bombardment ionization (FAB), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). Electrospray ionization is most widely used for both thermally labile substances and stable compounds.

The principle of electrospray ionization was first described by Dole et al.in 1968.[66] Dole et al. proposed the charge residue model that explained the formation of gas phase ions produced by electrospray in an experiment analyzing polystyrenes with ESI. In 1984, Fenn made a breakthrough by coupling ESI to a mass spectrometer for the detection of large biological molecules such as polyethylene glycol.[67] The accurate mass of large proteins was measured in Fenn's study.

The ESI can transfer ions from solution to the gas phase, from which the ions are directed to the mass analyzer. Unlike other ionization methods, such as FAB ion desolvation using highly localized heating,[68] ion desolvation of ESI gradually uses low thermal energy at atmospheric pressure. ESI is a soft ionization method that is useful for both non-volatile and thermally labile compounds. This thesis is based on ESI-MS analysis; thus, the following discussion will focus on ESI and nanoESI.

In electrospray ionization,[67] the sample solution is pumped through a stainless steel capillary (75–150 μm i.d.) at a flow rate of 1 μL/min–1 mL/min and a high voltage (2 or 3 kV) is applied to the tip of the capillary (Figure 1-5). The positive ions then build up at the liquid surface. When the repulsion of the positive ions at the liquid surface exceeds the liquid surface tension, a Taylor cone forms. When the electrical field is high enough, the Taylor cone becomes unstable and a liquid jet is formed. Small droplets with positive charge are sputtered and extracted to the mass analyzer. The solvent in the droplets is evaporated with the help of a curtain gas (nitrogen). Subsequently, the solvent evaporation reduces the size of the droplets while the their charge remains constant, which relatively increases the positive charge on the surface of the droplets. Eventually, the positive charge of the droplets increases the electrostatic repulsion and overcomes the surface tension of the droplets, leading to smaller charged droplets. This evaporation and droplet splitting process is repeated until finally gas phase ions of analytes are formed, which are subsequently extracted into the analyser of the mass spectrometer. The formation of gaseous ions between the capillary tip and the sampling cone occurs at atmospheric pressure with little thermal effects, so this method is considered to be a soft ionization process.

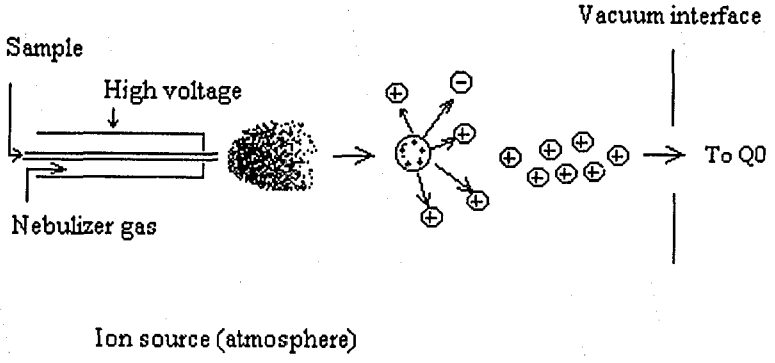


Figure 1-5. Schematic diagram of electrospray ionization

Nanospray ionization is an advanced form of ESI, and uses a small amount of the sample. Nanospray ionization was first introduced in 1994 as a low flow rate version of electrospray ionization.[69,70]

In nanospray ionization, the sample solution (1-4 μL) is offline-loaded into a very fine gold- or silver-coated capillary tip with an inner diameter of ~1 μm. The flow of sample solution is forced by syringe pressure. A reasonably high voltage (700–1000 V) is applied to the capillary resulting in sample ionization and spraying. The flow rate of the sample solution using this capillary is very low (20–40 nL/min).[69] Eventually, charged sample ions enter the analyzer of the mass spectrometer. NanoESI generates very small droplets with diameters 100–1000 times smaller than those of droplets generated by conventional ESI. As a result, the efficiency of ionization is improved. The lower flow rates consume less samples, which also allows for a longer analysis time. Cross-contamination is no longer a problem because each capillary tip is used only once. Fewer ion suppression

effects occur in this ionization compared with conventional ESI. On the other hand, this increases the cost of the coated tips (3.00 USD per tip).

1.3.2 Tandem mass spectrometry for identification

The mass analyzer separates the ions formed in the ionization source according to their mass-to-charge (m/z) ratios. There are many types of mass analyzers, such as quadrupoles, time-of-flight (TOF), magnetic sector, and quadrupole ion-trap.

Tandem (MS-MS) mass spectrometers are instruments that have more than one analyzer. They are useful for structural and sequencing studies. Two, three, and four analyzers have been combined into tandem mass spectrometers such as triple quadrupole, magnetic sector-quadrupole or TOF, magnetic sector-magnetic sector and quadrupole-TOF. In tandem MS, one analyzer can be used as a collision cell to generate fragmentation of analytes. Quadrupole-TOF mass spectrometry (QTOF) will be described here because only QTOF has been used in this research.

QTOF was introduced around 1995.[71] As the schematic diagram shows in Figure 1-6, three quadrupoles were combined with TOF. The radio frequency (RF)-only Q0 focuses and transfers ions from the vacuum interface to the high vacuum region of Q1. The mass filter Q1 (API QSTAR Pulsar-i instrument) in MS mode can be used in RF-only mode and as an ion transmission. In MS/MS mode, the mass filter Q1 transmits the selected parent ions. Q2 can be used in the RF-only mode for MS scans or in the MS/MS mode as a collision cell in which ions collide with nitrogen gas molecules forming product ions. The ions then move into the TOF section from Q2. Ions are accelerated upon the application of a high voltage and flight in a field-free region. The velocity of the ions is inversely related to their m/z. Ions with low m/z travel faster than ions with high m/z.

Therefore, ions with different m/z can be measured according to their different flight times in the field-free region. However, ions with the same m/z do not have exactly the same initial velocities when they are accelerated because of the kinetic energy difference given to the gas phase ions.[71] This effect can decrease the mass resolution. In the API QSTAR Pulsar-i instrument, the effect of the initial energy can be corrected by using an ion reflectron. The reflectron is an ion mirror that can reverse the direction of the ions. Ions with the same m/z and higher velocity fly through the reflectron further and spend a longer time in the reflectron than ions with the same m/z but lower velocity.[72] Therefore, the small energy difference is corrected, and high mass resolution can be achieved in the TOF analyzer, which is very useful for multi-component mixture analysis. The TOF analyzer has high ion transmission efficiency and can record all ions arriving at the detector during an ionization cycle. Therefore, the TOF analyzer has a very high spectrum acquisition rate and its sensitivity is much higher than that of the scanning analyzer.

The QTOF combination has high sensitivity, high mass resolution, and high mass accuracy for precursor and product ions, and can provide fragmentation information. Combining the advantages of nanoESI source and QTOF makes them especially suitable for the analysis of complex mixtures such as ginkgo products.

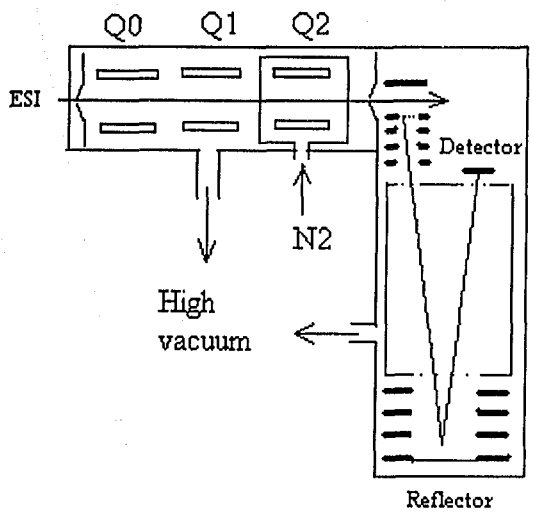


Figure 1-6. Schematic diagram of a quadrupole-time-of-flight mass spectrometer

1.4 The objectives of my thesis

The analytical techniques currently available for quality control of ginkgo products involve multiple steps and require different instrumentation and methods for different classes of compounds. These methods are laborious and time-consuming. In addition, the available methods cannot detect adulteration of the products. Thus, the objectives of my thesis research were: (1) to develop a rapid and reliable analytical technique for the simultaneous determination of the active and potentially toxic components in ginkgo products; (2) to obtain multiplex data from a single analysis for quality control; and (3) to demonstrate the application of this technique for screening commercial ginkgo products.

A nano-electrospray ionization mass spectrometry technique was explored to achieve these objectives. The instrumental and experimental conditions were studied for simultaneous analysis of the active and toxic components. The established method was used to analyze commercial ginkgo products from local stores to obtain composition and concentration information.

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Chapter 2

Development of a Nanoelectrospray Ionization Mass Spectrometry Technique for Rapid and Simultaneous Detection of Active and Potentially Toxic Compounds in Ginkgo Products*

2.1 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 of 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 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 as discussed in Chapter 1 (see Figures 1-1, 1-2, and 1-3 for their structures and Table 2-1 for their molecular weights). A number of studies have shown that the presence of both classes of compounds in ginkgo extracts at appropriate concentrations is required to achieve beneficial effects.[1,3,7,8] The accepted composition of "standardized ginkgo extracts" is

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Table 2-1. Molecular weights of the target compounds in ginkgo

Compounds		MW (amu)
Terpenes:		
Ginkgolide A	(GA)	408
Ginkgolide B	(GB)	424
Ginkgolide C	(GC)	440
Bilobalide	(BB)	326
Flavonol Glycosides:		
Kaempferol	(K)	286
Quercetin	(Q)	302
Isorhamnetin	(I)	316
3-O-(β-D-glucosyl)kaempferol	(GK)	466
3-O-(β-D-glucosyl) quercetin	(GQ)	482
3-O-(β-D-glucosyl)isorhamnetin	(GI)	496
3-O-(α-L-rhamnosyl)kaempferol	(RK)	432
3-O-(β-D-rhamnosyl) quercetin	(RI)	448
3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl)kaempferol	(RGK)	594
3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) quercetin	(RGQ)	610
3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl) isorhamnetin	(RGI)	624
3-O-(6''-O-(α-L-rhamnosyl) β-D-glucosyl)-3'-methylmyricetin	(RGMeM)	640
3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) kaempferol	(R_2GK)	740
3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) quercetin	(R_2GQ)	756
3-O-(2''-O, 6''-O-bis (α-L-rhamnosyl) β-D-glucosyl) isorhamnetin	(R_2GI)	770
Ginkgolic acids:		
Ginkgolic acid I		320
Ginkgolic acid II		346
Ginkgolic acid III		374

6% of terpenes and 24% of flavonol glycosides.[1,7] In addition to these active components, ginkgo plants also contain ginkgolic acids, which are toxic. It is recommended that the concentration of ginkgolic acids should be less than 5 μg/g 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 the flavonoids quercetin (Q), kaempferol (K), and isorhamnetin (I) (see Figure 2-1), 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 the bioavailability, pharmacodynamics, and pharmacokinetics of intact flavonol glycosides and the hydrolysis products (flavonoids) are different.[16-19]

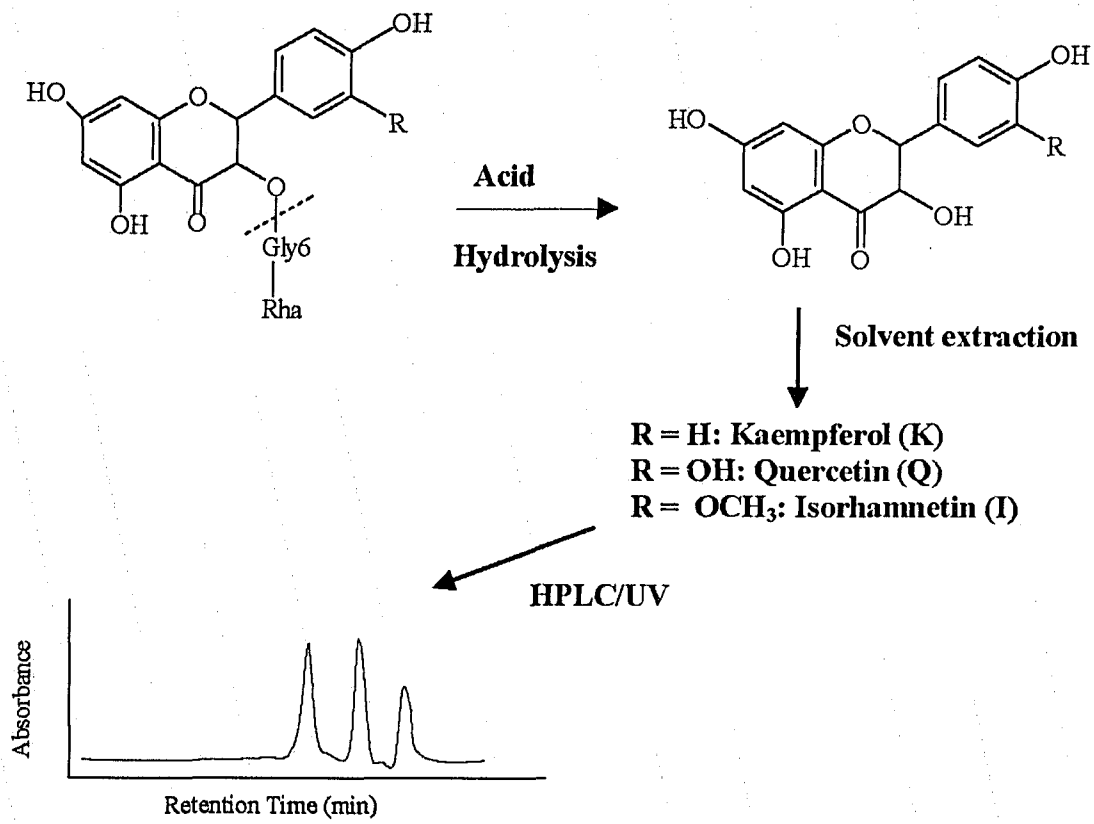

Figure 2-1. Conventional HPLC analysis of flavonoids
(after acid hydrolysis of flavonol glycosides)

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 the rapid analysis of ginkgo products. This method is useful for quality control and for detection of product adulteration.

2.2 Experimental

2.2.1 Reagents and materials

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

2.2.2 Samples

Stock standard solutions of ginkgolides A, B, and C, bilobalide, ginkgolic acids I and II, quercetin dehydrate (Q), kaempferol (K), and isorhamnetin (I) (1 mg/mL), and ginkgo extracts were separately prepared by dissolving appropriate quantities of these standards in methanol. Working standard solutions were diluted to 10-100 μg/mL with a buffer of 5 mM ammonium acetate in 1:1 methanol/water.

The extraction of ginkgo products was optimized by varying the percentage of methanol (20 % to 100%) in water. When the percentage of methanol increased from 20% to 100%, the amount of ginkgolic acids also increased, whereas other components

did not change significantly. Therefore, all the commercial samples (1 mg) were extracted using 1 mL of 100% methanol and diluted with 5 mM ammonium acetate in 1:1 MeOH/water to be 50 μg/mL for the subsequent nanoESI-MS analysis. Details of the results will be discussed in Chapter 3.

2.2.3 Instrumentation

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

In the single MS scan mode, the mass measurements (200-900 amu) were performed using the 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 (DP_1) of -5 V, a second declustering potential (DP_2) of -10 V, and a focusing potential (FP) of -5 V.

To obtain a reproducible mass spectrum of "standard" ginkgo extracts that can be used for fingerprinting, the fragmentation of analytes was minimized or eliminated in order to acquire a mass spectrum that closely represents the composition of the solution. The

ionization and fragmentation of the commercially available standards of bilobalide, ginkgolides A, B, and C, ginkgolic acids I and II, quercetin dehydrate, kaempferol, and rutin (RGQ) were studied individually under different conditions. After optimizing the instrument parameters with individual standards, we then optimized the conditions for ginkgo extracts (see Figure 2-2).

The electrospray voltage at -900 V was found to provide optimum ionization efficiency. Other instrumental parameters (such as DP_2 and FP) were also optimized in the same manner. A DP_2 of -10 V and FP of -5 V were selected.

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 10-40 eV and a collision gas setting of 5. The resulting product ions were analyzed by the TOF analyzer with a microchannel plate multiplier detector. Analyst QS software (Applied Biosystems, Foster City, California, USA) was used for the spectrum acquisition and data analysis. Igor Pro software (WaveMetrics, Lake Oswego, Oregon, USA) was used to plot the spectra.

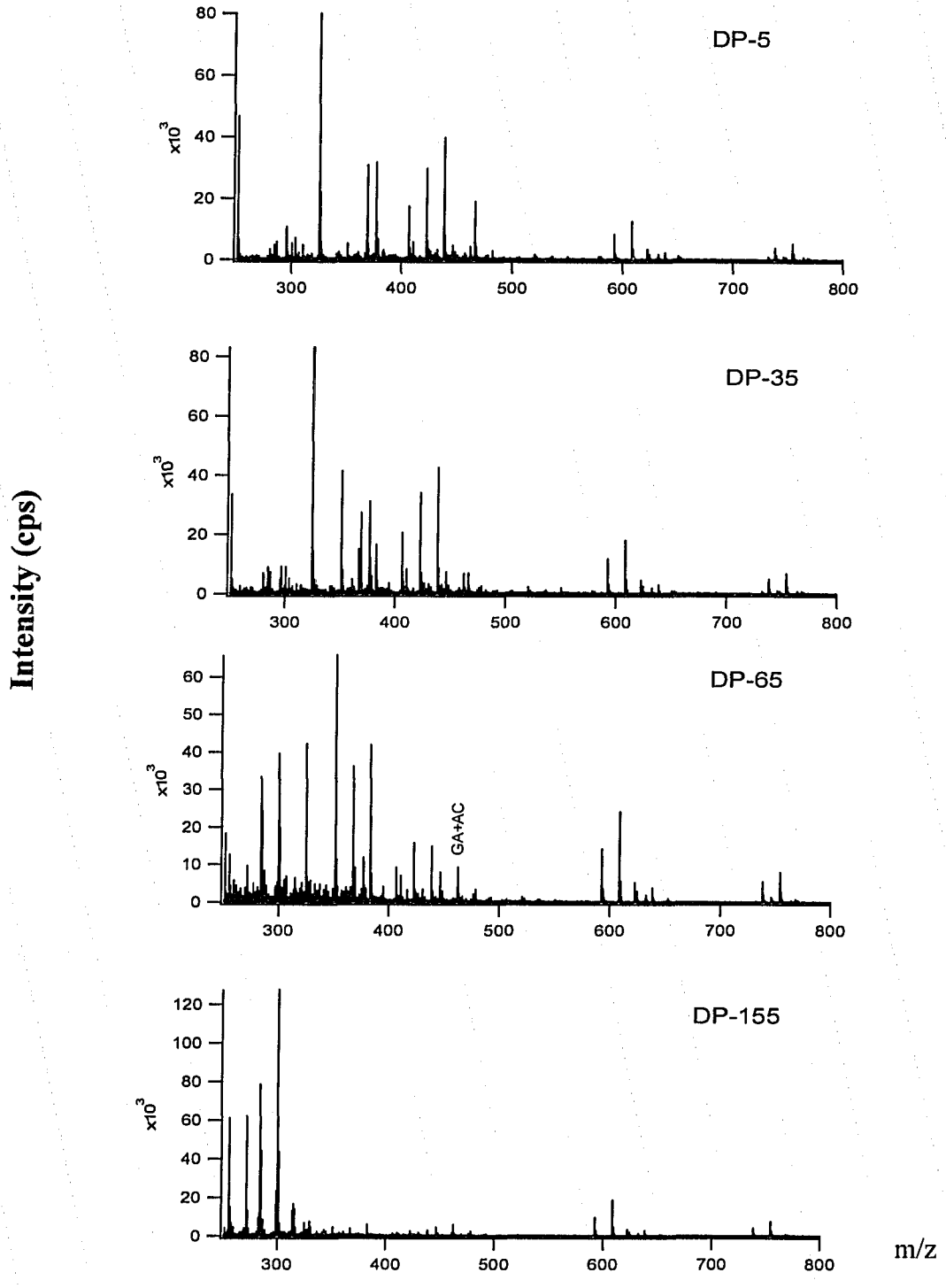

Figure 2-2. Fragmentation of components in the ginkgo extract at different DP_1
With the increase of DP_1 from -5 V to -155 V, fragmentation of terpenes increased. When DP_1 was set at -5 V, minimum fragmentation of these components in the ginkgo extract was achieved. Therefore, DP_1 at -5 V was used for the rest of this study.

2.3 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 nanoelectrospray mass spectrometry (nanoESI-MS) as the platform to develop a technique that can alleviate the two main problems: the hydrolysis of the flavonol glycosides to the flavonoids quercetin, kaempferol, and isorhamnetin, and the lack of chromophores 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 (bilobalide and ginkgolides A, B, and 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, $(M+H)^+$, $(M+Na)^+$, $(M+K)^+$, and $(M+NH_4)^+$. In comparison, the negative ionization produces a typical ion of $(M-H)^-$ for each of the target compounds under optimized conditions (Figure 2-3).

Figure 2-4A shows a typical negative ion mass spectrum of a "standard" ginkgo leaf extract using nanoESI-MS, demonstrating that ginkgo terpene trilactones and intact flavonol glycosides are simultaneously detected. The standard ginkgo leaf extract contains mainly these two classes of active compounds (Figure 2-4A). To confirm that the nanoESI-MS negative ionization technique can also determine ginkgolic acids, standards of ginkgolic acids I and II (10 μg/mL) were spiked into the same ginkgo extract. Figure 2-4B shows that all three classes of important ginkgo components, terpenes, intact flavonol glycosides, and ginkgolic acids, can be determined simultaneously with a single analysis.

The nano-ESI-MS method also offers good reproducibility (Figures 2-5) of the MS spectrum as demonstrated by repeated analysis of a ginkgo extract over 18 days. Similar spectra and peak intensities were obtained. Four ginkgo extracts (g1, g2, g3, and GK) produced by the standard procedures were analyzed using the nano-ESI-MS method (Figure 2-6). The spectra show that the four extracts have a similar composition: BB, GA, GB, GC, RGK, RGQ, RGI, RGMeM, R_2GK, and R_2GQ. Thus, this technique is useful for fingerprinting and quality control of complicated mixtures such as ginkgo products. These extracts were used as "standard" for the rest of the study.

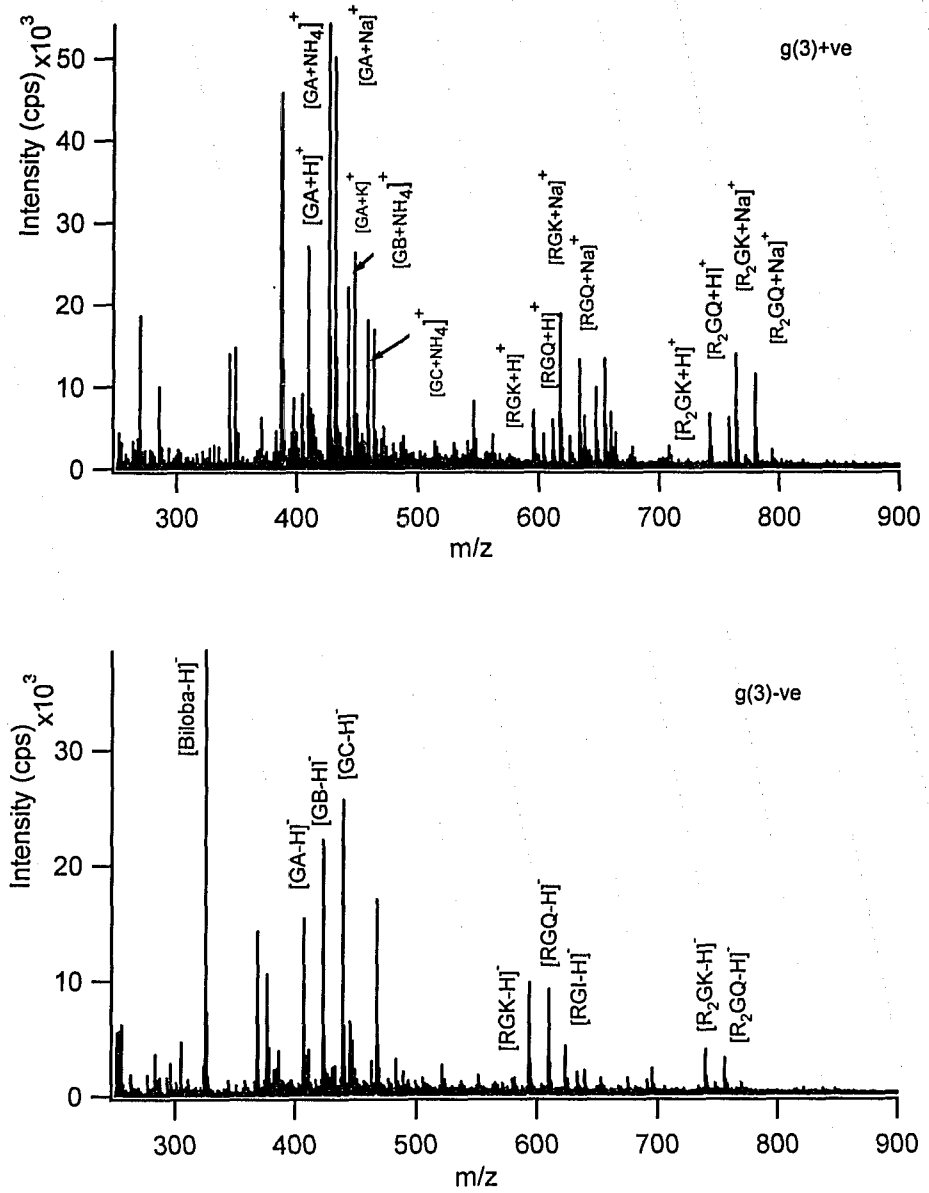

Figure 2-3. Mass spectra of ginkgo extract in positive and negative mode
50 μg/ml ginkgo extract in 5 mM ammonium acetate in 1:1 MeOH/water
DP_1 (+,-)5 V, FP(+,-) 5 V, electrospray voltage(+,-) 900 V
GA, GB, and GC are ginkgolides A, B, and C, respectively.
RGK, RGQ, RGI, R_2GK, R_2GQ, are intact flavonol glycosides.

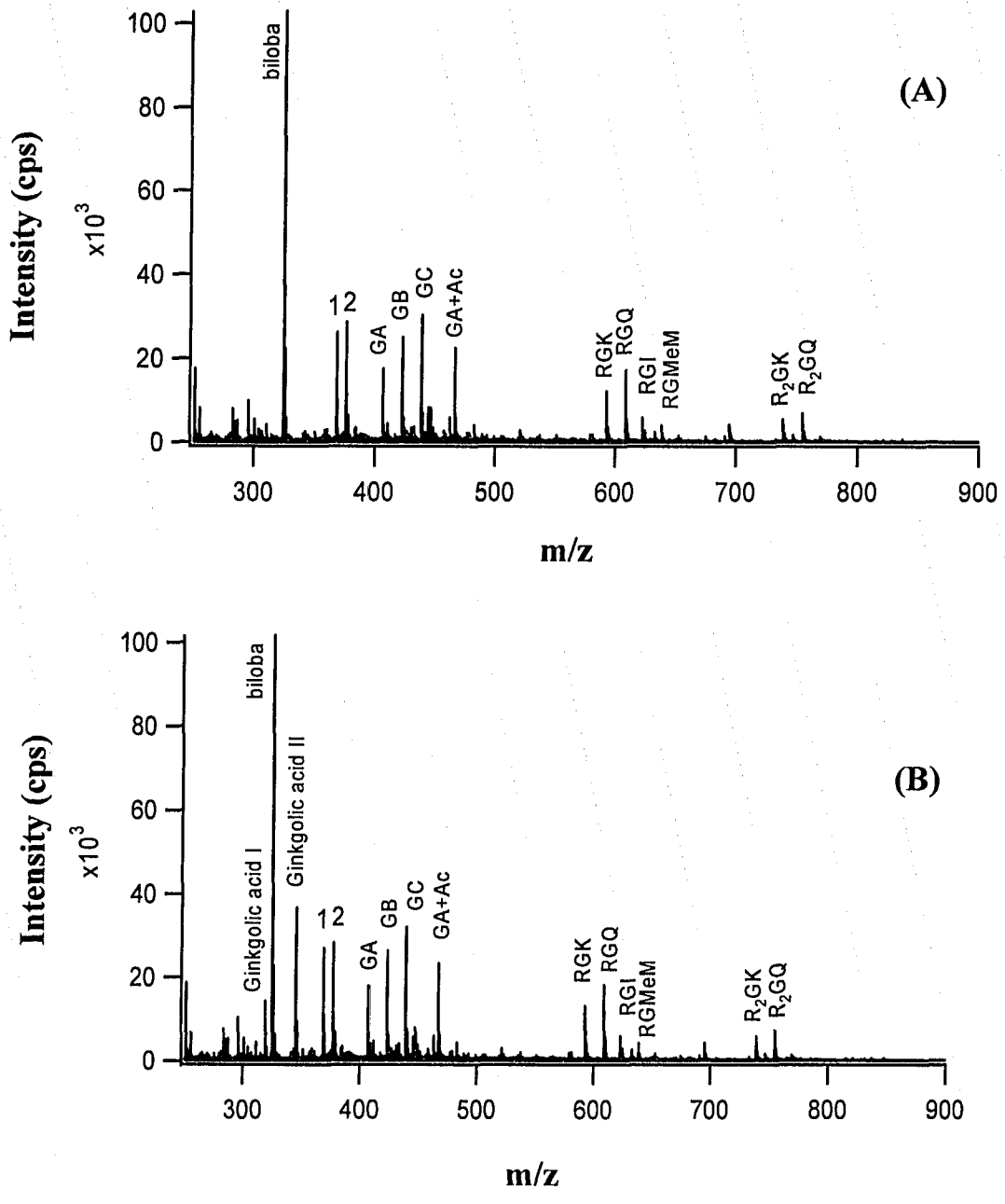

Figure 2-4. Simultaneous detection of three groups of components in ginkgo products (A) standard ginkgo extract (50 μg/mL), and (B) the same standard ginkgo extract spiked with 10 μg/mL standards of ginkgolic acids I and II. GA, GB, and GC are ginkgolides A, B, and C, respectively. GA+Ac corresponds to an acetate adduct of GA. RGK, RGQ, RGI, RGMEM, R_2GK, and R_2GQ, are intact flavonol glycosides. Peak 1 is an unidentified peak. Peak 2 represents GA-2CH_3 (m/z 377.0904, mass accuracy 0.18 ppm). Tandem mass spectrometry (MS/MS) of GA confirmed peak 2.

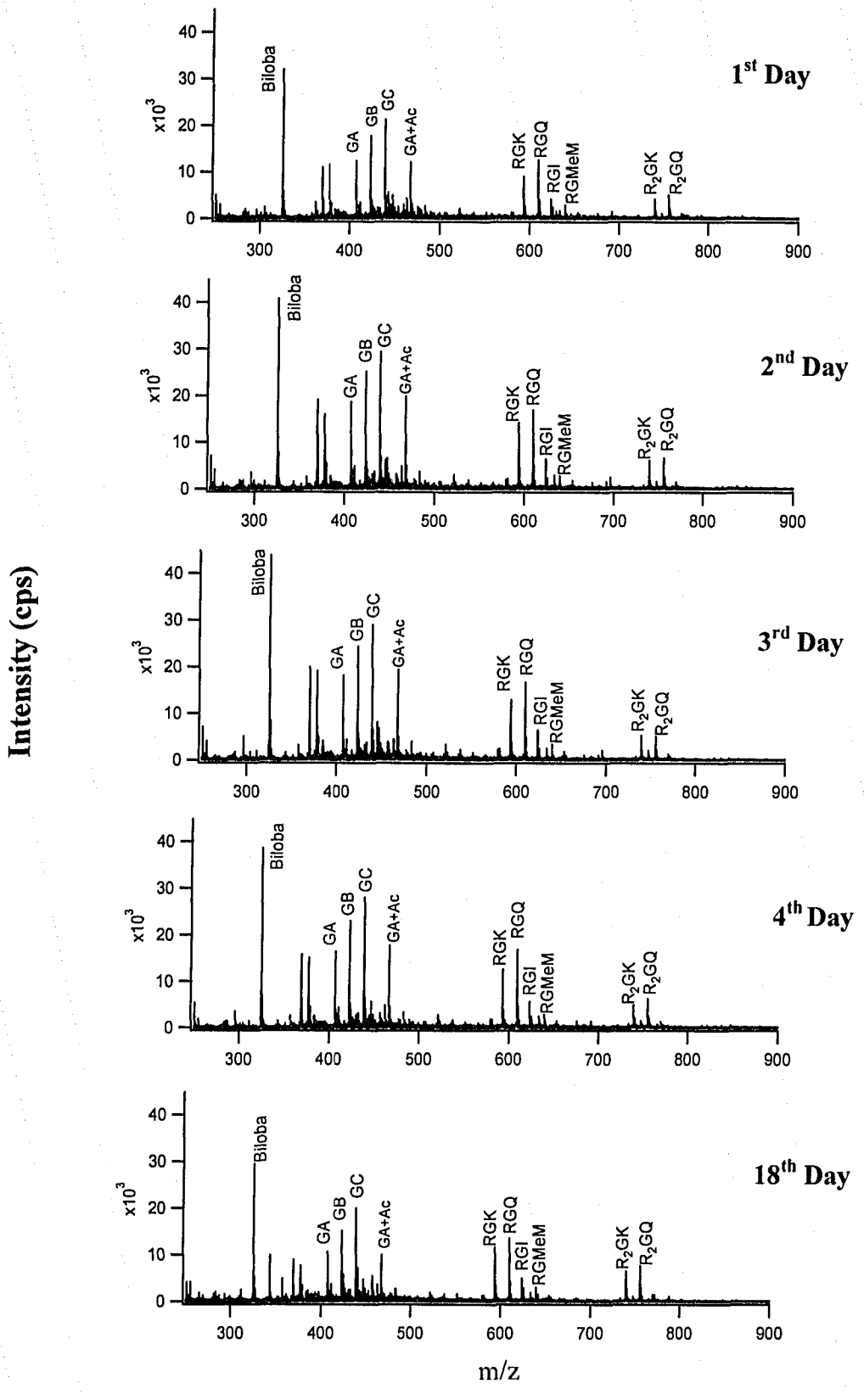

Figure 2-5. Reproducible mass spectra of a standard extract
Representative mass spectra obtained from repeated analysis of a standard extract over 18 days, showing day-to-day reproducibility

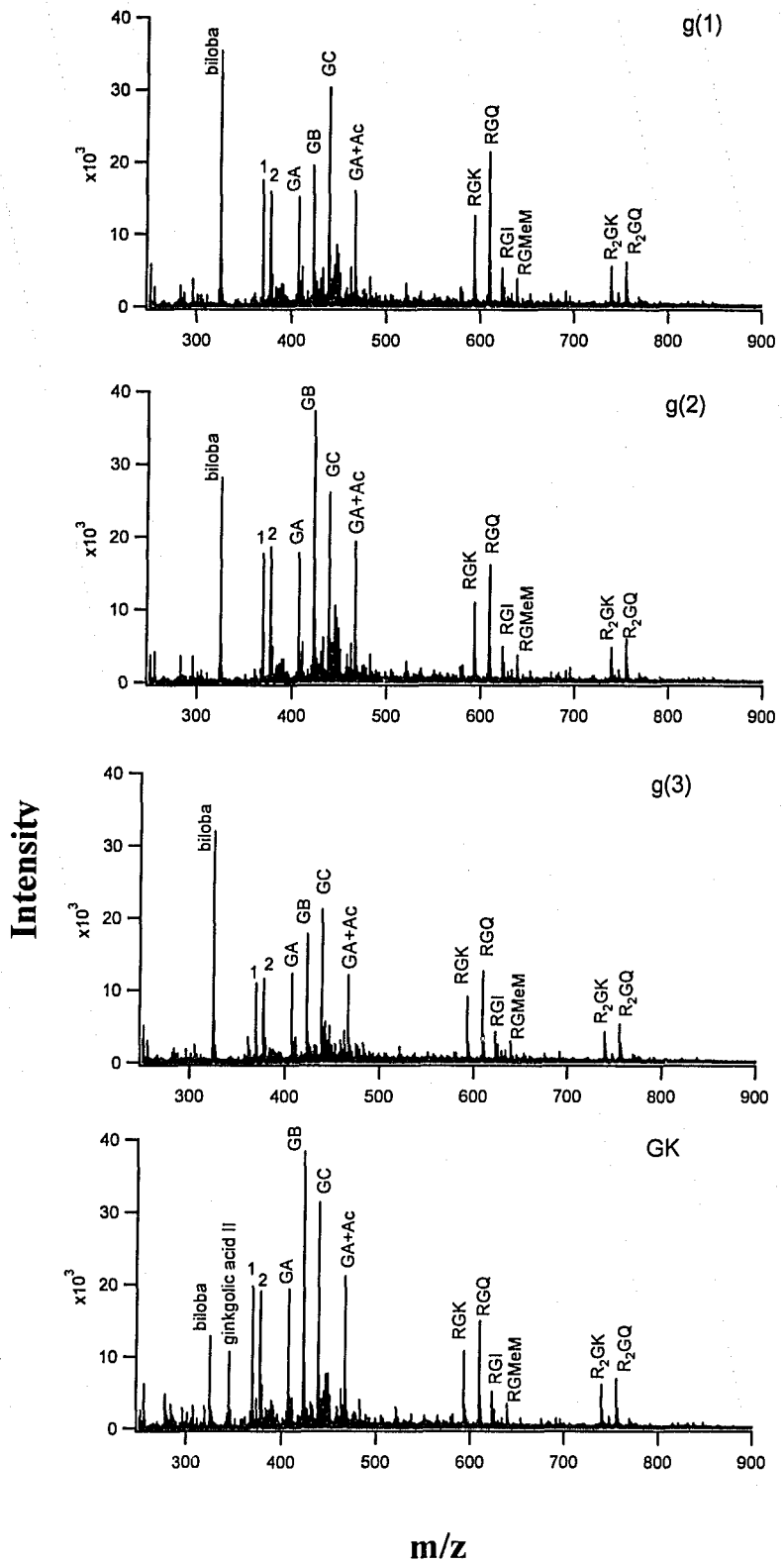

Figure 2-6. Negative ESI-MS spectra of four ginkgo "standard" extracts produced by the standard procedures showing consistency between the extracts. GA, GB, and GC are ginkgolides A, B, and C, respectively. GA+Ac corresponds to an acetate adduct of GA. RGK, RGQ, RGI, RGMEM, R_2GK, and R_2GQ, are intact flavonol glycosides. Peak identities are the same as in Figure 2-4.

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. Figure 2-7 shows three mass spectra from the analysis of (A) a "standard" ginkgo leaf extract (50 μg/mL), (B) the same extract fortified with 5 μg/mL of quercetin (Q), and (C) a commercial ginkgo product that is evidently fortified with synthetic flavonoids (K, Q, and I). RGK, RGQ, RGI, R_2GK, and R_2GQ are the primary intact flavonol glycosides detected in the "standard" ginkgo extract (Figure 2-7A). Their surrogate products of fortification, such as quercetin (Q), can be differentiated from the authentic flavonol glycosides (Figure 2-7B). These flavonoids (K, Q, and I) are not detectable in the "standard" ginkgo extract (Figure 2-7A). 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 (Figure 2-7C). This fortification (product adulteration) would not be recognized by the conventional methods that involve hydrolysis of intact flavonol glycosides followed by analysis of the 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.

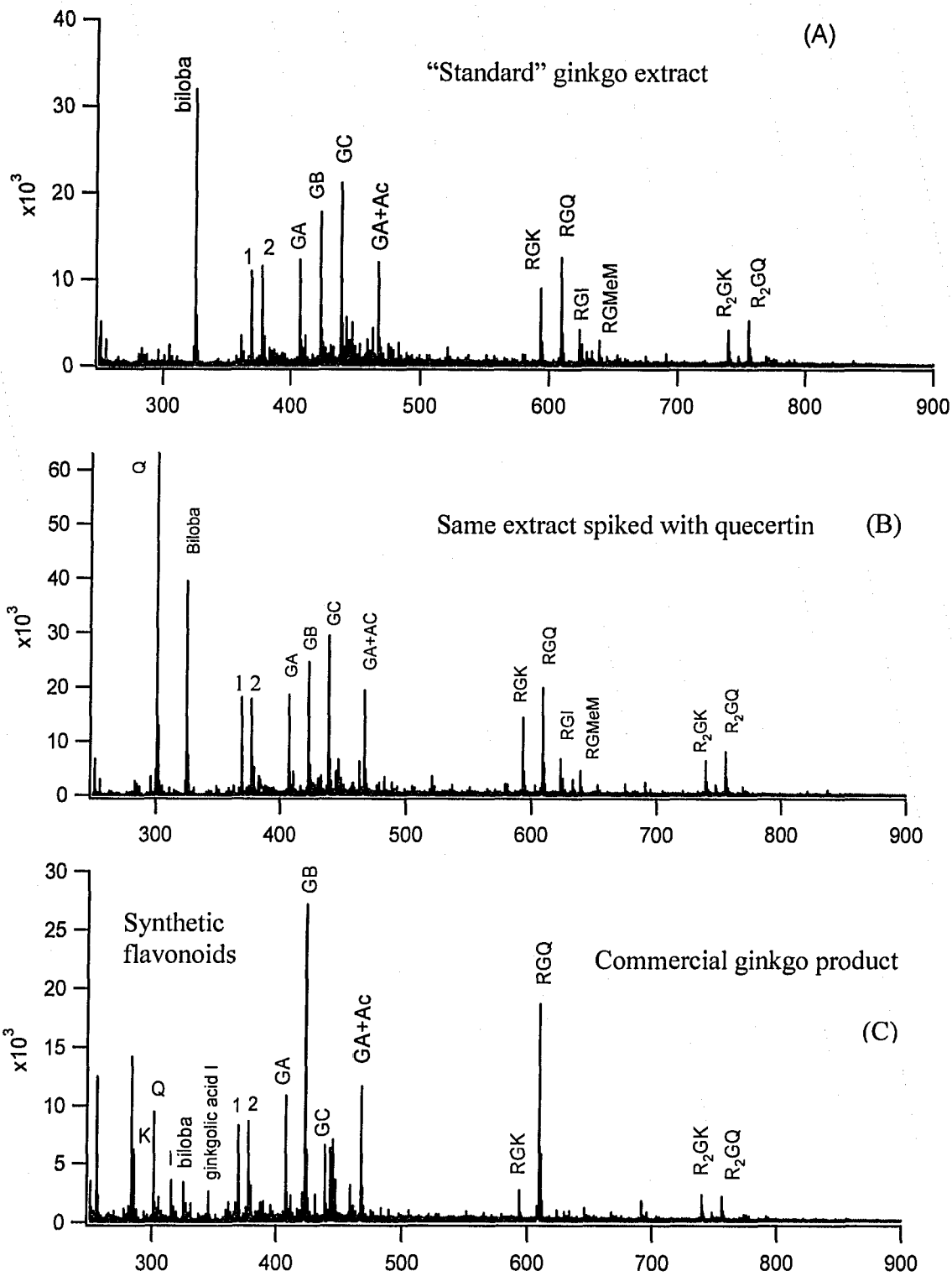

Figure 2-7. Detection of adulteration

(A) a "standard" ginkgo extract (50 µg/mL), (B) the same extract spiked with 5 µg/mL of standard quecertin, 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 Figure 2-4.

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 observed that the market products had 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 had composition and contents of the major active components closely resembling those in the "standard" extracts (Figure 2-8A). Five products did not meet the requirement of 5% terpenes and 24% flavonol glycosides (Figure 2-8B): two products contained very low levels of active ginkgo compounds (<1/4 of the "standard" extract); two samples contained less than 50% of the active components; and one sample contained some unknown components that were detected together with low levels of active components. Similar mass spectral profiles of these products suggest that there was no product fortification although the contents of active compounds varied in these products. Four products showed potential fortification with synthetic flavonoids (Q and/or K, I) and rutin (RGQ), such as those shown in Figures 2-7C and 2-8C. The product shown in Figure 2-8C contains very low levels of intact flavonol glycosides except for unusually high levels of Q and RGQ. The ratio of RGQ/RGK in this product is 4.4, more than four times higher than in "standard" extracts (Figure 2-4 A) and most other products (Figure 2-8A). 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 were detected in two of the 14 products. Figure 2-8D shows an example of a commercial ginkgo product obtained from a local food store that contained ginkgolic acids I, II, and III. Both MS/MS spectra and accurate mass confirmed the

identity of ginkgolic acids. Compared with the profiles of the "standard" extracts, the presence of ginkgolic acids and peak intensities of the two commercial samples suggest 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.

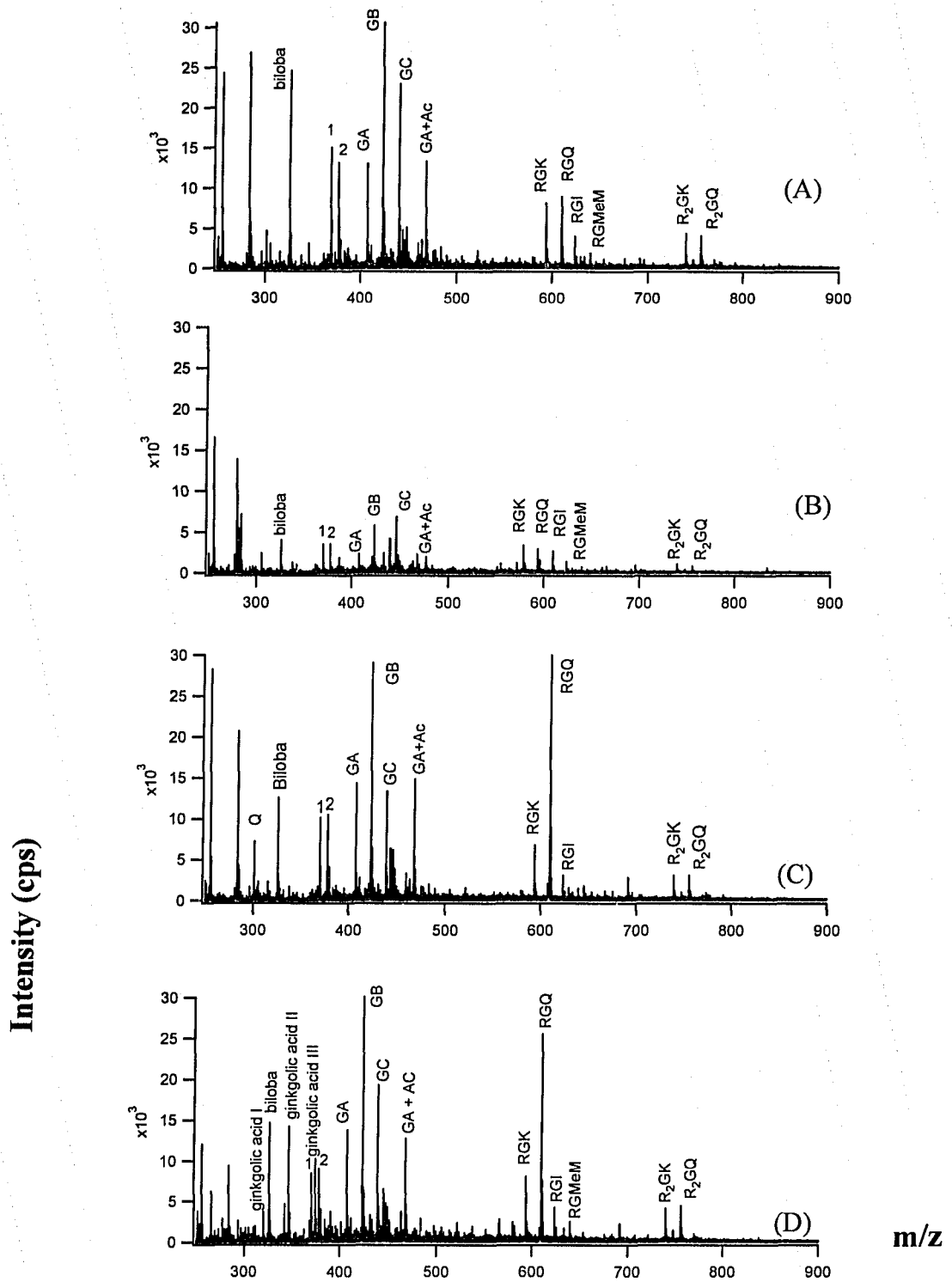

Figure 2-8. Representative mass spectra of selected commercial ginkgo products (100 μg/mL) showing large variations in the composition and concentrations of active and toxic components and fortification. Sample (A) meets the requirement of the standard ginkgo extract. Sample (B) contains low levels of active components. Sample (C) shows 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 Figure 2-4.

2.4 Discussion

The well-recognized composition of ginkgo products is based on the pioneering work of Dr. Willmar 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 optimizations, and pharmacological and toxicological investigations, a ginkgo extract consisting of 6% terpenes, 24% flavonol glycosides, and <5 μg/g ginkgolic acids has been accepted as the "standardized ginkgo extract."[20-21] Clinical trials using ginkgo products with this standardized extract composition have shown beneficial effects in treating various diseases, such as cerebrovascular and peripheral circulatory disorders, asthma, coughs, bladder inflammation, blennorrhagia, 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 agents for cancer because the 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 in this study. It is alarming that two of the 14 products contain a higher level of toxic ginkgolic acids compared with the "standard" extracts. 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.

The 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 of complicated sample pre-treatment processes. It takes less than 120 seconds to obtain a quality mass spectrum with accurate mass and MS/MS information for identification.

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 analysis of the 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-, and tri-glycosides). This further provides a means of detecting potential adulteration with synthetic flavonoids, alleviating the problems encountered by conventional methods.

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Chapter 3

Quantitative Analysis of Ginkgo Products Using NanoESI-MS

3.1 Introduction

Intact flavonol glycosides, terpenes and ginkgolic acids in ginkgo products can be simultaneously detected in a single run using the nanoESI-MS method as described in Chapter 2. This method is useful for detecting product fortification and fingerprinting of ginkgo products.

The quality of natural health products is relevant public health issue because of their widespread use. Single-compound medicines undergo vigorous toxicity testing and clinical trials before approval by the FDA for human use. Herbal products are often composed of a complex mixture of compounds. They are assumed to be safe without standardized testing. Recently, Health Canada approved new Natural Health Products Regulations, which are intended to ensure that all Canadians have ready access to natural health products that are safe, effective, and of high quality, and to protect the health of consumers.[1]

The health benefits of ginkgo products have been studied in a number of clinical trials.[2-5] Some studies suggest that the therapeutic efficacy of ginkgo extracts is not consistent because the composition of the active components varies from one manufacturer to another.[2,5-7] A number of studies have investigated the variation in the quality of ginkgo products and reported significant differences in the composition of the active components in different commercial ginkgo products.[8-14] Direct quantification of the active and toxic components is desirable for quality control of ginkgo products;[12]

however, as discussed in Chapters 1 and 2, there is no single method that is able to provide quantification of both the active and the toxic components in ginkgo products.

To explore the quantification of flavonol glycosides, terpenes, and ginkgolic acids in ginkgo products, the nano-ESI-MS method developed in Chapter 2 is further studied in this chapter. Two synthetic compounds were selected for evaluation of their suitability as internal standards for ginkgo analysis. The mass spectra and fragmentation of these two compounds and their interference with the identification of the ginkgo components were carefully examined. To develop a quantification method, it was necessary to understand how individual components in a mixture may fragment during analysis. Thus, the fragmentation of individual ginkgo components in a mixture during single MS scan and MS/MS analysis was studied. The fragmentation of individual components in single MS scan mode was compared with that in the MS/MS mode using the same collision energy. These fragmentation patterns were also used in the quantification of each compound that was based on the single scan spectrum. Calibration curves of different components were obtained according to the intensity ratio of each component versus the internal standard. The sample extraction methods were examined and the precision of the sample extraction was determined. This method was used to determine the concentration of each component in 14 commercial products of ginkgo extracts. The mass accuracy of the different compounds was also calculated and used for confirmation of the components in the commercial products.

3.2 Experimental

3.2.1 Reagents and materials

Ginkgolide A, B, and C, bilobalide, and ginkgolic acid I and II standards were purchased from LKT Laboratories. Quercetin dehydrate, kaempferol, isorhamnetin, rutin, barbaloin, and mangiferin were purchased from Sigma-Aldrich. Ammonium acetate and HPLC-grade methanol were purchased from Fisher Scientific. Commercial ginkgo products were purchased from local stores.

3.2.2 Instrumentation

Nanospray mass spectrometry experiments were performed using a QSTAR Pulsar i mass spectrometer with a nanospray ionization source. The mass spectrometer was operated in the negative ion mode. Sample solutions were introduced into the source by conductive capillaries. The details of the instrumentation are described in the instrumentation section of Chapter 2.

In the single MS scan mode, the mass range (mass-to-charge) was m/z 200 to 900. In MS/MS mode, the parent ions were fragmented with a collision energy of 11.2 eV and 20 eV.

3.2.3 Preparation of standard solutions and calibration curves

Stock solutions (1 mg/L) of the standards ginkgolide A, B, and C, bilobalide, ginkgolic acids I and II, quercetin dehydrate, kaempferol, rutin, mangiferin, and barbaloin were separately dissolved in methanol. Working solutions of these standards were diluted from the stock solutions with a buffer solution containing 5 mM ammonium acetate (NH_4Ac) in 1:1 MeOH/water. A set of working solutions of the mixed standards (0.1-20 μg/mL each) consisting of ginkgolides A, B, and C, bilobalide, kaempferol, quercetin,

and ginkgolic acids I and II with 0.6 μg/mL barbaloin as the internal standard was prepared in the same buffer solution.

The calibration curves of ginkgolides A, B, and C, bilobalide, kaempferol, quercetin, rutin, and ginkgolic acids I and II were obtained based on the relative intensity of the individual standard (analyte) over that of the internal standard versus the concentrations of the analyte. The total intensity counts of the individual standard include the main peak $(M-H)^-$ and its fragments.

Because no standard compounds are available for RGK, RGI, R_2GK, R_2GQ, R_2GI, and ginkgolic acid III, the quantification of these components was estimated using the standard RGQ for RGK, RGI, R_2GK, R_2GQ, and R_2GI, and ginkgolic acid I for ginkgolic acid III.

3.2.4 Extraction of samples

Sample S1 was used to examine the extraction efficiencies separately using vortex and ultra-sonication methods, because this sample contains all the groups of ginkgo components: ginkgolides, flavonol glycosides, and ginkgolic acids.

Vortex method: 1 mg of sample S1 was first mixed with 1 mL of 20% MeOH in water. The mixture was vortexed for 10 min, followed by centrifugation for 5 min, and the supernatant was collected. Subsequently, the supernatant was diluted to be equivalent to 100 μg/mL (dry weight of the ginkgo product) with 5 mM NH_4Ac in 1:1 MeOH/water for nanospray mass spectrometry analysis.

Ultra-sonication method: 1 mg of sample S1 was dissolved in 1 mL of 20% MeOH in water and extracted under ultra-sonication for 10 min. After centrifugation for 5 min, the supernatant of the sample solution was taken out and diluted to 100 μg/mL (dry

weight of the ginkgo product) with 5 mM NH_4Ac in 1:1 MeOH/water for nano-electrospray mass spectrometry analysis.

The initial results showed that extraction efficiency by both vortex and ultra-sonication was comparable. To process many samples more eficiently, we chose the ultra-sonication method for extraction of the commercial ginkgo products.

The extraction efficiency of the ultra-sonication method was further examined using different ratios of MeOH/water solvent. Sample S1 was extracted using 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% methanol in water under ultra-sonication for 10 min. The supernatant was diluted to 100 μg/mL (dry weight of the sample) with 5 mM NH_4Ac in 1:1 MeOH/water, and subsequently analyzed using nanoESI in negative ion mode. 100% MeOH was chosen to extract the ginkgo samples because more peaks (ginkgolic acid I, II, III) were detected and the extraction efficiency of the other components did not change significantly.

Seven replicates of sample S1 were prepared separately with the internal standard and extracted by ultra-sonication for 10 min using 100% methanol. The supernatant was diluted to 100 μg/mL (dry weight of the sample) with 5 mM NH_4Ac in 1:1 MeOH/water and analyzed by the mass spectrometer. The relative standard deviation of the peak intensities of the different components was calculated.

Commercial ginkgo samples analyzed in this chapter are listed below. Different lots of samples S1, S2, and S14 were obtained from the same suppliers.

Table 3-1. Different ginkgo commercial samples and "standard" extracts

Sample #	Supplier	Appearence	Lot no.
S1	1	Capsules	1
S1-1		Capsules	2
S1-2		Capsules	3
S2	2	Capsules	1
S2-1		Capsules	2
S2-2		Capsules	3
S3	3	Capsules	
S4	4	Capsules	
S5	5	Capsules	
S6	6	Capsules	
S7	7	Capsules	
S8	8	Softgels	
S9	9	Tablets	
S10	10	Tablets	
S11	11	Caplets	
S12	12	Caplets	
S13	13	Tablets	
S14-1	14	Softgels	1
S14-2		Softgels	2
g(1)	15	Powder	
g(2)	15	Powder	
g(3)	15	Powder	
GK	16	Powder	

3.3 Results

3.3.1 Evaluation of internal standards

An internal standard was used to account for the fluctuations in the mass spectrometry signal response and the variation in the sample preparation procedures. The internal standard should have similar chemical and physical properties or be structurally homologous to the analyte.[15] The best internal standard is a stable isotope-labelled analog of the analyte; however, no isotope-labelled standards of terpenes, flavonol glycosides, or ginkgolic acids were available. Mangiferin (2-β-D-Glucopyranosyl-1,3,6,7-tetrahydroxy-xanthen-9-one) and barbaloin (10-β-D-Glucopyranosyl-1,8-dihydroxy-3-(hydroxyl-

methyl)-anthracen-9-one) consist of structural moieties similar to ginkgo flavonol glycosides (Section 1.2.2). Their structures are shown in Figures 3-1A and B. They were examined as internal standards for analysis of the ginkgo extracts in this study.

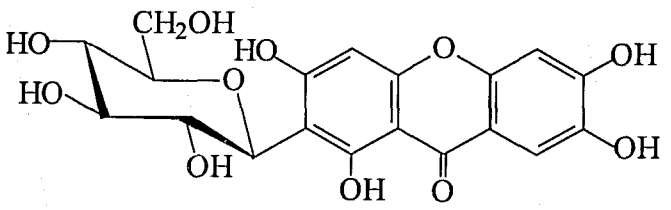


Figure 3-1A. Structure of mangiferin

(2-β-D-Glucopyranosyl-1,3,6,7-tetrahydroxyxanthen-9-one)

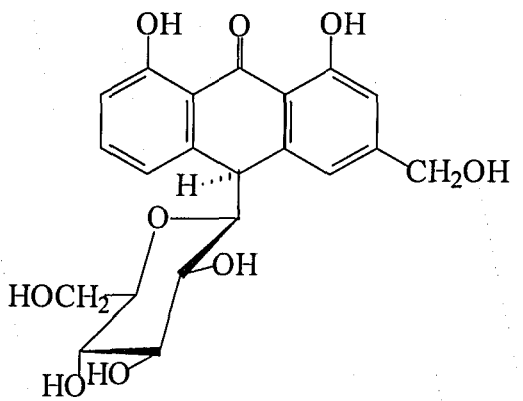


Figure 3-1B. Structure of barbaloin

(10-β-D-Glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-anthracen-9-one)

Figures 3-2A and 3-2B show the negative ionization mass spectra of mangiferin and barbaloin. The molecular ion of mangiferin was at m/z 421, $[422\text{-}H]^-$, in the negative

mode, which was close to the molecular ion of ginkgolide B (m/z 423, $[424\text{-}H]^-$). The mass spectrum of barbaloin shows m/z 417 as the major peak with small peaks of m/z 477 and 297, corresponding to barbaloin+Ac^- and barbaloin+$(C_6H_4OH\text{-}CO)^-$, respectively.

The suitability of mangiferin and barbaloin as internal standards was first evaluated with their effect on the mass spectrum of the ginkgo extracts. A ginkgo extract sample with mangiferin and barbaloin was analyzed by negative ionization nanoESI-MS and the mass spectrum is shown in Figure 3-2C. Neither mangiferin nor barbaloin interfered with the detection of the ginkgo extract. Considering that the main peak of mangiferin (m/z 421) was close to the main peak of ginkgolide B (m/z 423), barbaloin was chosen as the internal standard. Barbaloin produced two ions, m/z 417 and 477 (acetate adduct of barbaloin), whose relative intensities were consistent from run to run. These ions did not interfere with the identification of the ginkgo compounds. Therefore, barbaloin was chosen for the quantification of ginkgo products.

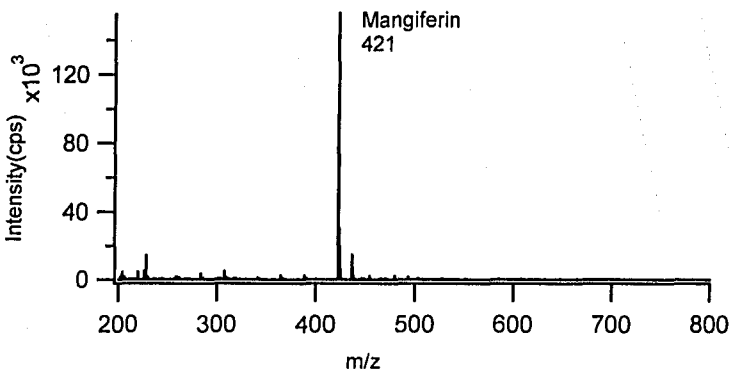


Figure 3-2A. Mass spectrum of mangiferin m/z 421
DP_1-5V, FP-5V, electrospray voltage -900V

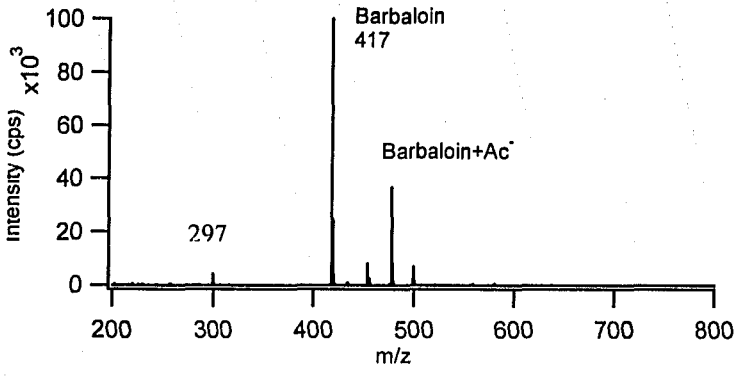


Figure 3-2B. Mass spectrum of barbaloin m/z 417
DP_1-5V, FP-5V, electrospray voltage -900V

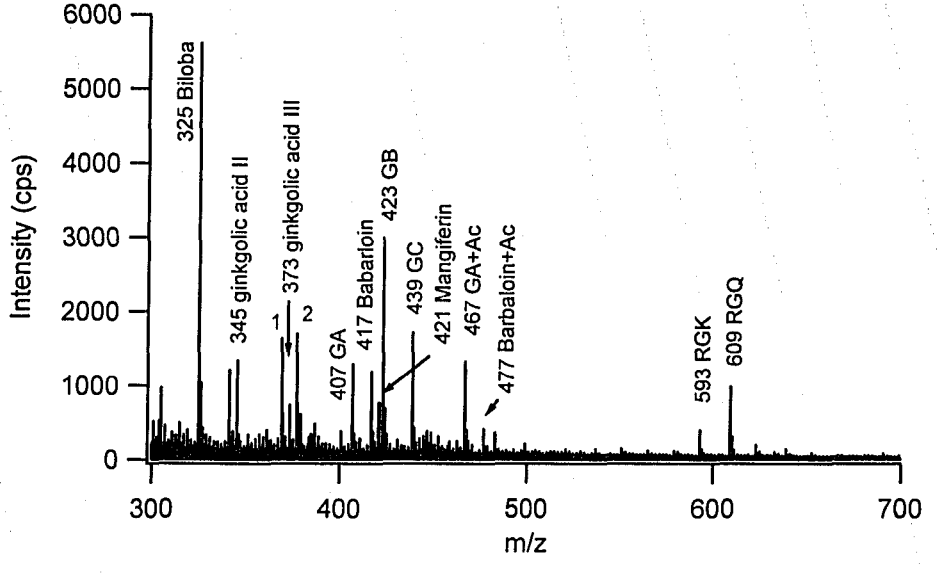


Figure 3-2C. Mass spectrum of ginkgo sample + mangiferin + barbaloin m/z
DP_1-5V, FP-5V, electrospray voltage -900V
Peak 1 is an unidentified peak; Peak 2 represents GA-$2CH_3$

3.3.2 Characterization of standards

3.3.2.1 Single MS scan spectrum, measured mass and mass accuracy of the mixed standards

The mixed standard solution contained 0.7 μg/mL of each ginkgolide A, B, and C, bilobalide, quercetin, kaempferol, and rutin, 15 μg/mL of each ginkgolic acids I and II with 0.6 μg/mL of barbaloin as the internal standard in 5 mM ammonium acetate in 1:1 MeOH/water. Figure 3-3 shows the single MS scan mass spectrum of this mixed standard solution. This spectrum was obtained using negative ionization nanospray mass spectrometry under the optimized conditions, which included a nanospray voltage of -900 V, DP_1 of -5 V, DP_2 of -10 V, and FP of -5 V. Ginkgolides A, B, and C, bilobalide, kaempferol, quercetin, rutin, ginkgolic acids I and II, and barbaloin were separated according to their different m/z ratios. All the standard compounds were detected as $(M-H)^-$ except GA, which showed both $(GA-H)^-$ and $(GA+Ac-H)^-$. The measured mass and mass accuracy of the standard components in Figure 3-3 are obtained and summarized in Table 3-2. The mass accuracy of all components is generally better than 35 ppm with or without the internal standard correction. The accuracy of the molecular masses obtained with the internal standard was relatively better than those obtained without the internal standard in this analysis.

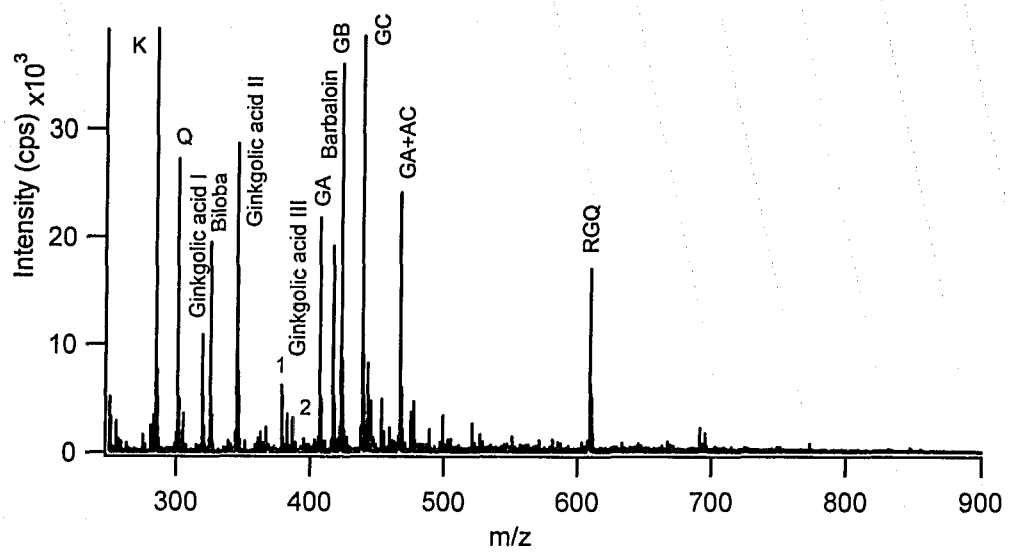


Figure 3-3. Mass spectrum of mixed standard
0.7 μg/mL mixed standards (ginkgolic acids 15 μg/mL) + 0.6 μg/mL barbaloin in 5 mM ammonium acetate in 1:1 MeOH/water
DP_1-5 V, FP-5 V, electrospray voltage -900 V
GA, GB, and GC are ginkgolides A, B, and C, respectively. GA+Ac corresponds to an acetate adduct of GA. RGK, RGQ, RGI, RGMeM, R_2GK, and R_2GQ are intact flavonol glycosides. Peak 1 is an unidentified peak; Peak 2 represents GA-$2CH_3$.

Table 3-2. Mass accuracy of terpenes, flavonol glycosides, and ginkgolic acids in mixed standards

	Compound	Theoretical Molecular Weight (amu)	Experimental Molecular Weight (amu)		Mass Accuracy (ppm)	
			(no I.S.)	(with I.S.)	no I.S.	with I.S.
Terpenes	Bilobalide	326.1002	326.1002	326.1002	0.00	0.00
	Ginkgolide A	408.1420	408.1432	408.1404	-4.41	-3.9
	Ginkgolide A+Ac	468.1631	468.1614	468.1617	-3.63	-3.00
	Ginkgolide B	424.1369	424.1341	424.1339	-6.60	-7.07
	Ginkgolide C	440.1319	440.1292	440.1294	-6.13	-5.68
Ginkgolic acids	Ginkgolic acid I	320.2351	320.2299	320.2340	-1.62	-3.43
	Ginkgolic acid II	346.2508	346.2388	346.2442	-34.7	-19.1
Aglycone	K	286.0477	286.0422	286.0431	-19.2	-16.1
	Q	302.0427	302.0416	302.0457	-3.64	1.00
Flavonol glycosides	RGQ	610.1534	610.1534	610.1534	0.00	0.00

3.3.2.2 Fragmentation patterns of individual components in the mixed standard solution

The individual ions detected in the single MS scan spectrum shown in Figure 3-3 were further studied using tandem mass spectrometry.

In the single MS scan mode, the experimental conditions used for obtaining Figure 3-3, the collision energy is calculated based on CE $=Q_0$-RO_2, where Q_0 was -20.0 V and RO_2 was -8.8 V; therefore, the offset collision energy in single MS scan mode CE was -11.2 eV.

To obtain the MS/MS spectra of the peaks shown in Figure 3-3, individual ions were selected and fragmented using the same collision energy (-11.2 eV) that was used in the

full scan, to examine the fragmentation of each component that might occur in the full scan. Figures 3-4 to 3-16 demonstrate the MS/MS spectra of the standard compounds that are shown in Figure 3-3. Kaempferol, quercertin and rutin are mainly present as molecular ions $[M-H]^-$ in the spectra, as shown in Figures 3-4, 3-5 and 3-12, suggesting that these compounds had minimum fragmentation under these conditions. Figure 3-6 shows the fragment pattern of bilobalide containing $[M-H]^-$, $[M-OH-C_4H_9]^-$, and $[M-OH-C_4H_9-CH_2]^-$. For ginkgolides A, B and C, the main peak $[M-H]^-$ and fragment peaks of $[M-H-CO]^-$ and $[M-H-CO-CO]^-$ were observed in Figures 3-7 to 3-9 respectively. In addition, a ginkgolide A adduct (m/z 467: $[GA+Ac]^-$ from GA and NH_4Ac in the buffer) and a ginkgolide C adduct (m/z 499: $[GC+Ac]^-$ from GC and NH_4Ac in the buffer) were observed in Figures 3-10 and 3-11. Ginkgolic acids I and II produced fragments of $[M-H]^-$ and $[M-COO]^-$ as shown in Figures 3-13 to 3-16. Ginkgolic acids I and II were also fragmented using higher collision energy CE of 20 eV for identification, as shown in Figures 3-14 and 3-16.

The fragment ions of individual compounds are summarized in Table 3-3. The tandem MS/MS spectra showed that bilobalide and ginkgolide A were more fragile than other components. The fragmentation patterns were useful for qualitative identification and quantitative determination.

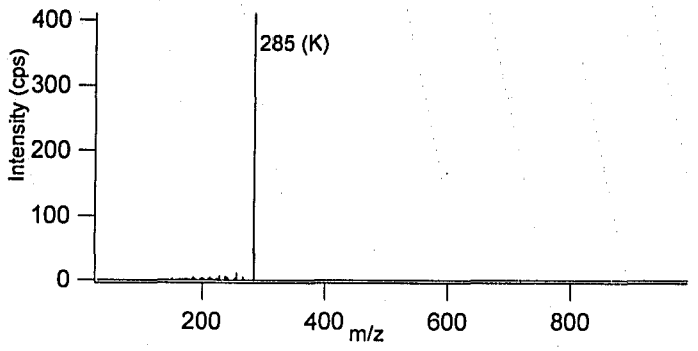


Figure 3-4. The MS/MS spectrum of m/z 285 (K) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

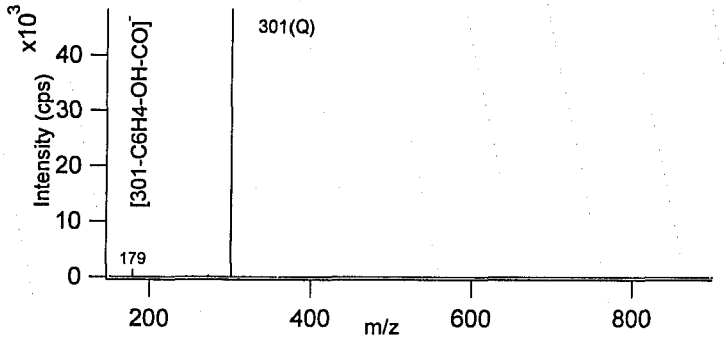


Figure 3-5. The MS/MS spectrum of m/z 301(Q) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

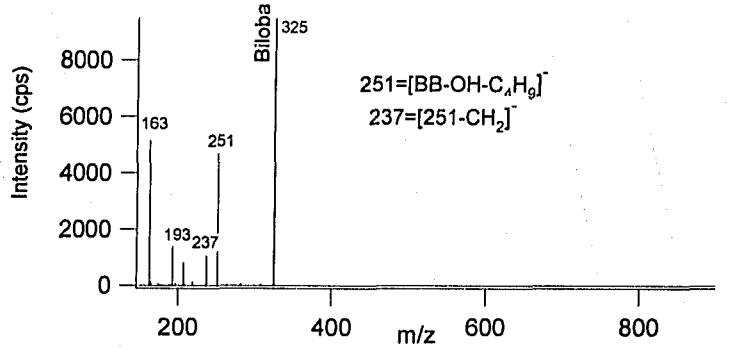


Figure 3-6. The MS/MS spectrum of m/z 325 (BB) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

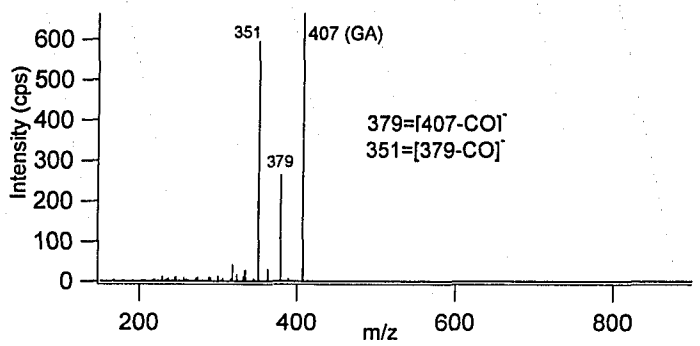


Figure 3-7. The MS/MS spectrum of m/z 407 (GA) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

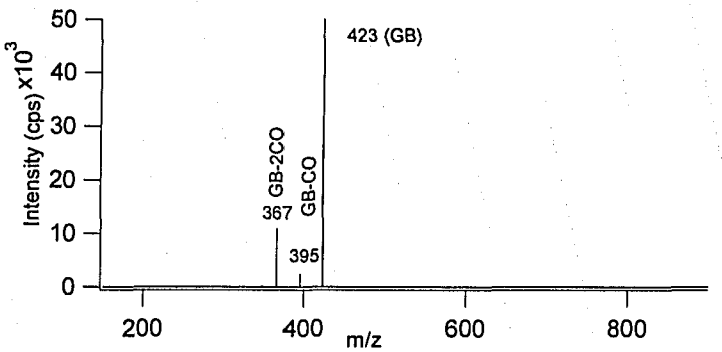


Figure 3-8. The MS/MS spectrum of m/z 423 (GB) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

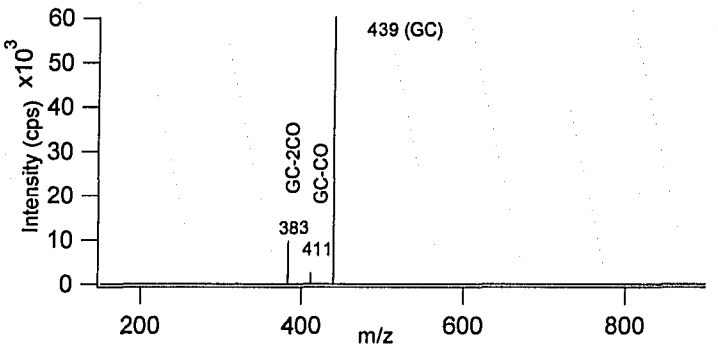


Figure 3-9. The MS/MS spectrum of m/z 439 (GC) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

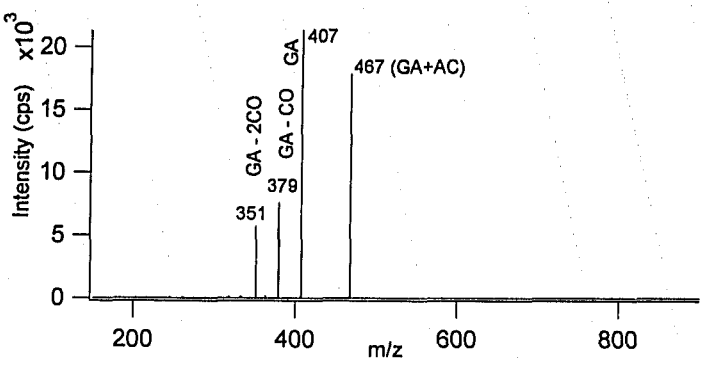


Figure 3-10. The MS/MS spectrum of m/z 467 (GA+AC) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

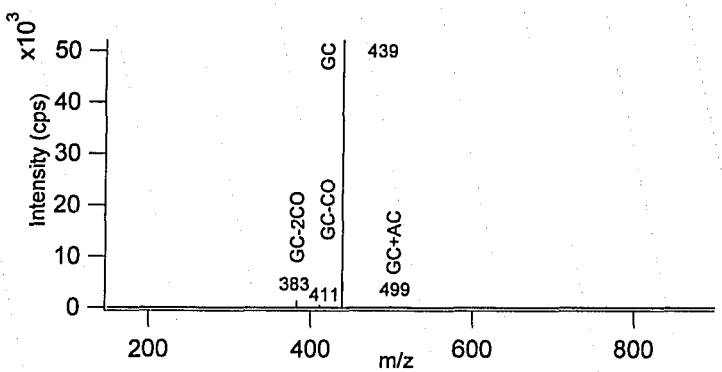


Figure 3-11. The MS/MS spectrum of m/z 499 (GC+AC) from mixed standards
DP_1-5 V, FP-5 V, electrospray voltage -900 V, CE -11.2 eV

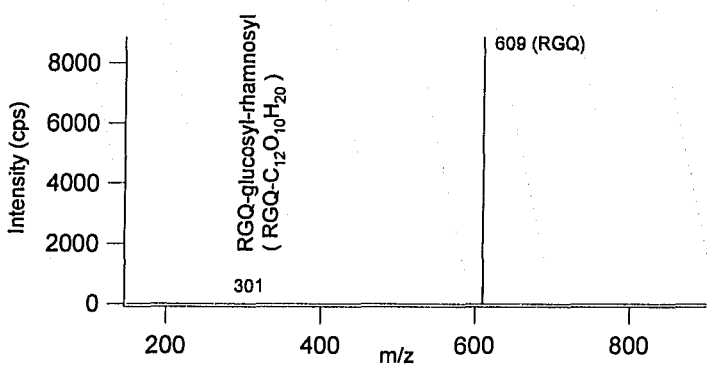


Figure 3-12. The MS/MS spectrum of m/z 609 (RGQ) from mixed standards
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

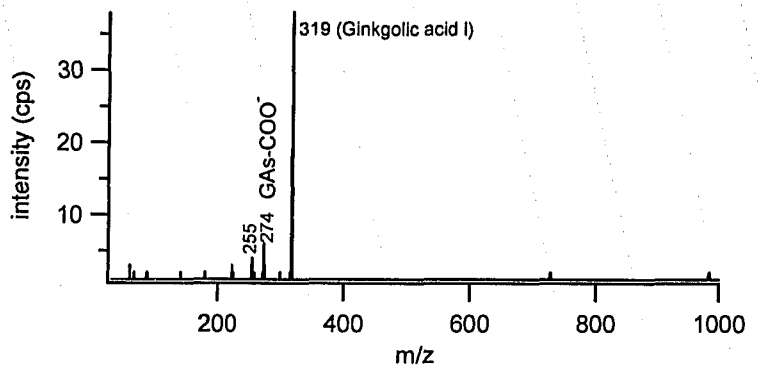


Figure 3-13. The MS/MS spectrum of m/z 319 (ginkgolic acid I)
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

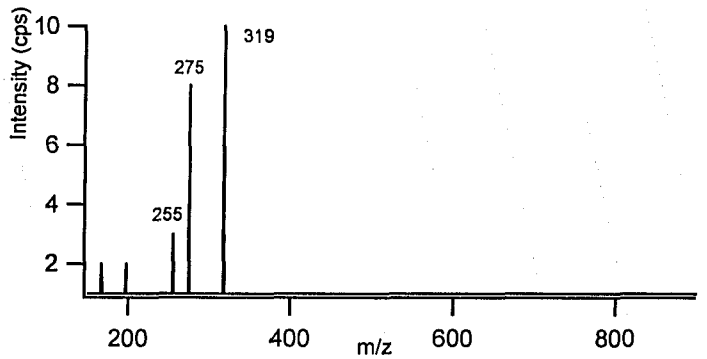


Figure 3-14. The MS/MS spectrum of m/z 319 (ginkgolic acid I) for identification
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -20 eV

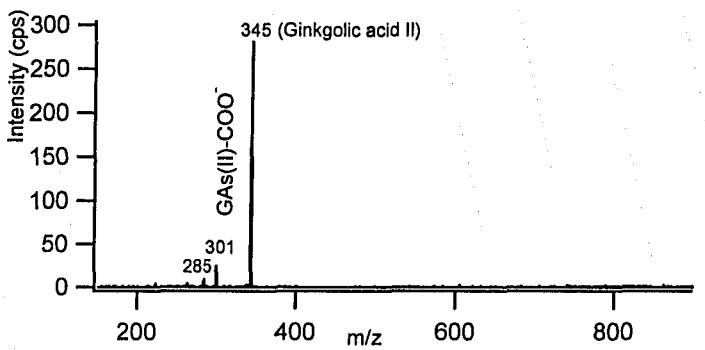


Figure 3-15. The MS/MS spectrum of m/z 345 (ginkgolic acid II)
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -11.2 eV

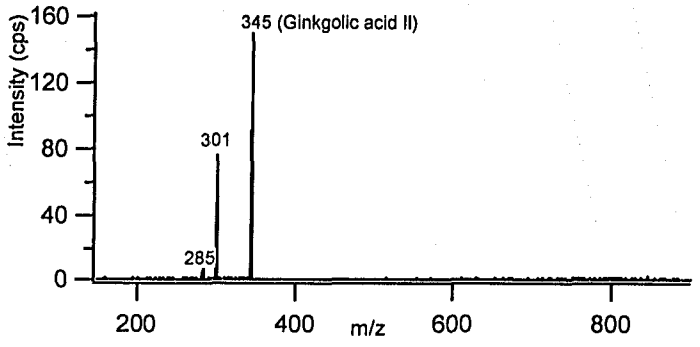


Figure 3-16. The MS/MS spectrum of m/z 345 (ginkgolic acid II) for identification
DP_1 -5 V, FP -5 V, electrospray voltage -900 V, CE -20 eV

Table 3-3. A list of MS/MS ions of each component in the mixed standard solution
Mode: negative IS: -900 V, DP_1 -5 V, FP -5 V, CE -11.2 eV

Compound	Parent Ions $[M-H]^-$ m/z	Fragment Ions (MS/MS) m/z	Features
K	285		
Q	301	179	
Ginkgolic acid I	319	275, 255	
Ginkgolic acid II	345	301, 285	
Bilobalide	325	251, 237, 193, 163	Fragile compound
Ginkgolide A	407	379, 351	Fragile compound
[a]Ginkgolide A+Ac	467	407, 379, 351	
Barbaloin	417	297	
[b]Barbaloin+Ac	477	417, 297	
Ginkgolide B	423	395, 367	
Ginkgolide C	439	411, 383	
[c]Ginkgolide C+Ac	499	439, 383, 411	
RGQ	609	301	More stable

a: acetate adduct of GA, **b**: acetate adduct of barbaloin, **c**: acetate adduct of GC

3.3.2.3 Calibration curves

A set of the mixed standard solutions was prepared to consist of 0.1 to 4 μg/mL each of ginkgolides A, B, and C, bilobalide, kaempferol, quercetin, and rutin and 5-15 μg/mL of ginkgolic acids I and II with a constant concentration (0.6 μg/mL) of the internal standard barbaloin in a buffer of 5 mM ammonium acetate in 1:1 methanol/water. The

calibration curves of ginkgolides A, B, and C, bilobalide, kaempferol, quercetin, rutin, and ginkgolic acids I and II were obtained based on the relative intensity of individual standards to that of the internal standard. Figure 3-17(A-K) shows the linear relationship between the concentration of individual components and the relative intensity (individual component over internal standard). The linear ranges and correlation coefficients of ginkgolide A, B, and C, bilobalide, kaempferol, quercetin, rutin, and ginkgolic acid I and II are summarized in Table 3-4. The linear range is limited to one order of magnitude, and the correlation coefficients are better than 0.98 for all components. This may be useful, in combination with sample dilution, because the ginkgo products usually contain higher levels of these compounds.

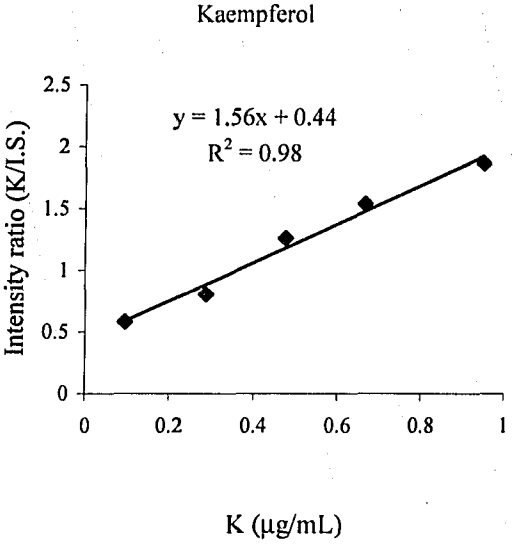


Figure 3-17A. Calibration curve of kaempferol
(Intensity counts using peak m/z 285)

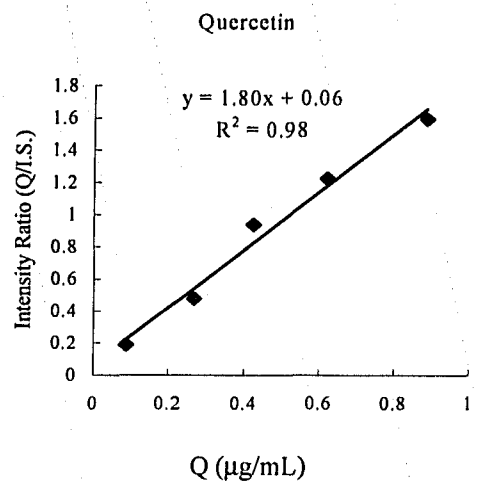


Figure 3-17B. Calibration curve of quercetin
(Intensity counts using peak m/z 301)

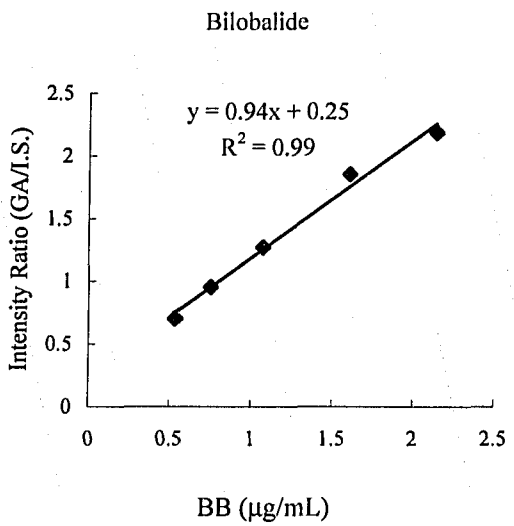


Figure 3-17C. Calibration curve of bilobalide
(Intensity counts using the total counts of peak m/z 325, 251, 237)

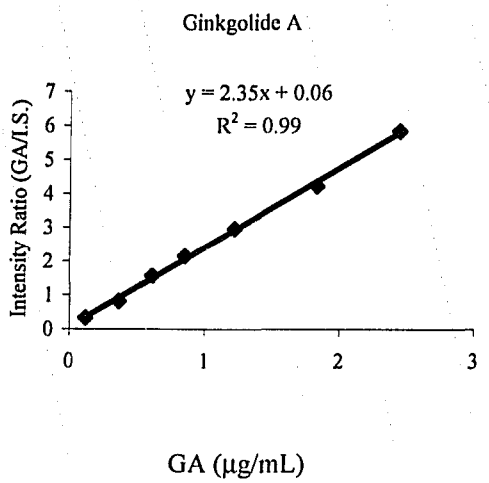


Figure 3-17D. Calibration curve of ginkgolide A
(Intensity counts using the total counts of peak m/z 407, 467, 379, 351)

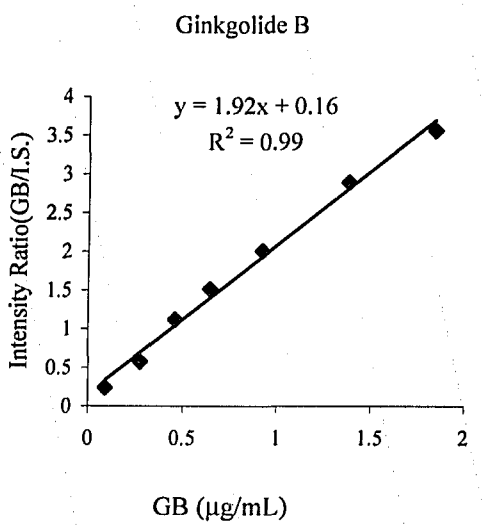


Figure 3-17E. Calibration curve of ginkgolide B
(Intensity counts using the total counts of peak m/z 423, 395, 367)

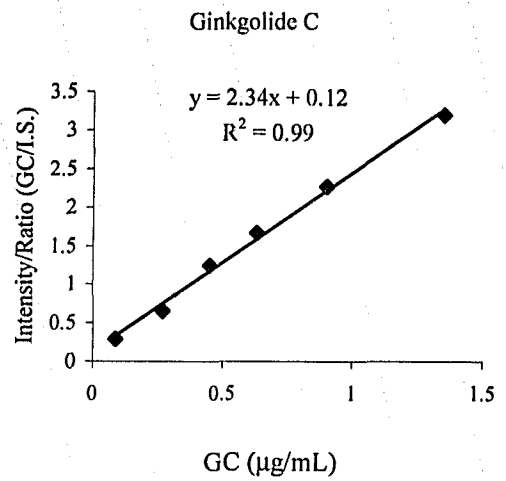


Figure 3-17F. Calibration curve of ginkgolide C
(Intensity counts using the total counts of peak m/z 439, 411, 383)

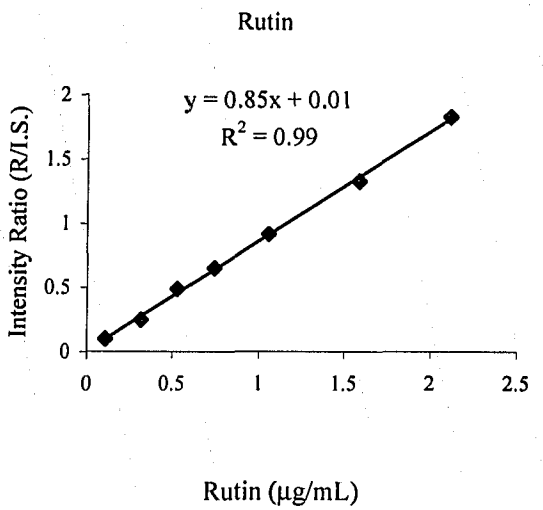


Figure 3-17G. Calibration curve of rutin
(Intensity counts using peak m/z 609)

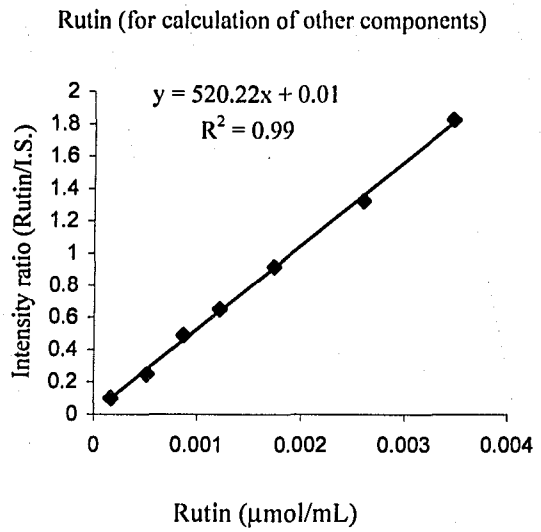


Figure 3-17H. Calibration curve of rutin used for the calculation of other flavonol glycosides
(Intensity counts using peak m/z 609)

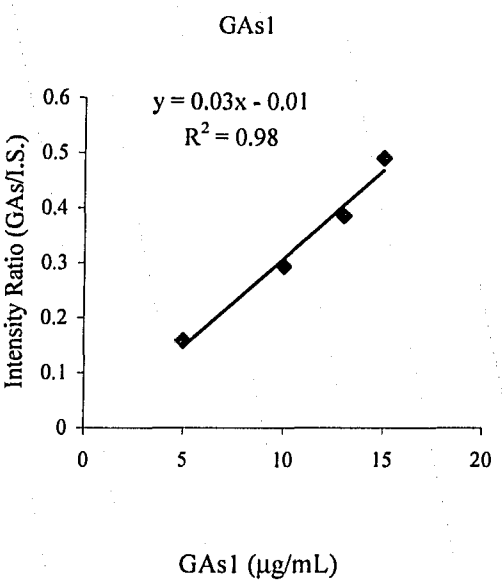


Figure 3-17I. Calibration curve of ginkgolic acid I
(Intensity counts using peak m/z 319)

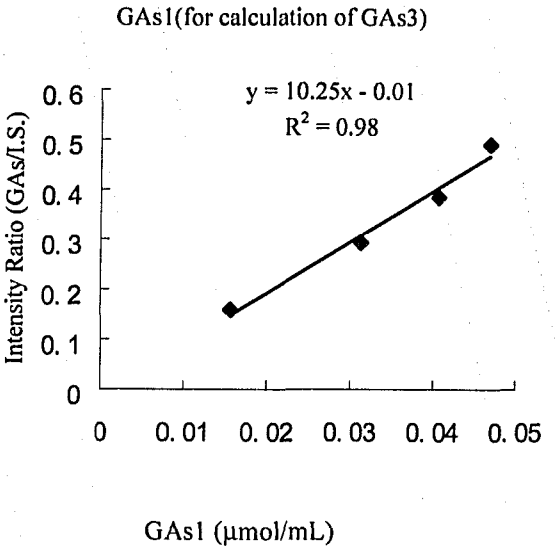


Figure 3-17J. Calibration curve of ginkgolic acid I used for the calculation of ginkgolic acid III
(Intensity counts using peak m/z 319)

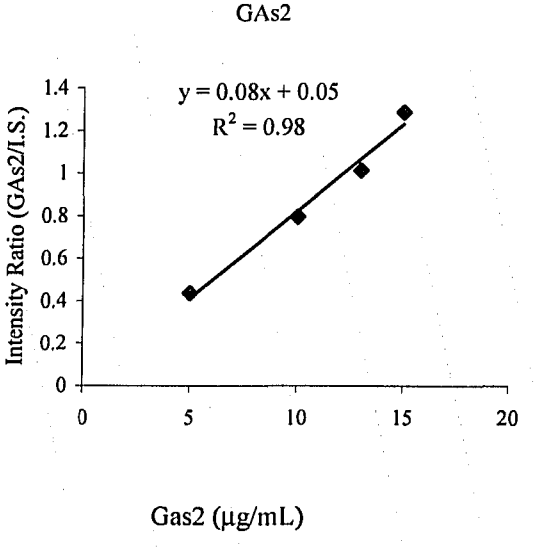


Figure 3-17K. Calibration curve of ginkgolic acid II
(Intensity counts using peak m/z 345)

Table 3-4. Linear ranges and correlation coefficients of individual calibration curves

Components	Linear Range (μg/mL)	Correlation Coefficient (R^2)
Kaempferol	0.1-1.0	0.9982
Quercetin	0.1-1.0	0.9813
Bilobalide	0.5-2.5	0.9886
Ginkgolide A	0.1-2.5	0.9980
Ginkgolide B	0.1-2.0	0.9917
Ginkgolide C	0.1-1.5	0.9947
Rutin	0.1-2.0	0.9984
Ginkgolic acid I	5-15	0.9800
Ginkgolic acid II	5-15	0.9810

3.3.3 Evaluation of sample extraction methods

3.3.3.1 Sample extraction using vortex and ultra-sonication

Vortex and ultra-sonication methods were used to examine the extraction efficiencies. Sample S1 was chosen for this evaluation because it contained three groups of ginkgo components: ginkgolides, flavonol glycosides, and ginkgolic acids.

One mg of sample S1 was dissolved in 20% MeOH/water and extracted separately using vortex or ultra-sonication following the procedures in Section 3.2.4. When sample S1 was vortexed for 1 min, the spectrum of S1 analyzed by nanoESI-MS showed that small amounts of ginkgolide A, B, and C and bilobalide were detected, as shown in Figure 3-18. When sample S1 was vortexed for 10 min, the spectrum of S1 analyzed using the same nanoESI-MS method showed that the amounts of ginkgolide A, B, and C, bilobalide, and flavonol glycosides detected had significantly increased, as shown in Figure 3-19. Figure 3-20 shows the spectrum of sample S1 obtained using ultra-sonication for 10 min and analyzed using the same nanoESI-MS method. Comparable results for extraction efficiency are shown in Figures 3-19 and 3-20. Therefore,

considering the convenience of sample pre-treatment of multiple samples, ultra-sonication for 10 minutes was chosen to extract the ginkgo samples.

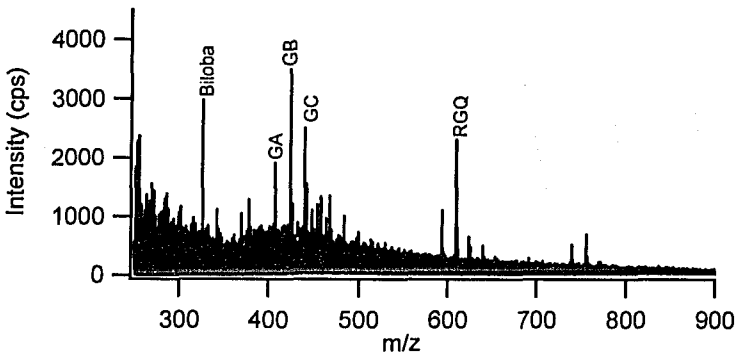


Figure 3-18. Extraction using vortex for 1 min
Mass spectrum of the extract of commercial ginkgo sample S1 using 20% MeOH after vortexing for 1 min
DP_1 -5 V, FP -5 V, electrospray voltage -900 V

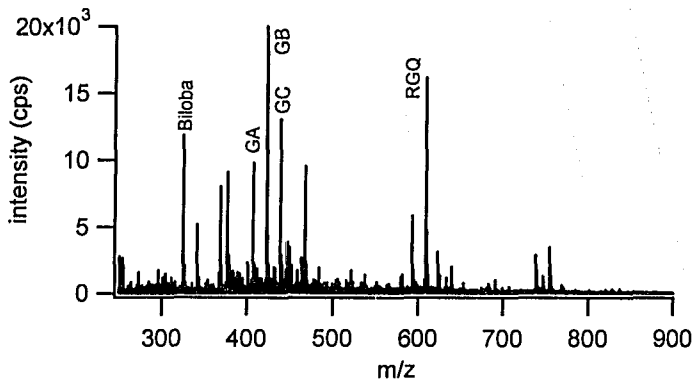


Figure 3-19. Extraction using vortex for 10 min
Mass spectrum of the extract of commercial ginkgo sample S1 using 20% MeOH after vortexing for 10 min
DP_1 -5 V, FP -5 V, electrospray voltage -900 V

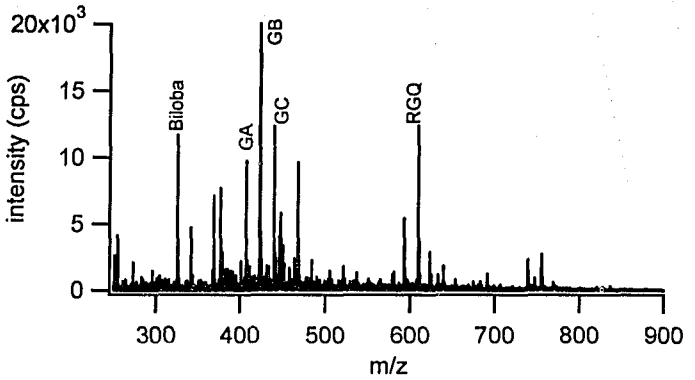
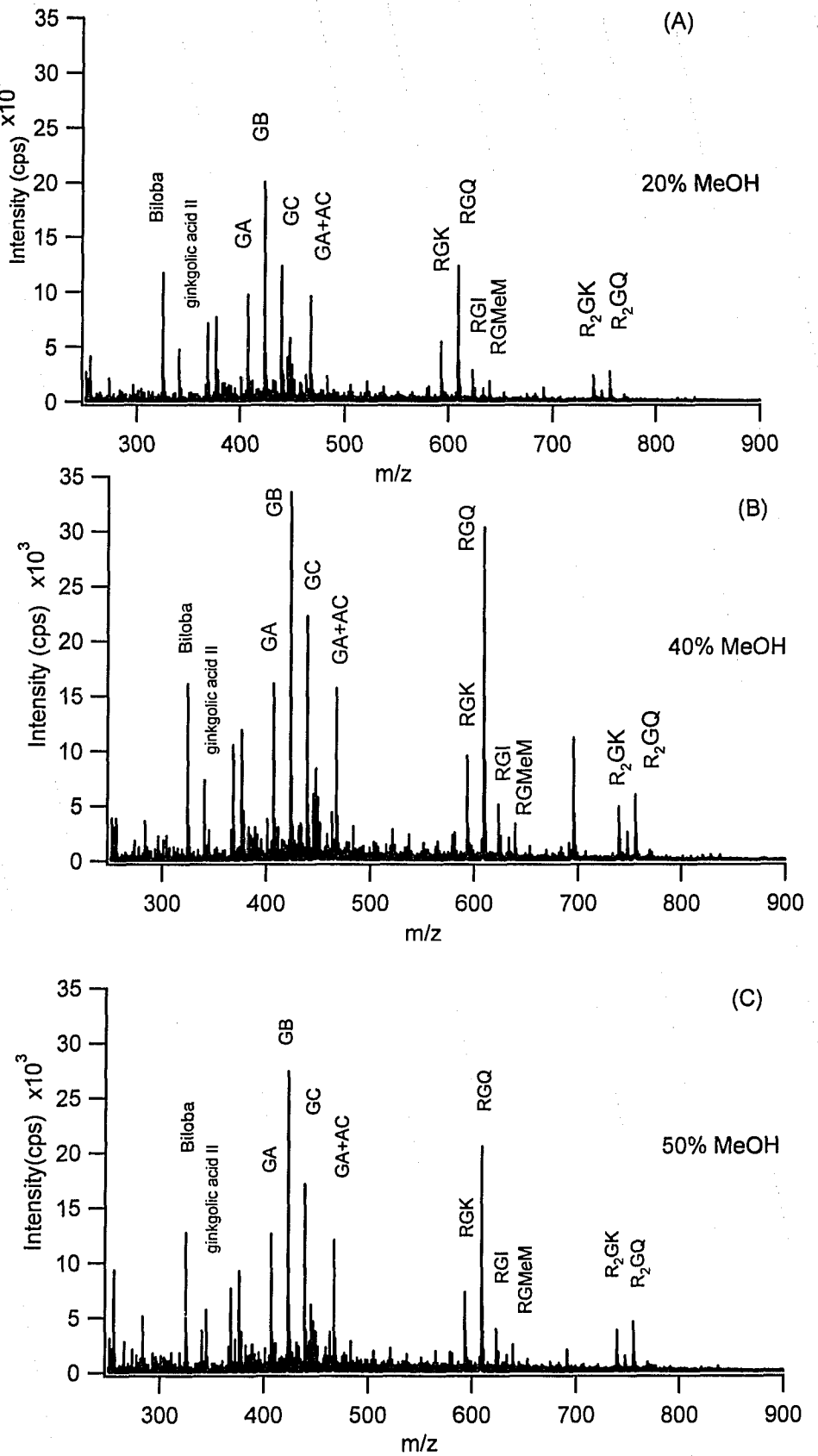


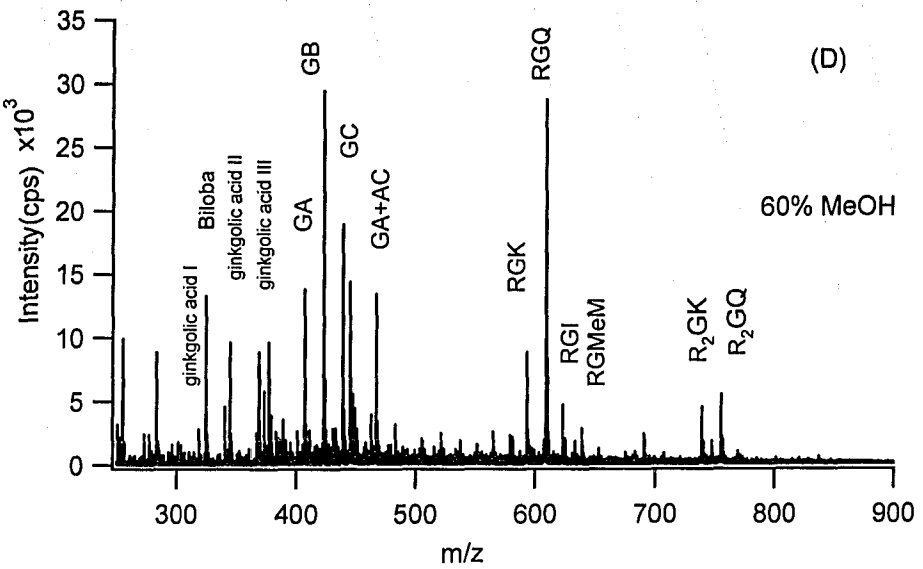
Figure 3-20. Extraction using ultra-sonication for 10 min
Mass spectrum of the extract of commercial ginkgo sample S1 using 20% MeOH after ultra-sonication for 10 min
DP_1 -5 V, FP -5 V, electrospray voltage -900 V

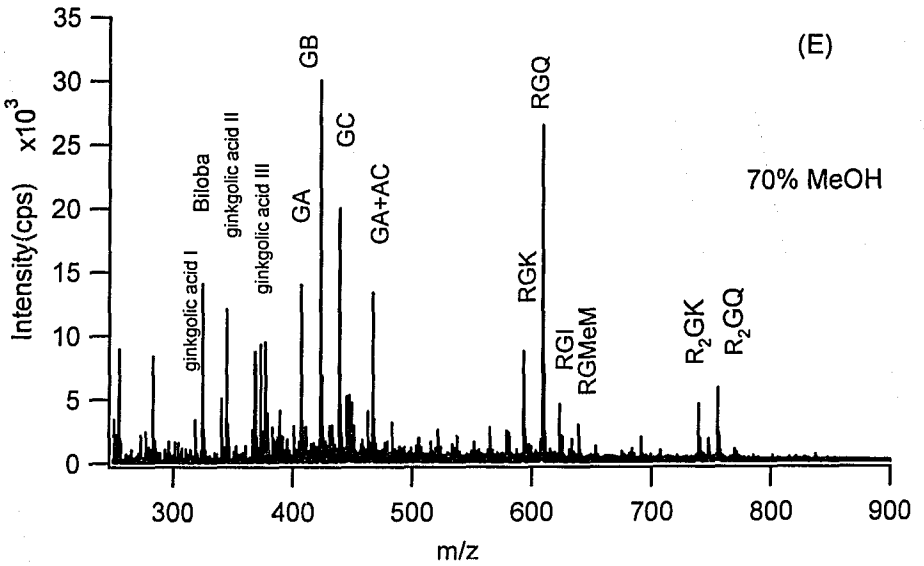
3.3.3.2 Effect of MeOH on extraction efficiency

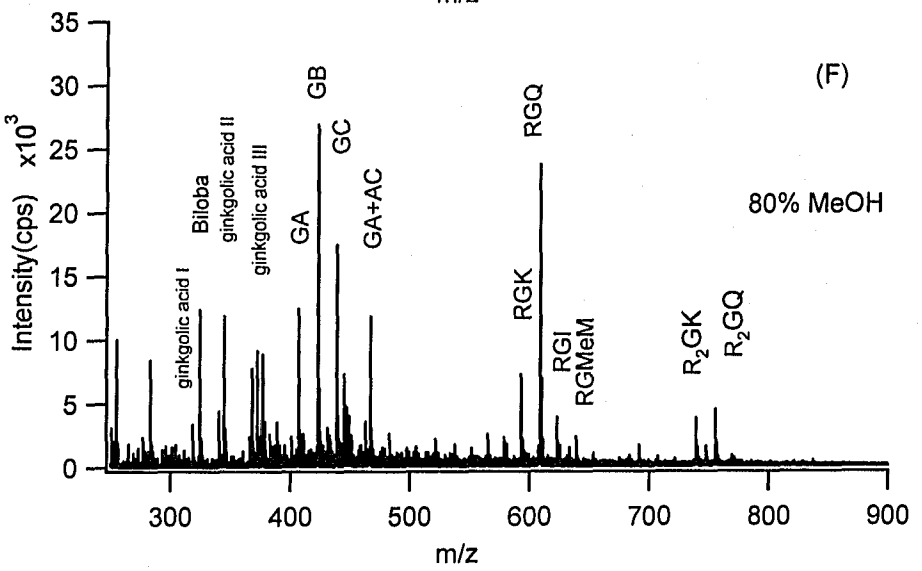
Solvents containing 10-100% MeOH in water were used to extract sample S1, which was then analyzed by the nanoESI-MS method. The sample preparation was described in Section 3.2.4. Figure 3-21 (A-C) shows the results of the extraction efficiency. When MeOH was increased from 20 to 50% in the extraction solvent, the peak intensities of ginkgolides and flavonol glycosides did not change significantly. The amounts of ginkgolic acids detected in the extraction using 20 to 50% MeOH were very small. When MeOH was increased from 60 to 100% in the extraction solvent, the amounts of ginkgolides and flavonol glycosides detected did not change significantly while the amounts of ginkgolic acids detected increased significantly, as shown in Figure 3-21 (D-H). Therefore, 100% MeOH was chosen to extract the commercial ginkgo samples.

In addition, several 1 mg of sample S1 from different capsules were extracted using 100% MeOH in sonication 10 min and analyzed by the nanoESI-MS method. The results showed similar intensities indicating that the sample was homogenous. Two examples were shown in Figures 3-22 and 3-23.









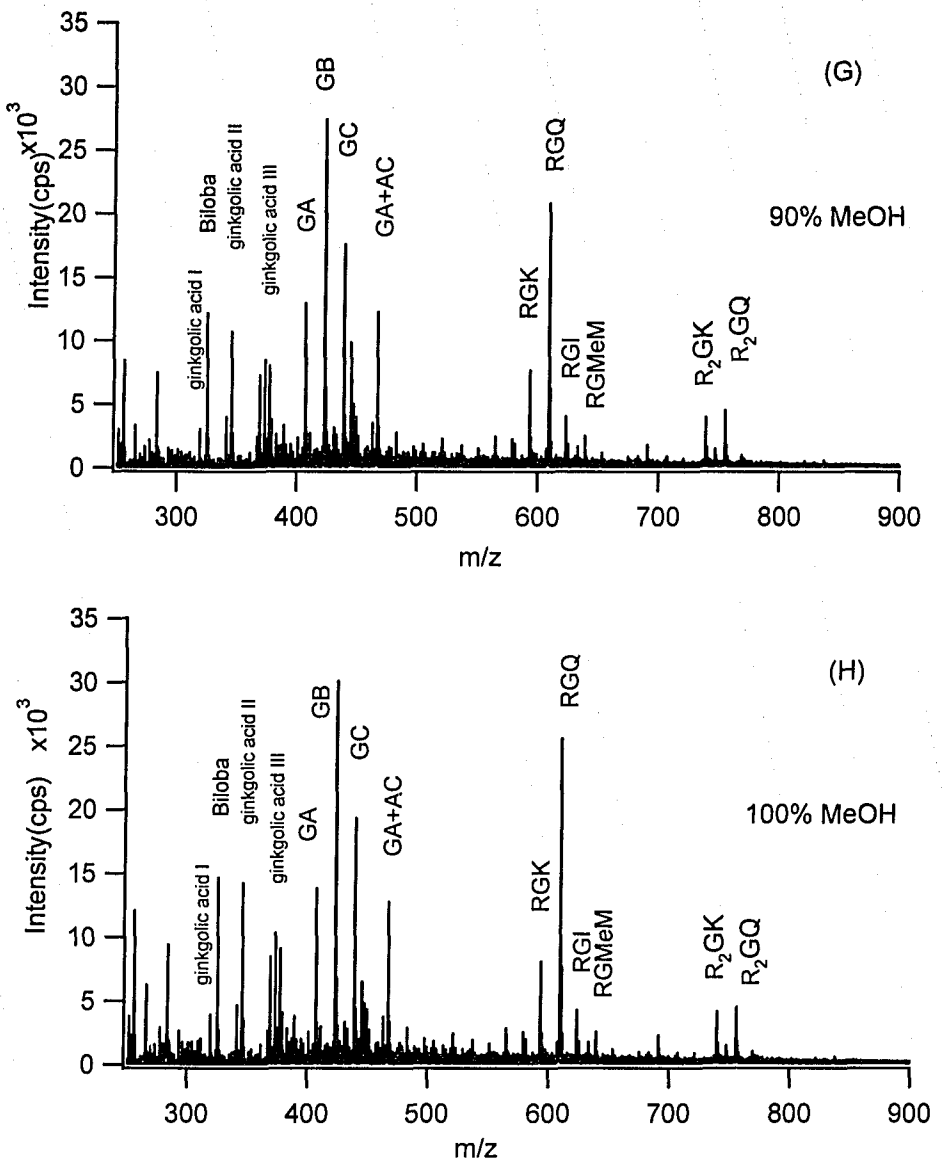


Figure 3-21. Extraction using different ratios of MeOH/water
Commercial sample S1 was extracted using 10-100% methanol and diluted to 100 μg/mL in 5 mM NH_4Ac, 1:1 MeOH/water. DP_1 -5 V, FP -5 V, electrospray voltage -900 V

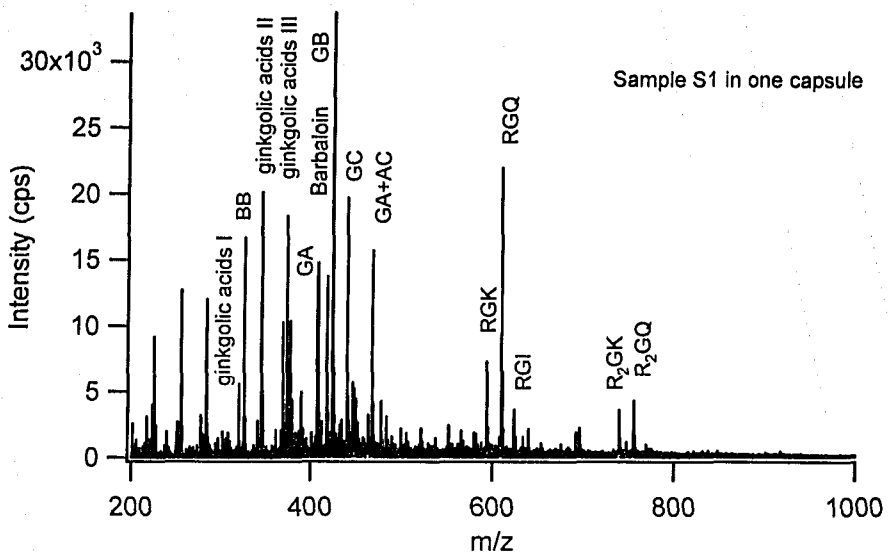


Figure 3-22. Extraction of sample S1 from one capsule
1 mg sample S1 was extracted using 100% methanol
and diluted to 100 μg/mL in 5 mM NH_4Ac, 1:1 MeOH/water
DP_1 -5 V, FP -5 V, electrospray voltage -900 V
The peak intensity ratio (each peak intensity (cps)/ internal standard):
BB 1.21; GA 1.07; GB 2.63; GC 1.43; GAs2 1.46; GAs3 1.33;
RGK 0.53; RGQ 1.59; R_2GQ 0.31

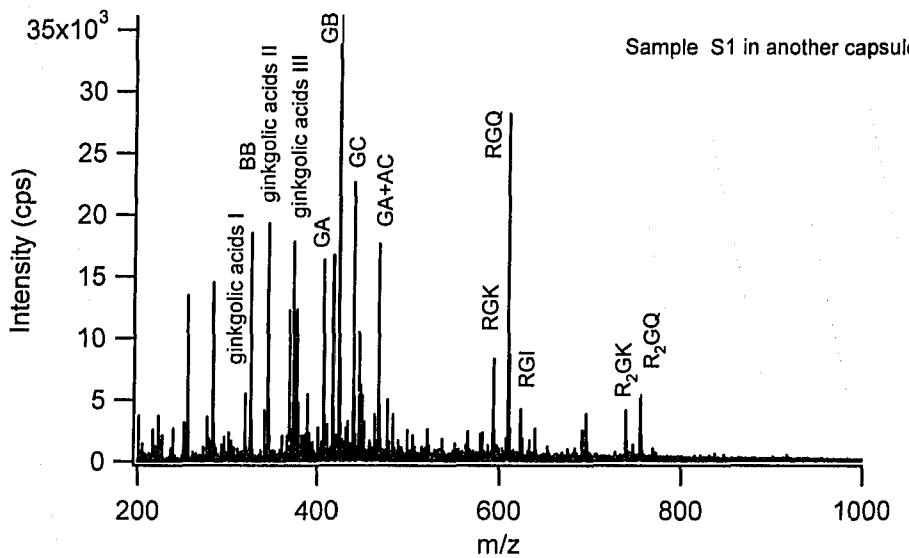


Figure 3-23. Extraction of sample S1 from another capsule
1 mg sample S1 in second capsule was extracted using
100% methanol and diluted to 100 μg/mL in 5 mM NH_4Ac,
1:1 MeOH/water DP_1 -5 V, FP -5 V, electrospray voltage -900 V
The peak intensity ratio (each peak intensity (cps)/ internal standard):
BB 1.21; GA 1.07; GB 2.63; GC 1.43; GAs2 1.46; GAs3 1.33;
RGK 0.53; RGQ 1.59; R_2GQ 0.31

3.3.4 Characterization of identified ions and mass accuracy in commercial samples

Sample S1 was extracted using 100% MeOH under ultra-sonication for 10 min, diluted to 100 μg/mL in 5 mM NH_4Ac in 1:1 MeOH/water, and analyzed by the nano-ESI-MS method. The identified ions and mass accuracy in Figure 3-21 are summarized in Tables 3-5 and 3-6.

In addition, blank samples were also extracted using identical procedures as sample S1 and analyzed by the nano-ESI-MS method. The results showed that the components in ginkgo samples did not exist in the background (Figure 3-24).

Table 3-5. Identified ions in the commercial ginkgo sample S1

Negative mode, DP_1 -5 V, FP -5 V, electrospray voltage -900 V

m/z	Identified Compounds		Ion Adducts
	MW (amu)	Compound Name	
285.04	286.05 $C_{15}H_{10}O_6$	Kaempferol	-H
301.04	302.04 $C_{15}H_{10}O_7$	Quercetin	-H
315.05	316.06 $C_{16}H_{12}O_7$	Isorhamnetin	-H
319.22	320.24 $C_{20}H_{29}O_3$	Ginkgolic acid I	-H
325.09	326.10 $C_{15}H_{18}O_8$	Bilobalide	-H
345.25	346.25 $C_{22}H_{33}O_3$	Ginkgolic acid II	-H
374.28	374.28 $C_{24}H_{38}O_3$	Ginkgolic acid III	-H
407.14	408.14 $C_{20}H_{24}O_9$	Ginkgolide A	-H
423.13	424.14 $C_{20}H_{24}O_{10}$	Ginkgolide B (Ginkgolide J)	-H
439.12	440.13 $C_{20}H_{24}O_{11}$	Ginkgolide C	-H
593.15	594.16 $C_{27}H_{30}O_{15}$	3-O-(6''-O-(α-L-rhamnosyl)-β-D-glycosyl) Kaempferol	-H
609.15	610.15 $C_{27}H_{30}O_{16}$	(1)3-O-(6''-O-(α-L-rhamnosyl)-β-D-glycosyl) Quercetin (2)3-O-(2''-O-(β-D-glycosyl)-α-L-rhamnosyl) Quercetin	-H
623.16	624.17 $C_{28}H_{32}O_{16}$	3-O-(6''-O-(α-L-rhamnosyl)-β-D-glycosyl) Isorhamnetin	-H
639.16	640.16 $C_{28}H_{32}O_{17}$	3-O-(6''-O-(α-L-rhamnosyl)-β-D-glycosyl)-3'-methylmyricetin	-H
739.20	740.22 $C_{33}H_{40}O_{19}$	3-O-(2''-O,6''-O-bis(α-L-rhamnosyl)-β-D-glycosyl) Kaempferol	-H
755.20	756.21 $C_{33}H_{40}O_{20}$	3-O-(2''-O,6''-O-bis(α-L-rhamnosyl)-β -D-glycosyl) Quercetin	-H
769.22	770.23 $C_{34}H_{42}O_{20}$	3-O-(2''-O, 6''-O-bis(α-L-rhamnosyl-β-D-glycosyl) Isorhamnetin	-H

Table 3-6. Accurate mass measurement of terpenes, flavonol glycosides, and ginkgolic acids in the commercial ginkgo sample S1

	Compound	Theoretical Molecular Weight (amu)	Experimental Molecular Weight (amu)	Mass Accuracy (ppm)
Terpenes	Bilobalide	326.1002	326.1002	0.00
	Ginkgolide A	408.1420	408.1448	6.86
	Ginkgolide B	424.1369	424.1379	2.36
	Ginkgolide C	440.1319	440.1298	-4.77
Ginkgolic acids	Ginkgolic acid I	320.2351	320.2353	0.63
	Ginkgolic acid II	346.2508	346.2533	7.23
	Ginkgolic acid III	374.2821	374.2783	-10.2
Aglycone	K	286.0477	286.0480	1.05
	Q	302.0427	302.0510	27.5
	I	316.0583	316.0644	19.3
Flavonol glycosides	RGK	594.1585	594.1586	0.17
	RGQ	610.1534	610.1534	0.00
	RGI	624.1690	624.1719	4.65
	RGMeM	640.1640	640.1698	9.06
	R_2GK	740.2164	740.2071	-12.6
	R_2GQ	756.2113	756.2053	-7.94
	R_2GI	770.2269	770.2316	6.10

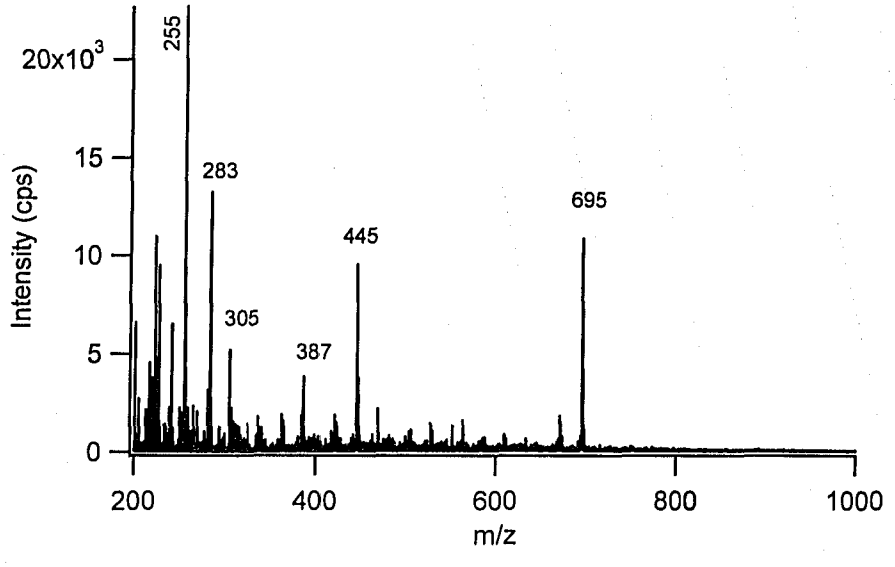


Figure 3-24. Mass spectrum of blank sample
5 mM ammonium acetate in 1:1 MeOH/water
DP_1 -5 V, FP -5 V, electrospray voltage -900 V

3.3.5 Reproducibility

Seven replicates of sample S1 were extracted by 100% MeOH, diluted to 100 μg/mL with 5 mM ammonium acetate in 1:1 MeOH:water with 0.6 μg/mL barbaloin as an internal standard, and analyzed by the nanoESI-MS method. The relative peak intensities of the individual peaks over that of the internal standard peak for different components were obtained and the relative standard deviations (RSD) of the peak intensities were calculated (Table 3-7). The relative standard deviations were 5-14 %, except for the low concentration of kaempferol, which was 31 %. These results demonstrate good reproducibility of the method.

Table 3-7. Reproducibility of relative peak intensities of sample S1

Sample	Replicates / Relative Intensity (cps)							Standard	Average	RSD
S1	1	2	3	4	5	6	7	Deviation		%
K	0.210	0.123	0.198	0.276	0.108	0.232	0.204	0.06	0.19	31
Q	0.170	0.124	0.153	0.124	0.168	0.127	0.147	0.02	0.14	14
I	0.034	0.030	0.033	0.030	0.029	0.030	0.031	0.00	0.03	6
BB	0.937	0.811	0.948	0.882	0.791	0.919	0.932	0.06	0.89	7
GAs1	0.226	0.197	0.234	0.226	0.180	0.240	0.221	0.02	0.22	10
GAs2	0.795	0.721	0.864	0.818	0.653	0.865	0.766	0.08	0.78	10
GAs3	0.734	0.648	0.794	0.759	0.605	0.818	0.635	0.08	0.71	12
GA	1.947	1.656	1.914	1.809	1.760	1.825	1.952	0.11	1.84	6
GB	1.643	1.502	1.693	1.574	1.440	1.654	1.734	0.11	1.61	7
GC	1.340	1.169	1.335	1.228	1.167	1.276	1.349	0.08	1.27	6
RGK	0.343	0.300	0.319	0.294	0.326	0.306	0.321	0.02	0.32	5
RGQ	1.161	0.996	0.933	0.925	1.027	0.924	1.014	0.08	1.00	8
RGI	0.175	0.151	0.158	0.150	0.168	0.151	0.159	0.01	0.16	6
RGMeM	0.109	0.093	0.100	0.092	0.105	0.094	0.101	0.01	0.10	7
R2GK	0.171	0.154	0.161	0.148	0.163	0.156	0.158	0.01	0.16	5
R2GQ	0.221	0.197	0.196	0.178	0.207	0.188	0.200	0.01	0.20	7
R2GI	0.044	0.037	0.040	0.038	0.038	0.041	0.040	0.00	0.04	6

3.3.6 Quantitative analysis of commercial ginkgo products

Commercial ginkgo samples were obtained from 14 different suppliers (S1-S14). Different lots of samples S1, S2, and S14 (S1-1, S1-2, S1-3, S2-1, S2-2, S2-3, S14-1, S14-2) as well as four standard ginkgo extracts were also obtained (Table 3-1). Each sample was extracted using 100% MeOH under ultra-sonication for 10 min, diluted to 100 μg/mL with 5 mM ammonium acetate in 1:1 MeOH:water, and analyzed by the nanoESI-MS method using 0.6 μg/mL barbaloin as the internal standard. Quantification

of individual components was accomplished using calibration curves, which were constructed based on the ratio of the intensity of the individual components to the internal standard versus the individual components concentration. The intensity ratio of each component over the internal standard is shown in Table 3-8. Commercial samples S6 and S8 were diluted to 400 μg/mL because the concentrations of those two samples were low. Four "standard" extracts (g(1), g(2), g(3), and GK) were diluted to 50 μg/mL.

The relative standard deviations of the peak intensity of the different lot samples (S1, S2, and S14) are calculated in Table 3-9 to 3-11. The results showed that samples were consistent in composition.

Table 3-8. The peak intensity ratios of different components in ginkgo products

	Ginkgolides				**Ginkgolic acids**			**Aglycones**		
Sample	BB /I.S.	GA /I.S	GB/ I.S.	GC/ I.S.	GAs1 /I.S.	GAs2 /I.S.	GAs3 /I.S.	K/I.S.	Q/I.S.	I/I.S.
S1	0.89	1.63	1.67	1.13	0.24	0.89	0.80	0.05	0.08	0.03
S1-1	0.83	1.35	0.96	0.75	0.21	0.74	0.65	0.11	0.15	0.06
S1-2	0.76	1.82	2.05	1.18	0.16	0.43	0.41	0.06	0.08	0.03
S2	0.77	1.69	1.59	0.89	ND	ND	ND	0.18	0.34	0.10
S2-1	0.61	1.30	1.26	0.66	ND	ND	ND	0.19	0.33	0.11
S2-2	0.50	1.11	1.08	0.56	ND	ND	ND	0.08	0.27	0.10
S3	0.39	1.06	1.14	0.63	ND	ND	ND	0.12	0.18	0.06
S4	0.33	1.04	1.94	0.68	ND	ND	ND	0.38	0.60	0.21
S5	0.94	1.16	1.32	0.78	ND	ND	ND	0.04	0.05	0.03
S6	1.04	1.09	1.15	1.15	ND	ND	ND	0.07	ND	ND
S7	0.48	1.04	1.11	0.68	0.25	0.58	0.56	0.20	0.34	0.11
S8	1.18	1.10	1.24	1.20	ND	ND	ND	0.11	0.05	0.04
S9	0.48	1.41	1.21	0.73	ND	ND	ND	0.05	0.07	0.03
S10	0.79	1.47	1.38	0.96	ND	ND	ND	0.05	0.07	0.03
S11	1.14	1.15	1.26	1.05	ND	ND	ND	0.14	0.17	0.08
S12	0.74	0.75	0.59	0.76	ND	ND	ND	0.06	0.08	0.03
S13	0.67	1.05	1.24	0.59	ND	ND	ND	0.03	0.02	ND
S14-1	0.16	0.56	0.57	0.44	ND	ND	ND	ND	ND	0.02
S14-2	0.29	0.75	0.67	0.47	ND	ND	ND	0.03	0.03	0.02
g(1)	1.41	1.27	0.77	1.36	ND	ND	ND	0.04	0.05	ND
g(2)	1.35	1.89	1.92	1.54	ND	ND	ND	0.04	0.06	ND
g(3)	1.72	1.71	1.15	1.51	ND	ND	ND	ND	0.06	ND

GK	1.04	1.43	1.34	1.17	0.08	0.29	0.13	0.05	0.05	0.02

ND: not detected

Table 3-8 (cont.): The peak intensity ratios of different components in ginkgo products

Sample	Glycosides						
	RGK /I.S.	RGQ /I.S.	RGI /I.S.	RGMeM /I.S.	R2GK /I.S.	R2GQ /I.S.	R2GI /I.S.
S1	0.35	1.06	0.18	0.11	0.18	0.21	0.04
S1-1	0.30	0.73	0.15	0.08	0.15	0.14	0.03
S1-2	0.30	1.13	0.15	0.09	0.15	0.16	0.03
S2	0.37	1.55	0.16	0.07	0.16	0.17	0.03
S2-1	0.35	1.69	0.15	0.07	0.14	0.14	0.03
S2-2	0.28	1.30	0.13	0.05	0.11	0.12	0.03
S3	0.21	0.81	0.09	0.04	0.09	0.10	ND
S4	0.23	1.44	0.07	0.03	0.16	0.18	0.03
S5	0.58	0.58	0.31	0.13	0.19	0.18	0.04
S6	0.66	0.63	0.31	0.15	0.25	0.22	ND
S7	0.17	0.45	0.06	ND	0.10	0.10	ND
S8	0.60	0.53	0.27	0.13	0.25	0.19	0.04
S9	0.32	0.70	0.17	0.10	0.14	0.14	0.04
S10	0.33	0.83	0.16	0.10	0.15	0.17	0.04
S11	0.33	0.36	0.16	0.08	0.17	0.16	0.04
S12	0.30	0.42	0.15	0.09	0.13	0.15	0.03
S13	0.28	0.34	0.15	0.08	0.13	0.12	0.03
S14-1	0.15	0.23	0.07	0.05	0.06	0.06	0.02
S14-2	0.11	0.20	0.05	0.03	0.05	0.05	ND
g(1)	0.48	0.77	0.22	0.15	0.20	0.22	0.06
g(2)	0.52	0.78	0.24	0.18	0.22	0.28	0.06
g(3)	0.60	0.90	0.30	0.22	0.22	0.30	0.07
GK	0.37	0.49	0.18	0.12	0.19	0.22	0.04

Table 3-9. Reproducibility of different components of sample S1 in different lots

	Ginkgolides				Ginkgolic acids			Aglycones		
Sample	BB /I.S.	GA /I.S	GB/ I.S.	GC/ I.S.	GAs1 /I.S.	GAs2 /I.S.	GAs3 /I.S.	K/I.S.	Q/I.S.	I/I.S.
S1	0.893	1.632	1.671	1.128	0.238	0.889	0.800	0.052	0.079	0.034
S1-1	0.834	1.345	0.965	0.748	0.206	0.741	0.651	0.105	0.147	0.059
S1-2	0.761	1.817	2.051	1.176	0.157	0.426	0.414	0.055	0.078	0.028
Average	0.829	1.598	1.562	1.017	0.200	0.686	0.622	0.071	0.101	0.040
RSD%	8	15	35	23	20	34	31	42	39	40

	Glycosides						
	RGK /I.S.	RGQ /I.S.	RGI /I.S.	RGMeM /I.S.	R2GK /I.S.	R2GQ /I.S.	R2GI /I.S.
S1	0.351	1.058	0.184	0.112	0.178	0.205	0.043
S1-1	0.296	0.734	0.151	0.077	0.151	0.136	0.032
S1-2	0.299	1.134	0.151	0.086	0.148	0.159	0.035
Average	0.315	0.975	0.162	0.091	0.159	0.167	0.037
RSD%	10	22	12	20	10	21	16

Table 3-10. Reproducibility of different components of sample S2 in different lots

	Ginkgolides				Ginkgolic acids			Aglycones		
Sample	BB /I.S.	GA /I.S	GB/ I.S.	GC/ I.S.	GA s1 /I.S /I.S .	GAs2 /I.S.	GAs3 /I.S.	K/I.S.	Q/I.S.	I/I.S.
S2	0.771	1.689	1.595	0.892	ND	ND	ND	0.176	0.337	0.098
S2-1	0.611	1.298	1.263	0.663	ND	ND	ND	0.189	0.330	0.109
S2-2	0.497	1.113	1.082	0.557	ND	ND	ND	0.078	0.275	0.101
Average	0.626	1.366	1.313	0.704				0.147	0.314	0.103
RSD%	22	22	20	24				41	11	6

	Glycosides						
	RGK /I.S.	RGQ /I.S.	RGI /I.S.	RGMeM /I.S.	R2GK /I.S.	R2GQ /I.S.	R2GI /I.S.
S2	0.369	1.551	0.161	0.072	0.162	0.166	0.034
S2-1	0.345	1.688	0.153	0.068	0.139	0.141	0.033

S2-2	0.282	1.304	0.129	0.053	0.114	0.120	0.028
Average	0.332	1.515	0.148	0.064	0.138	0.142	0.032
RSD%	14	13	11	16	17	16	10

Table 3-11. Reproducibility of different components of sample S14 in different lots

Sample	Ginkgolides				Ginkgolic acids			Aglycones		
	BB /I.S.	GA /I.S	GB/ I.S.	GC/ I.S.	GAs1 /I.S.	GAs2 /I.S.	GAs3 /I.S.	K/I.S.	Q/I.S.	I/I.S.
S14-1	0.160	0.561	0.571	0.442	ND	ND	ND	ND	ND	0.023
S14-2	0.290	0.753	0.667	0.472	ND	ND	ND	0.025	0.033	0.025
Average	0.225	0.657	0.619	0.457				0.025	0.033	0.024
RSD%	41	21	11	5						

	Glycosides						
	RGK /I.S.	RGQ /I.S.	RGI /I.S.	RGMeM /I.S.	R2GK /I.S.	R2GQ /I.S.	R2GI /I.S.
S14-1	0.152	0.228	0.074	0.046	0.060	0.058	0.017
S14-2	0.110	0.195	0.047	0.032	0.049	0.050	ND
Average	0.131	0.212	0.060	0.039	0.054	0.054	0.017
RSD%	23	11	32	25	13	10	

The concentration of each aglycone in the commercial ginkgo products and "standard" extracts is shown in Table 3-12. The total concentration of aglycones in the samples ranged from 0.00 to 0.5%. The total concentration of aglycones in the "standard" extracts was lower than in the commercial samples. Quercetin presented a higher concentration than other aglycones in the commercial samples. Samples S2, S4, and S7 had a higher concentration of quercetin (S2: 1.4 μg/mg; S4: 2.9 μg/mg; S7: 1.4 μg/mg) while the "standard" extracts contained undetectable quercetin. The higher concentration of quercetin demonstrated the possibility of adulteration with synthetic quercetin. The possibility of adulteration with synthetic flavonoids can also be seen in the spectrum of sample S2 in Figure 3-25. Similar results were reported in the literature.[13]

Table 3-12. The concentration of aglycones in ginkgo products

Sample	K (μg/mg)	Q (μg/mg)	I (μg/mg)	Sum (K+Q+I) (μg/mg)	(%)
S1	ND	ND	ND	ND	ND
S1-1	ND	0.2	ND	0.2	0.0
S1-2	ND	ND	ND	ND	ND
S2	ND	1.4	ND	1.4	0.1
S2-1	ND	1.3	0.04	1.3	0.1
S2-2	ND	1.0	ND	1.0	0.1
S3	ND	0.5	ND	0.5	0.1
S4	ND	2.9	1.59	4.5	0.5
S5	ND	ND	ND	ND	ND
S6	ND	ND	ND	ND	ND
S7	ND	1.4	0.09	1.5	0.2
S8	ND	ND	ND	ND	ND
S9	ND	ND	ND	ND	ND
S10	ND	ND	ND	ND	ND
S11	ND	0.4	ND	0.4	0.0
S12	ND	ND	ND	ND	ND
S13	ND	ND	ND	ND	ND
S14-1	ND	ND	ND	ND	ND
S14-2	ND	ND	ND	ND	ND
g(1)	ND	ND	ND	ND	ND
g(2)	ND	ND	ND	ND	ND
g(3)	ND	ND	ND	ND	ND
GK	ND	ND	ND	ND	ND

(I) was calculated using the calibration curve of quercetin
ND: not detected

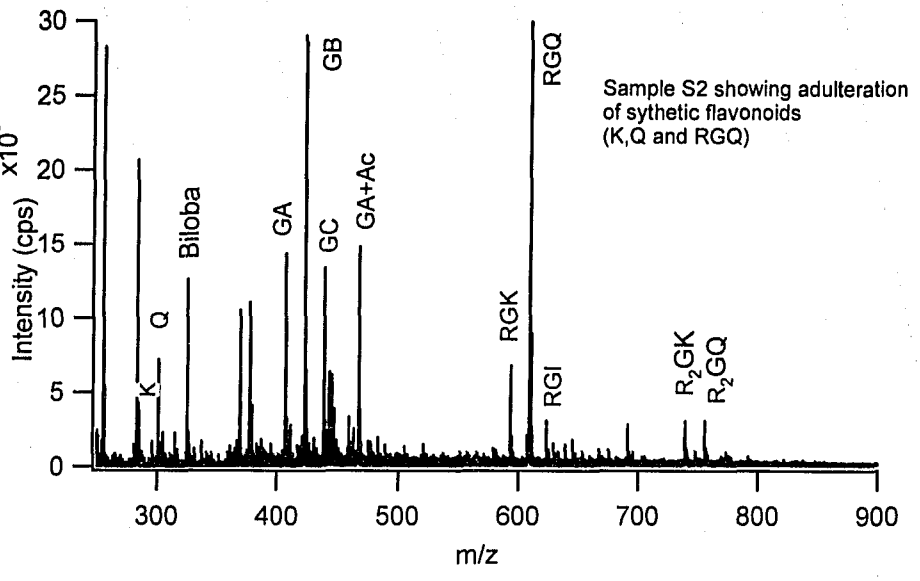


Figure 3-25. Mass spectrum of sample S2
5 mM ammonium acetate in 1:1 MeOH/water
DP_1 -5 V, FP -5 V, electrospray voltage -900 V

The concentrations of di- and tri-glycosides in commercial ginkgo products and "standard" extracts were calculated as shown in Table 3-13A. The total concentration of di- and tri-glycosides in commercial ginkgo products ranged from 0.5 to 3.0%, while in "standard" extracts it ranged from 7.8 to 12.5%. The quantification results need to be validated by other methods. The concentration ratios of each flavonol glycoside over RGK were useful, as shown in Table 3-13B. Samples S1, S2, and S4 had a relatively high RGQ/RGK ratio (S1: RGQ/RGK=3.1; S2: RGQ/RGK=4.4; S4: RGQ/RGK=6.7; "standard" extract g(3): RGQ/RGK=1.5) demonstrating the possibility of adulteration with the synthetic flavonoids (see the spectrum of sample S2 in Figure 3-22). This is consistent with the published data[14] that showed the adulteration of one commercial sample, which contained a rutin concentration that was six times higher than the other products.

Table 3-13A. The concentration of di- and tri-glycosides in ginkgo products

Sample	RGK	RGQ	RGI	RGMeM	R2GK	R2GQ	R2GI	Sum	
	μg/mg	μg/mg	μg/mg	μg/mg	μg/mg	μg/mg	μg/mg	μg/mg	%
S1	3.9	12.3	2.1	1.3	2.4	2.9	0.5	25.4	2.5
S1-1	3.3	8.5	1.7	0.9	2.0	1.9	0.4	18.6	1.9
S1-2	3.3	13.2	1.7	1.0	2.0	2.2	0.4	23.8	2.4
S2	4.1	18.1	1.8	0.8	2.2	2.3	0.4	29.7	3.0
S2-1	3.9	19.7	1.8	0.8	1.9	1.9	0.4	30.2	3.0
S2-2	3.1	15.2	1.5	0.6	1.5	1.6	0.3	23.8	2.4
S3	2.4	9.4	1.0	0.5	1.2	1.3	0.0	15.7	1.6
S4	2.5	16.7	0.8	0.3	2.2	2.6	0.3	25.5	2.6
S5	6.5	6.7	3.6	1.5	2.6	2.6	0.5	24.0	2.4
S6	1.9	1.8	0.9	0.5	0.9	0.8	0.0	6.7	0.7
S7	1.9	5.2	0.6	0.0	1.4	1.3	0.0	10.2	1.0
S8	1.7	1.5	0.8	0.4	0.9	0.7	0.1	6.0	0.6
S9	3.5	8.1	2.0	1.2	1.9	2.0	0.4	19.1	1.9
S10	3.6	9.7	1.9	1.2	2.0	2.4	0.4	21.1	2.1
S11	3.7	4.1	1.8	0.9	2.3	2.3	0.5	15.6	1.6
S12	3.3	4.8	1.7	1.0	1.8	2.1	0.4	15.0	1.5
S13	3.1	3.9	1.7	0.9	1.7	1.6	0.3	13.3	1.3
S14-1	1.7	2.6	0.8	0.5	0.7	0.7	0.1	7.1	0.7
S14-2	1.2	2.2	0.5	0.3	0.6	0.6	0.0	5.4	0.5
g(1)	21.5	35.7	10.2	6.8	11.1	12.5	3.0	100.7	10.1
g(2)	23.2	36.4	11.1	8.4	12.3	15.8	3.0	110.2	11.0
g(3)	26.9	41.7	13.8	10.4	11.9	17.1	3.5	125.3	12.5
GK	16.4	22.7	8.4	5.3	10.6	12.7	2.2	78.1	7.8

ND: not detectable

RGK, RGI, RGMeM, R_2GK, R_2GQ and R_2GI were calculated using the calibration curve of RGQ

Table 3-13B. The concentration ratio of each flavonol glycoside over RGK in ginkgo products

Sample	RGQ/RGK	RGI/RGK	RGMcM/RGK	R2GK/RGK	R2GQ/RGK	R2GI/RGK
S1	3.14	0.54	0.33	0.62	0.73	0.14
S1-1	2.58	0.52	0.26	0.62	0.56	0.11
S1-2	3.96	0.52	0.29	0.60	0.66	0.12
S2	4.38	0.45	0.19	0.53	0.56	0.09
S2-1	5.10	0.45	0.19	0.48	0.50	0.10
S2-2	4.85	0.46	0.18	0.48	0.52	0.10
S3	3.98	0.42	0.19	0.49	0.56	ND
S4	6.66	0.32	0.13	0.89	1.02	0.13
S5	1.03	0.56	0.22	0.40	0.39	0.08
S6	0.98	0.49	0.24	0.47	0.41	ND
S7	2.71	0.31	0.05	0.72	0.68	ND
S8	0.91	0.46	0.23	0.50	0.38	0.08
S9	2.31	0.57	0.33	0.53	0.57	0.12
S10	2.66	0.51	0.32	0.56	0.65	0.12
S11	1.09	0.49	0.23	0.62	0.61	0.14
S12	1.45	0.51	0.31	0.53	0.62	0.12
S13	1.25	0.55	0.29	0.55	0.50	0.11
S14-1	1.56	0.48	0.28	0.45	0.45	0.08
S14-2	1.88	0.40	0.26	0.50	0.53	ND
g(1)	1.66	0.47	0.32	0.52	0.58	0.14
g(2)	1.57	0.48	0.36	0.53	0.68	0.13
g(3)	1.55	0.51	0.39	0.44	0.64	0.13
GK	1.38	0.51	0.32	0.65	0.77	0.13

The concentration of ginkgolides in commercial ginkgo products and "standard" extracts was calculated and is shown in Table 3-14A. The total concentration of ginkgolides in commercial ginkgo products ranged from 0.6 to 2.7%, while in "standard" extracts it ranged from 8.3 to 15.1%. The total concentration of ginkgolides in commercial ginkgo products was lower than in "standard" extracts. The quantification data needs to be validated by other methods. The concentration ratios of each ginkgolide over ginkgolide C were useful, as shown in Table 3-14B. Bilobalide presented the highest

concentration of ginkgolides in the "standard" extracts and some of the commercial samples. This is consistent with published results.[14] The ratios of bilobalide/ginkgolide C in the "standard" extracts and some of the commercial samples were close to 10:4 (2.5), which are similar to published results.[14,16] The ginkgolide components of sample S11 from the commercial ginkgo products have a similar pattern to those of the "standard" extracts, showing good quality control.

Table 3-14A. The concentration of ginkgolides in ginkgo products

Sample	BB	GA	GB	GC	Sum	
	μg/mg	μg/mg	μg/mg	μg/mg	μg/mg	%
S1	6.1	6.7	8.1	4.3	25.2	2.5
S1-1	5.4	5.5	4.4	2.7	17.9	1.8
S1-2	4.4	7.5	10.1	4.5	26.5	2.7
S2	4.5	6.9	7.7	3.3	22.5	2.3
S2-1	2.5	5.3	6.0	2.3	16.0	1.6
S2-2	1.0	4.5	5.0	1.9	12.4	1.2
S3	ND	4.3	5.3	2.2	11.8	1.2
S4	ND	4.2	9.5	2.4	16.1	1.6
S5	6.8	4.7	6.3	2.8	20.6	2.1
S6	2.0	1.1	1.4	1.1	5.6	0.6
S7	0.8	4.2	5.2	2.4	12.5	1.3
S8	2.4	1.1	1.5	1.2	6.2	0.6
S9	0.8	5.8	5.7	2.6	14.9	1.5
S10	4.8	6.0	6.6	3.6	21.0	2.1
S11	9.4	4.6	5.9	4.0	23.9	2.4
S12	4.1	2.9	2.5	2.8	12.3	1.2
S13	3.3	4.2	5.9	2.0	15.4	1.5
S14-1	ND	2.1	2.4	1.4	5.9	0.6
S14-2	ND	3.0	2.9	1.5	7.3	0.7
g(1)	51.4	20.7	13.7	21.4	107.1	10.7
g(2)	48.2	31.2	37.6	24.4	151.4	15.1
g(3)	67.3	28.2	21.5	23.8	140.8	14.1
GK	16.0	23.4	25.5	18.2	83.0	8.3

ND: not detected

Table 3-14B. The concentration ratio of each ginkgolide over ginkgolide C in ginkgo products

Sample	BB/GC	GA/GC	GC/GC
S1	1.42	1.56	1.88
S1-1	2.00	2.04	1.63
S1-2	0.98	1.67	2.24
S2	1.36	2.09	2.33
S2-1	1.09	2.30	2.61
S2-2	0.53	2.37	2.63
S3	ND	1.95	2.41
S4	ND	1.75	3.96
S5	2.43	1.68	2.25
S6	1.82	1.00	1.27
S7	0.33	1.75	2.17
S8	2.00	0.92	1.25
S9	0.31	2.23	2.19
S10	1.33	1.67	1.83
S11	2.35	1.15	1.48
S12	1.46	1.04	0.89
S13	1.65	2.10	2.95
S14-1	ND	1.50	1.71
S14-2	ND	2.00	1.93
g(1)	2.40	0.97	0.64
g(2)	1.98	1.28	1.54
g(3)	2.83	1.18	0.90
GK	0.88	1.29	1.40

ND: not detected

The concentration of ginkgolic acids in commercial ginkgo products and "standard" extracts are shown in Table 3-15. Samples S1 and S7 contained ginkgolic acids. Ginkgolic acid III had the highest concentration of the ginkgolic acid components. The total concentration of ginkgolic acids in these commercial products ranged from 26.5 to 48.3%. Sample S1 has three lot samples and contained 48.3, 40.0, and 26.5% total

ginkgolic acids, showing consistency in composition for detecting ginkgolic acids. The "standard" extract GK has a total ginkgolic acids concentration of 45.6%. Samples S1, S7, and GK contained very high concentrations of ginkgolic acids, 50000–90000 times over the 5 μg/g (0.0005% in the extract) limit, and far beyond the quality control requirement for ginkgo products.

Table 3-15. The concentration of ginkgolic acids in ginkgo products

Sample	GAs1	GAs2	GAs3	Sum	
	μg/mg	μg/mg	μg/mg	μg/mg	%
S1	78.4	108.1	296.5	483.0	48.3
S1-1	68.1	90.1	242.1	400.3	40.0
S1-2	53.1	51.6	155.8	260.5	26.5
S2	ND	ND	ND	ND	ND
S2-1	ND	ND	ND	ND	ND
S2-2	ND	ND	ND	ND	ND
S3	ND	ND	ND	ND	ND
S4	ND	ND	ND	ND	ND
S5	ND	ND	ND	ND	ND
S6	ND	ND	ND	ND	ND
S7	82.5	70.0	209.7	362.2	36.2
S8	ND	ND	ND	ND	ND
S9	ND	ND	ND	ND	ND
S10	ND	ND	ND	ND	ND
S11	ND	ND	ND	ND	ND
S12	ND	ND	ND	ND	ND
S13	ND	ND	ND	ND	ND
S14-1	ND	ND	ND	ND	ND
S14-2	ND	ND	ND	ND	ND
g(1)	ND	ND	ND	ND	ND
g(2)	ND	ND	ND	ND	ND
g(3)	ND	ND	ND	ND	ND
GK	117.2	137.7	201.3	456.2	45.6

ND: not detected
The concentration of ginkgolic acid III was calculated using the calibration curve of ginkgolic acid I

3.4 Discussion and conclusions

The quantification of terpenes, flavonol glycosides, and ginkgolic acids in ginkgo products has been further developed in this chapter.

The MS/MS fragmentation pattern for each compound was obtained and was used for identification. Internal standards were examined and barbaloin was chosen as the internal standard for quantification of ginkgo products. The mass accuracy of the mixed standard with the internal standard was less than 19 ppm, which shows accurate mass. The correlation coefficient values of the linear calibration for K, Q, bilobalide, GA, GB, GC, rutin, and ginkgolic acid I and II are better than 0.98, which shows a good linear relationship. The linear ranges of each component were one order of magnitude. Therefore, samples containing a higher concentration of ginkgo components should be diluted. This demonstrates the possibility of quantification by nanoESI-MS when standards are available.

The amount of MeOH used to extract the sample was studied and 100% MeOH was found to be suitable for extracting three groups of ginkgo components: terpene, flavonol glycosides, and ginkgolic acids. The precision of sample extraction with the internal standard was less than 14% RSD, which shows good reproducibility.

The quantitative analysis of commercial ginkgo samples using this nanoESI-MS method showed that large variations existed between different brands and that the same pattern existed in different lots from the same supplier, indicating that the same extraction process resulted in similar products.

Samples S2, S4, and S7 had higher concentrations of quercetin, demonstrating the possibility of adulteration with synthetic quercetin. The quantification of di- and tri-

glycosides in commercial ginkgo products and "standard" extracts was compared. Samples S1, S2, and S4 had a relatively high RGQ/RGK ratio, which also showed the possibility of adulteration with rutin. These results are consistent with published data.[14] The total concentration of di- and tri-glycosides in commercial ginkgo products ranged from 0.5 to 3.0%, and in "standard" extracts from 7.8 to 12.5%. The concentration data needs further validation.

The quantification of ginkgolides in commercial ginkgo products and "standard" extracts was also compared. The total concentration of ginkgolides in commercial ginkgo products ranged from 0.6 to 2.7%, and in "standard" extracts from 8.3 to 15.1%, which was higher than that in commercial ginkgo products. These results also need validation by other methods.

A high quantity of ginkgolic acids in commercial ginkgo products causes quality control concerns. Samples S1 and S7 and the "standard" extract GK contained ginkgolic acids from 26.5 to 48.3%. The results show that the levels of ginkgolic acids were 50000-90000 times higher than the requirement described in Chapter 1.

It is evident that the different manufacturers did not provide ginkgo products of the same quality. The commercial ginkgo products must be safe, effective, and of high quality to protect consumers. An effective analytical method is important to control and monitor the commercial products. This nano-ESI/MS method shows its advantages in enabling fast and effective quality control.

3.5 References

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Chapter 4

Summary and Future Work

4.1 Summary of the thesis research

The conventional methods for analysis of active and toxic components in ginkgo products require different instruments. The analysis usually involves multiple pre-treatment processes, making it time-consuming and expensive. State-of-the-art mass spectrometry techniques are widely used for large molecules such as proteins and small compounds. However, their potential for the analysis complex mixtures such as ginkgo products without chromatographic separation has not been fully demonstrated. This thesis describes the development of a nanoelectrospray mass spectrometry technique that is capable of simultaneous determination of both the active and the toxic components in ginkgo products and demonstrates its application in the analysis of the complex mixtures, which are ginkgo products.

A negative ionization nanoelectrospray mass spectrometry (nanoESI-MS) technique was successfully developed for simultaneous detection of three different classes of ginkgo compounds: ginkgolides, flavonol glycosides and ginkgolic acids. Various instrumental parameters (such as the declustering potential, the focusing potential, the nanospray voltage, and the positive or negative ion scan) were systematically optimized. The optimized conditions included a nanospray voltage of -900 V, DP_1 of -5 V, DP_2 of -10 V, and FP of -5 V. Under the optimized conditions, the negative ionization mass spectrum of a "standard" ginkgo extract was reproducibly obtained. This technique was then used to fingerprint "standard" ginkgo extracts and 14 commercial samples. The nanoESI-MS method can directly detect intact flavonol glycosides, enabling the

differentiation between the glycosides originating from ginkgo plants and synthetic flavonoids (Q, K and I). The fingerprinting profile of the "standard" ginkgo extract was compared with the profiles of commercial ginkgo products. The variation in the different mass spectrum profiles from the different ginkgo products indicates the varying composition of the active and toxic compounds in different products. In some products, unexpectedly high levels of ginkgolic acids were detected, while some products contained high levels of quercetin or rutin, indicating addition of quercetin and rutin to the products. This technique is useful for detecting adulteration and incomplete elimination of ginkgolic acids from ginkgo leaves, which cannot be detected by conventional methods. The simultaneous detection of terpenes, flavonol glycosides and ginkgolic acids, and the capability to differentiate intact flavonol glycosides from aglycones for detection of adulteration are the two main advantages of the nanoESI-MS method.

This study further explored the potential of the nanoESI-MS technique for quantification of these three classes of ginkgo compounds. In order to develop quantification methods for these compounds, the fragmentation of different components occuring in the full scan mode was studied. Barbaloin was used as the internal standard for the quantification. The linear correlation coefficient values of the calibration curves of K, Q, bilobalide, GA, GB, GC, rutin and ginkgolic acids I and II were better than 0.98. The sample extraction efficiency was studied and components present in the extraction solution of the commercial samples were identified by the tandem mass spectra and accurate mass (better than 30 ppm). The relative standard deviation of the relative peak

intensity of each compound in the commercial samples was less than 14%, except for the low concentration of kaempferol, demonstratinging good reproducibility.

The nanoESI-MS method was used to quantify active and toxic components in commercial ginkgo products from different brands and different batches from the same supplier. A large variations in composition and concentration was found between different brands and consistency in detecting ginkgolic acids was found in different batch samples from the same supplier. Samples S2, S4, and S7 had a higher concentration of quercetin (S2: 1.4 μg/mg; S4: 2.9 μg/mg; S7: 1.4 μg/mg) and samples S1, S2, and S4 had a relatively high RGQ/RGK ratio, which indicated the possibility of adulteration with RGQ. Sample S11 had a pattern of ginkgolides similar to "standard" extracts showing the good quality control of this product. Samples S1 and S7 contained a higher concentration of ginkgolic acids (S1: 483.0 μg/mg; S1-1: 400.3 μg/mg; S1-2: 260.5 μg/mg; S7: 362.2 μg/mg), far beyond the 5μg/g requirement, which shows the importance of quality control.

This nanoelectrospray mass spectrometry (nanoESI-MS) technique has high sensitivity, requires minimal sample preparation, and uses only microlitres of sample, and the analysis only requirs a few minutes. The nanoESI-MS technique is useful for fast quality control of ginkgo products. However, the quantification of this technique requires further improvement in the linear dynamic range, and more standards and standard reference material are needed. Simultaneous detection of terpenes, flavonol glycosides, and ginkgolic acids, and of any adulteration by the nanoESI-MS method can provide better quality control of ginkgo products. The nanoESI-MS method overcomes some shortcomings of the conventional methods.

4.2 Future work

Some potential research can be extended from this study. A few projects are briefly described below.

1. Validation of the quantification method. This requires proper standards to ensure the accuracy of the quantification. It would be very useful to develop standard reference material using the standardized procedures used to produce ginkgo extracts, because producing individual standards for all compounds would be difficult. When the quantification with the standard reference material is established, this method should be validated against other methods.

2. Application of this technique to assess human exposure to toxic compounds in ginkgo products. Little information is available on the human exposure to these toxic compounds. The nanoESI-MS technique can be used to measure the toxic ginkgo components in urine samples of a population that regularly uses natural ginkgo products. The data could be used in human exposure and risk assessment.

3. The nanoESI-MS technique may be applied to analyze important compounds in other natural health products. This study is a demonstration that this technique can analyze complex mixtures with minimum sample preparation.

4. Working with regulatory agencies such as Health Canada, it may be possible to establish a standardized method for quality control of natural products using this technique. A reliable quality control and monitoring program will protect consumers from harmful effects due to consumption of uncontrolled doses of active components and from exposure to potentially harmful compounds in natural products. This is an important part of public health.