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THE IDENTIFICATION AND QUANTIFICATION OF GINGKOLIDES AND BILOBALIDES IN CHINESE HERBAL MEDICINES

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OVERVIEW

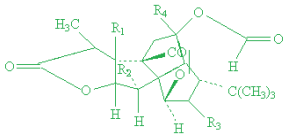
- *Ginkgo biloba* is a tree that has, since ancient times, been used as a component of traditional Chinese herbal medicines.
- Ginkgolides, the main components of preparations of Ginkgo leaf and root, are now being developed as therapeutic agents.
- In order to quantify known components of Ginkgo biloba leaf, fast gradient HPLC separations were coupled to MRM analyses on a Quattro Micro triple quadrupole mass spectrometer (Micromass UK Ltd., Manchester, UK).
- Quantification methods show good sensitivity, dynamic range and precision.
- In order to ascertain the identity of other components in the leaf preparation a slow HPLC gradient was coupled to high-resolution, accurate-mass analysis using a Q-Tof Ultima hybrid quadrupole/oaToF mass spectrometer (Micromass UK Ltd., Manchester, UK).
- A range of compounds and structural isomers were identified in the mixture.

INTRODUCTION

Medical treatment has long been connected with the natural products and actives extracted from plants. This has lead to many advances in medicines and has long been the pivotal force in fighting aliments. It has been known and appreciated for many years that certain plants can exhibit healing effects when prepared as part of a mixture, but when taken in isolation can cause detrimental effects. This knowledge is now being used more and more in sophisticated biological and bioanalytical studies of natural products.

Despite advances both in utilising synthetic approaches to drug design, and in sophisticated structure-activity studies, there is still a great need for compounds with a unique mechanism of action. Major breakthroughs have resulted primarily from the study of natural products. Some of the most important drugs have been isolated from plant sources; for example most antibiotics and anti-cancer drugs.

The maidenhair tree, *Ginkgo biloba*, is an ancient Chinese plant that has been cultivated for its health-promoting properties. Ginkgolides, the main active ingredients of *Ginkgo biloba*, have not only helped to explain the pharmacological basis of several traditional medicines, but have also provided a valuable new class of therapeutic agents. Research on the biochemical effects of *Ginkgo biloba* extracts is still at a very early stage. Although the terpene fraction of *Ginkgo biloba*, which contains the ginkgolides, may contribute to the neuroprotective properties of the *Ginkgo biloba* leaf, it is also likely that the flavonoid fraction, containing free radical scavengers, is important in this respect. The structures of the ginkolides are shown (**Figure 1**).



Gingkolide	R1, R2, R3, R4
Ginkgolide A	H, HO, H, HO
Ginkgolide B	H, HO, HO, HO
Ginkgolide C	HO, HO, HO, HO
Ginkgolide J	HO, HO, H, HO
Ginkgolide M	HO, H, HO, HO

Figure 1.

MICROMASS MS TECHNOLOGIES

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EXPERIMENTAL

Extraction Procedure

The extraction procedure used by the China Pharmaceutical Company is described as follows.

- 50g Ginkgo Biloba leaf powder was refluxed with 200ml 60% acetone for 1hr, then filtered.
- Extraction solvent was recovered and remaining residue was extracted with ethyl acetate.
- Ethyl acetate was recovered and remaining residue was extracted with water.
- Water was extracted with diethyl ether.
- Acetone, ethyl acetate and diethyl ether fractions were combined and evaporated.
- Samples were reconstituted in 60% acetone.

For the commercial samples obtained from the UK the method above was not necessary as the samples were obtained in tablet form. They were simply dissolved in 10mL of mobile phase and centrifuged to remove all the stabilisers. The resulting solvent was decanted and filtered. The samples where then ready for injection.

Quattro Micro

HPLC Pump Conditions

Mobile Phases

A	Acetonitrile
B	Water

The gradient Timetable contains 6 entries, which are:

Time	A%	B%	Flow (ml/min)
0.00	95.0	5.0	0.300
1.00	50.0	50.0	0.300
2.50	50.0	50.0	0.300
3.00	5.0	95.0	0.300
7.00	5.0	95.0	0.300
7.50	95.0	5.0	0.300

Injection Volume (µl)	5.0
Stop Time (mins)	12.00

Symmetry 100 x 2.1mm C_{18} 3.5µ HPLC column (Waters Corp., Milford, MA, USA).

Mass Spectrometer Conditions

Polarity	ES-
Capillary (kV)	3.00
Cone (V)	20.00
Extractor (V)	3.00
RF Lens (V)	0.5
Source Temperature (°C)	150
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	64
Desolvation Gas Flow (L/Hr)	671

Q-Tof Ultima

HPLC Pump Conditions

Mobile Phases

A	Acetonitrile
B	Water

The gradient Timetable contains 6 entries, which are:

Time	A%	B%	Flow (ml/min)
0.00	95.0	5.0	0.300
2.00	95.0	5.0	0.300
60.0	40.0	60.0	0.300
61.0	5.0	95.0	0.300
65.0	5.0	95.0	0.300
66.0	95.0	5.0	0.300

Injection Volume (µl)	5.0
Stop Time (mins)	72.00

Symmetry 100 x 2.1mm C_{18} 3.5µ HPLC column (Waters Corp., Milford, MA, USA).

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Mass Spectrometer Conditions

The instrument was operated using a Lockspray ionisation source and W mode ToF ion optics.

Polarity	ES-
Capillary (kV)	3.00
Cone (V)	60.00
Source Temperature (°C)	120
Desolvation Temperature (°C)	350
Cone Gas Flow (L/Hr)	50
Desolvation Gas Flow (L/Hr)	600
Resolution (FWHM)	20,000
Scan Range (m/z)	100 to 900
Acquisition Mode	Centroid
Reference Mass	Leucine Enkephalin at m/z 554.261

RESULTS

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Calibration lines were constructed over the range 0.5pg/uL to 5000pg/uL for a mixture of the three standards, with each individual calibration point being injected in duplicate. The resulting coefficients of correlation are shown below:

Gingko_A	R^2=0.999370
Gingko_B	R^2=0.997926
Bilobalide	R^2=0.999375
Gingko_A_isomer	R^2=0.999726

Each calibration line showed a good linear response with a high degree of precision for the method. The calibration graphs are shown (**Figures 2, 3, 4** and **5**).

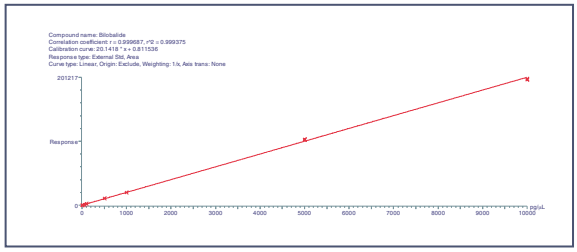

Figure 2. Bilobalide

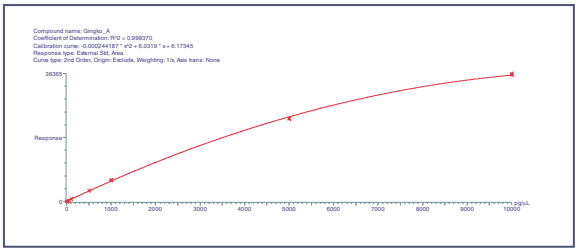

Figure 3. Ginkgolide A

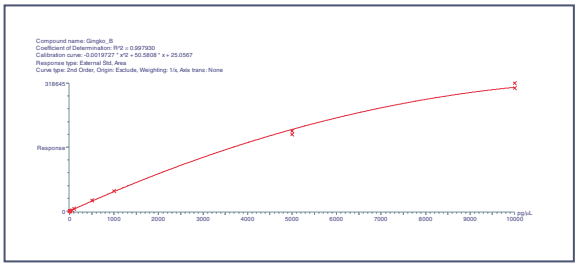

Figure 4. Ginkgolide B

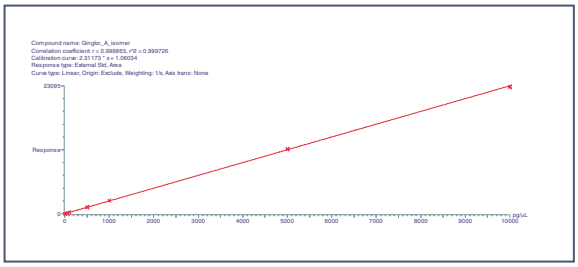

Figure 5. Ginkgolide A (isomer)

The samples where then injected in duplicate and the mean amount of active ingredients are shown (**Table 1**)

Samples	Gingko A	Gingko B	Bilobalide	Gingko A isomer
Commercial sample1	1722.1	884.6	889.3	31.5
Commercial sample2	328.0	185.9	172.4	5.8
Nanjing sample 1	1366.2	1122.9	N/A	58.1
Nanjing sample 2.	1323.3	1138.0	N/A	56.2

Table 1.

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Q-Tof Ultima

The base peak intensity (BPI) chromatogram for the analysis of an extract of gingko leaf is shown (**Figure 6**).

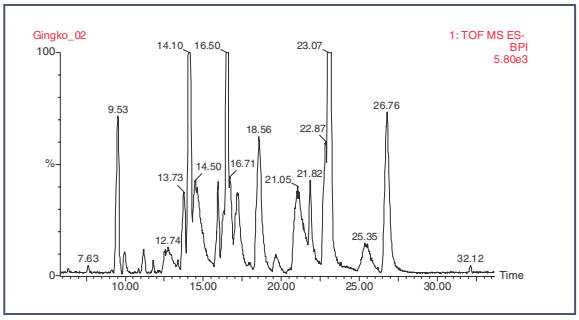

Figure 6.

A variety of components can be seen and their accurately determined masses, together with matched elemental compositions are given (**Table 2**). This allows many of the peaks to be identified. **Table 2**.

Retention Time/min	Measured Mass	Elemental Composition	ppm deviation	Identity
6.36	445.21	C21H33O10	6	
6.53	455.1202	C20H23O12	2.7	Gingkolide D?
7.63	325.0921	C15H17O8	-0.6	Isomer of Bilobalide
9.15	429.2134	C21H33O9	2.3	
9.53	441.1395	C20H25O11	-0.4	
9.97	441.1808	C14H33O15	-2.6	
10.62	465.2339	C21H37O11	0.7	
11.19	425.1465	C20H25O10	4	
11.79	451.255	C21H39O10	1.6	
12.56	325.0918	C15H17O8	-1.8	Isomer of Bilobalide
13.75	425.1454	C20H25O10	1.4	
14.1	325.0918	C15H17O8	-1.6	Bilobalide
14.5	609.1453	C27H29O16	-0.5	
15.95	423.129	C20H23O10	-0.2	Isomer of Gingkolide B (Gingkolide J or M?)
16.5	439.1239	C20H23O11	-0.3	Gingkolide C
17.18	447.0936	C21H19O11	2	
18.56	593.1474	C34H25O10	4.4	
19.65	431.0984	C21H19O10	1.4	
21.05	377.0859	C18H17O9	-3.5	
21.82	411.1651	C20H27O9	-1	
22.15	423.1296	C20H23O10	1.1	Isomer of Gingkolide B (Gingkolide J or M?)
23.03	423.1295	C20H23O10	0.9	Gingkolide B
23.07	407.1331	C20H23O9	-1.6	Gingkolide A
26.76	551.3066	C26H47O12	-0.3	
32.12	405.1201	C20H21O9	3.8	

Table 2.

CONCLUSION

Gingko Biloba's main components of interest, Gingkolide A (including the isomer), B and the Bilobalide were quantified using the methodology described. The results showed that commercial samples and the extracted Nanjing samples can be quantified using this method using the Quattro Micro tandem mass spectrometer.

The data from the Q-Tof highlighted the accurate mass measurements use in identifying the main components of the Gingko Biloba. The active ingredients were accurately mass measured to within 5ppm and the unknown compounds were also accurately mass measured and a structure was proposed. Further analysis of the unknowns can result in viable structures being proposed. With intense data mining possible metabolism, breakdowns and transformations can be determined.

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