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Fingerprint profile of *Ginkgo biloba* nutritional supplements by LC/ESI-MS/MS

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Abstract

Ginkgo biloba is one of the most popular herb nutrition supplements, with terpene lactones and flavonoids being the two major active components. A fingerprint profile method was developed using a capillary HPLC/MS method which can identify more than 70 components from the *G. biloba* product. The method allows the flavonoids and terpene lactones to be detected simultaneously and information of both the parent ion and its fragmentation can be obtained in just one HPLC/MS run. Targeted post-acquisition analysis allows mass spectrometric information regarding the identification of flavonoid components to be easily distinguished from other data, however the same approach for terpene lactones was less successful due to dimer formation and requires further development. The fingerprint profiles of five commercial *G. biloba* nutritional supplements were obtained and compared; variation of some components among the samples was observed and fortification could be detected. In the quality control analysis of the *G. biloba* product this method could be viewed as complementary to specific quantitative analysis of some bioactive components of the herb.

Keywords: Fingerprint; *Ginkgo biloba*; Flavonoids; Terpene lactones

1. Introduction

"Chemical Fingerprint analysis" of traditional Chinese herbs has received much attention in past reports as there are many varying factors in herbal medicine, raising concerns in quality control. Plant extracts are complex mixtures whose therapeutic effect is often attributed to the cumulative effects of many components (Xue and Roy, 2003) and so it is important to have an overall view of all the components in the extract to evaluate the quality of the plant product as many factors affect their quality and efficacy. As not all the components have reference standards for quantitation, QC (quality control) analysis is sometimes achieved by a process of "fingerprint analysis" in which the experimental data from the chemical analysis of different extracts is compared without accurately quantification or identifying the individual compounds. Chemical fingerprinting has previously been carried out using high-pressure liquid chromatography (HPLC), gas chromatography (GC), thin layer chromatography (TLC) GC/MS and nuclear magnetic resonance (NMR) and is accepted by the World Health Organization for the quality control of herbal medicines (WHO, 1991). It has attracted much interest in herbal extract research in recent years (Kerns et al., 1998) particularly in traditional Chinese medicines and their raw materials as stated by the Chinese Pharmacopoeia (Committee of National Pharmacopoeia, 2000) and is also now a demand of the European Economic Council (EEC) guideline 75/318 "Quality of Herb Drugs"

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(UNICEF/UNDP/World Bank/WHO, 2004). More recently HPLC-UV or HPLC/MS have been used for the detailed profiling of active components of trace marker compounds (Cai et al., 2002). Standardized extracts of *Ginkgo biloba* leaves are considered a drug in many European countries and China whilst being listed as a dietary supplement in North America. It is used in the treatment of peripheral and cerebral circulation disorders and is among the top selling dietary supplements in the herbal remedy market (Blumenthal, 2001). The major active components of *G. biloba* extract are the flavonoids and terpene lactones (Li and Fitzloff, 2002) and HPLC-UV has previously been used to monitor 33 flavonoids (Hasler and Stitcher, 1992), however HPLC-UV is not suitable for terpene lactone analysis. Mauri et al. (1999) obtained a typical fingerprint-like spectrum by direct infusion in ESI-MS mode and assigned 9 flavonol glycosides whilst nano-electrospray MS by direct infusion has been used for the detection of both active components (Liu et al., 2005). HPLC coupled with ion trap tandem mass spectrometry (LC/ITMS) is a powerful tool for compositional and structural analysis of active constituents in plant extracts (Ying et al., 2004). In this study, a HPLC/ITMS system using a capillary column (0.3 mm i.d.) was utilised in order to produce a fingerprint profile of *G. biloba* extract. The use of data-dependent settings to study active components more specifically was also investigated.

2. Experimental

2.1. Materials

Ginkgolide A (**GA**), ginkgolide B (**GB**), ginkgolide C (**GC**), bilobalide (**BL**), quercetin dehydrate (**QD**), quercetin-3-β-D-glucoside (**QG**), quercetin-3-rhamnoside (**QH**), kaempferol (**KF**), isorhamnetin (**IR**) and rutin (**RH**) were purchased from Sigma (St. Louis, MO, USA). HPLC grade solvents: methanol, acetonitrile, formic acid and acetic acid were purchased from Fisher chemicals (Loughborough, UK) and were used without further purification. Water was purified with a Milli-Q deionization unit (Millipore, Bedford, MA, USA). Gases used included oxygen free nitrogen and helium which were purchased from BOC Ltd. (Surrey, UK). Five standardized *G. biloba* extract products were obtained in this study, samples 1 and 2 were bought from a local market, sample 3 was bought from a nationwide health food shop and samples 4 and 5 were obtained from Obsidian Research Ltd. (Port Talbot, UK). Sample 3 was characterised as being of the best quality in our previous study (Ding et al., 2006a) and thence used in this study as a reference standard extract.

2.2. G. biloba sample preparation

Samples of commercial *Ginkgo* extract products were prepared by combining the contents of 10 capsules or 10 tablets then pulverizing into powder. 100 mg of the above powder were accurately weighed into a 20 mL vial. 20 mL of methanol were added to the sample and shaken briefly to mix. The sample vial was then sonicated in an ultrasonic bath Bransonic 2510 (Branson, Danbury, CT, USA) at a frequency of 42 kHz at 25 °C for 50 min, 1 mL of sample was then removed and centrifuged for 10 min at 17,000*g* to pellet insoluble material. The supernatant was removed into a sample vial and 5 μL of it analysed by HPLC/MS.

2.3. Capillary LC/MS of G. biloba nutrition supplement

The column used in this experiment was a capillary C_{18} column (300 μm i.d. × 15 cm) made in-house as previously described (Ding et al., 2006b). The HPLC/MS system was a LC Packings Ultimate Capillary LC system (Dionex, Amsterdam, Netherlands) linked with an LCQ Deca XP ion trap (Thermo Finnigan, Hemel Hempstead, UK). The mobile phases were composed of 0.1%(v/v) acetic acid in water (A), 1:1(v/v) mixture of ACN and methanol (B), the flow rate was 4 μL min^{-1}. The gradient ran from 10%B to 60%B over 120 min then to 98%B by 125 min. LC/ESI-MS was carried out in the negative ion mode from *m/z* 200–1600, the heated capillary temperature set to 200 °C, the electrospray voltage to 2.5 kV and the sheath and auxiliary gas flows to 60 and 15 arbitrary units respectively. The mass spectrometer was used in a data-dependent mode (DDA) composed of 3 scan events. The full scan mass spectrum was first obtained and followed by collision-induced dissociation of the highest abundant ion selected from the full scan. Next, in the third scan event, the highest abundant ion from the MS/MS analysis was chosen for further fragmentation (MS^3).

3. Result and discussion

The full scan limit of detection (LoD) determined using standard compounds available ranged from 8 to 25 ng mL^{-1} for flavonoids whilst terpene lactone LoDs ranged from 40 to 150 ng mL^{-1}. The overall sensitivity is more than 10 times higher than normal bore HPLC/MS full scan obtained in our pervious work indicating the benefit of the minituarisation of the combined HPLC/MS setup (Ding et al., 2006a). The base peak MS full scan of the *G. biloba* extract (Sample 3, our reference sample which was proven to be the best quality one) shows more than 70 peaks (Fig. 1). The advantage of the utilised DDA mass spectrometry setting is that it can obtain the full scan spectrum of components of the sample and the MS/MS and MS^3 spectra at the same time without the mass of the compounds being known. Although the HPLC separation was a lengthy process, shorter HPLC run times resulted in much co-elution of components and this led to reduced mass spectrometric information being obtained and fewer compounds being identified. Using the information gained

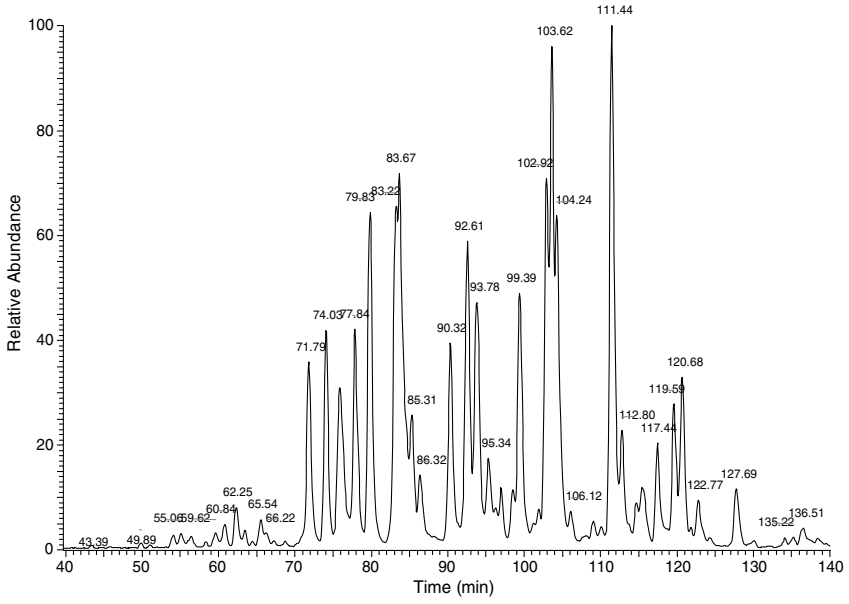

Fig. 1. Full scan base peak of capillary LC/MS of *Ginkgo biloba* supplement sample.

from data-dependent HPLC/MS/MS and the known fragmentation pathways of the major active compounds, (from previous literature and our own studies (Ding et al., 2006b; Fabre and Rustan, 2001; Ma et al., 1997; Hasler and Sticher, 1992; Hasler et al., 1992; Tang et al., 2001), definite or tentative identifications of peaks in the resulting mass spectrum could be obtained. Some examples are discussed indicating commonly detected losses for the classes of compounds considered. The rationale behind the characterization is that upon fragmentation, flavonoid glycosides will lose the bound sugar groups consecutively and produce the corresponding aglycone, the major sugar groups being glycosyl, rhamnosyl and coumaroyl groups. A difficulty in the characterization of flavonoids is that the coumaroyl group is the same mass as the rhamnosyl group, both having M_r 146 amu. According to the literature (Hasler and Sticher, 1992; Tang et al., 2001), all the flavonoids containing coumaroyl ester groups have them positioned as the outermost group in the flavonoid, so when fragmented, the first group to be lost should be *m/z* 146 (indicating a possible coumaroyl group) and this has assisted the identification in this paper.

3.1. Compound *1*

Compound **1** occurs at a retention time of 43.4 min and shows a base peak at *m/z* 917 (Fig. 2). Fragmentation of the *m/z* 917 ion yields a product ion at *m/z* 755 arising from the loss of 162 Da, representing a loss of a glucosyl ($-C_6H_{10}O_5$) unit (Fig. 3). Further fragmentation of *m/z* 755 caused a loss of 146 Da representing a loss of a rhamnosyl ($-C_6H_{10}O_4$) unit and produced a spectrum with product ions *m/z* 609, 343 and 301 as shown in Fig. 4. It should be noted that *m/z* 300 which corresponds to a radical quercetin anion is also present in this spectrum. According to literature (Hvattum and Ekeberg, 2003), the relative abundance of the radical aglycone to the aglycone product ion is dependent on the collision energy with a relative increase in radical aglycone product ion formation with an increase in collision energy. It was found in our study that, for this type of compounds, using the collision energy at 28% the aglycone product ions are more abundant than the radical aglycone ions (data not shown). The *m/z* 609 and corresponding fragment ions match the ions of the commercial standard rutin and its product ions (data not shown) and so the compound is thought to be a derivative of rutin (glucosyl–rhamnosyl quercetin). In the MS^3 spectrum the base peak product ion at *m/z* 301 together with the peak at *m/z* 300 are characteristic peaks of quercetin sugar complex fragment peak clusters. Since the mass of the glycoside functional groups glucosyl ($-C_6H_{10}O_5$) and rhamnosyl ($-C_6H_{10}O_4$) are both lost during fragmentation, Compound **1** is thought to consist of the quercetin moiety with two each of the glucosyl and rhamnosyl groups, as opposed to rutin which contains only one of each group. Compound **1** is therefore tentatively identified as 2-rhamnosyl–2-glucosyl quercetin, however it should be recognised that the sequence of the sugar moieties can not be distinguished by the data obtained.

3.2. Compound *42*

Compound **42** has a retention time from 75.3 min, its mass spectrum shows a base peak at *m/z* 325 and the MS/MS spectrum of this peak is identical to that previously observed for the standard terpene lactone bilobalide (data not shown). Further comparison of the retention time and MS^3 spectrum of ion (*m/z* 325 via *m/z* 251) of this compound with standard bilobalide confirms the identity as bilobalide.

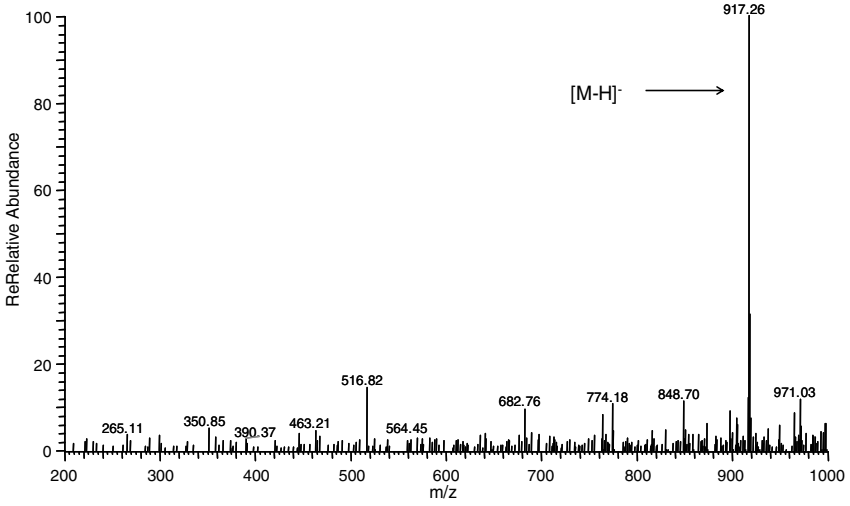

Fig. 2. Full scan spectrum of Compound 1 of *Ginkgo biloba* sample 3.

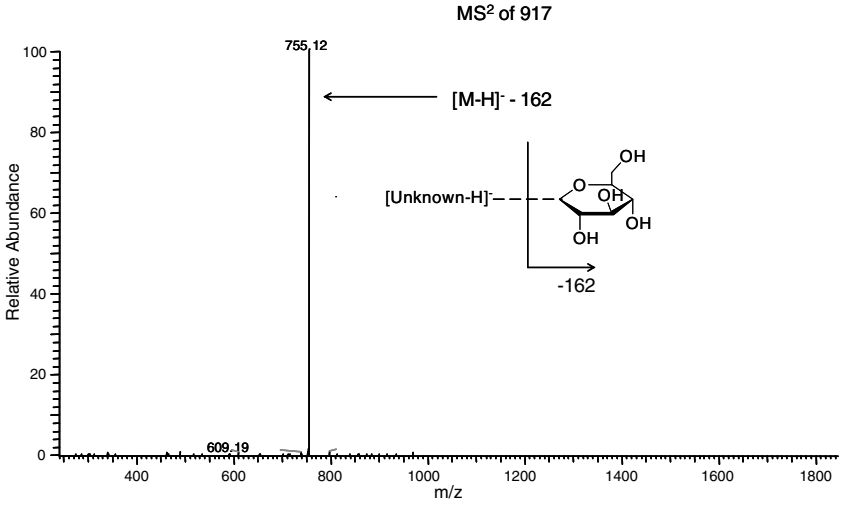

Fig. 3. MS/MS scan of compound **1** *m/z* 917.

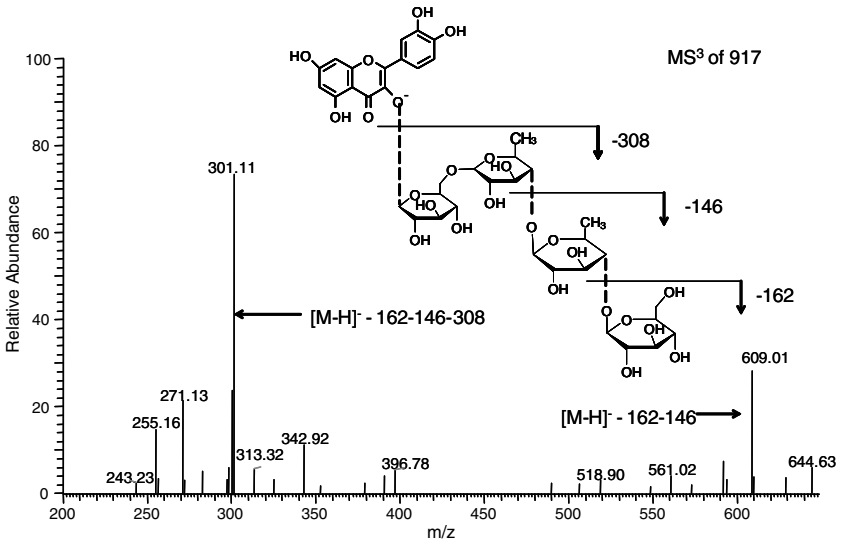

Fig. 4. MS^3 scan of compound **1** *m/z* 917 → 755.

3.3. Compound **52**

Compound **52** shows a retention time of 90.2 min and a base peak of 609, isobaric to that of compound **48** (identified by comparison with standards as rutin), MS/MS analysis produced a different fragmentation spectrum compared to the standard rutin, however, as shown in Fig. 5, it shows a characteristic fragment ions of *m/z* 301 and *m/z* 300 which indicates that the aglycone present is quercetin. The further fragmentation of *m/z* 301 (MS^3 of peak 52) is identical to MS^3 (609@301) spectrum obtained with the commercial standard rutin (data not shown). The compound is therefore considered to be similar in structure to rutin however the sugar moieties must be bound to the quercetin structure in a different manner compared to rutin in order to explain the differences in MS/MS data obtained and are currently the subject of further investigation.

3.4. Compound **53**

Compound **53** shows a retention time of 92.6 min and base peak at *m/z* 593; upon fragmentation, a major product ion *m/z* 285 is obtained representing a loss of 308 Da which is thought to occur via the loss of a rhamnosyl–glucosyl moiety. The MS^3 spectrum of *m/z* 285 is identical to that of the kaempferol standard ($M_W = 286$), and so Compound 53 is tentatively determined to be rhamnosyl–glucosyl kaempferol.

3.5. Compound **54**

Compound **54** shows a retention time of 93.7 min and base peak at *m/z* 623; up on fragmentation, a product ion at *m/z* 315 is obtained. This is again thought to represent a loss of a rhamnosyl–glucosyl moiety. The MS^3 spectrum of *m/z* 315 is identical to the MS/MS spectrum of standard isorhamnetin (data not shown), Compound 54 is therefore thought to be rhamnosyl–glucosyl isorhamnetin.

It worth noting that in this analysis, there are 6 peaks whose *m/z* is 755, two of which (Compounds **21** and **30**) are kaempferol derivatives, and four of which (Compounds **39**, **64**, **67** and **71**) arise from quercetin. Compounds **21** and **30**, losing 162 first, are thereby considered to be composed of 2-glucosyl groups and one rhamnosyl group. By comparing the obtained data with the elution sequences of similar compounds reported in the literature (Hasler and Sticher, 1992), compound **71** can be tentatively characterised as coumaroyl–glucosyl–rhamnosyl quercetin, and compounds **39**, **64** and **67** can be tentatively named as 2-rhamnosyl–glucosyl quercetins, however, further work needs to be done in order to clarify the *m/z* 146 sugar ring (whether it is a rhamnosyl group or a coumaroyl group). Furthermore, there are 5 peaks whose *m/z* is 739, and these have been tentatively characterised according to the rules explained above and comparisons with the literature. This degree of complexity and number of similar mass compounds is not surprising as there can be many combinations of sugar structures with high mass flavonoids and more experimentation would be needed to confirm the structures fully.

Using the principles and initial information regarding these compounds described above, tentative identifications could be obtained for compounds for which no commercial available standards could be obtained from information provided by the data-dependent analysis. Table 1 summarises the MS and MS^n data obtained for the peaks and their proposed identifications. The four terpene lactones (BL, GA, GB, GC) can be identified by comparing the full scan and MS^n data with standards, among these, GA, GB, and GC were detected with the dimer as the base peak in the

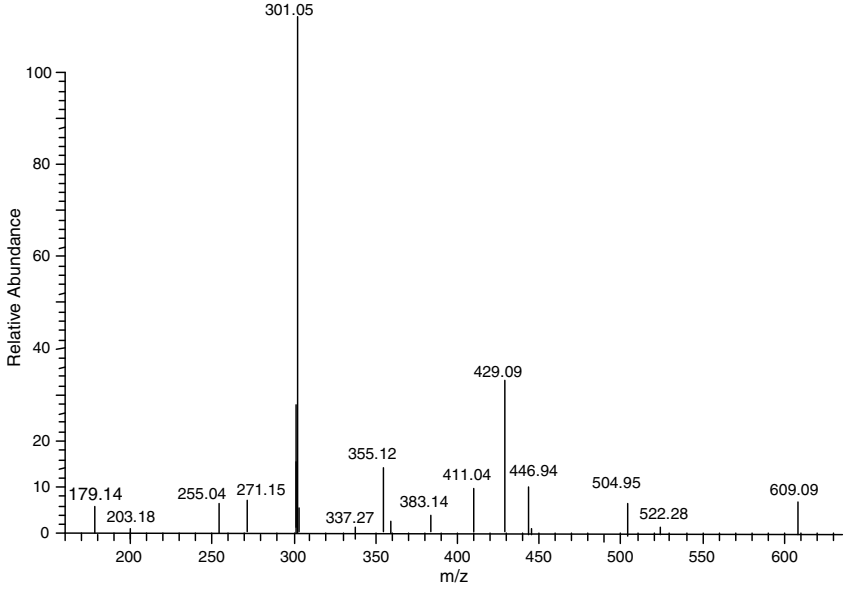

Fig. 5. MS/MS spectrum of compound 52, *m/z* 609.

Table 1
MS, MS/MS and MS^n of the peaks in Fig. 1 and the proposed identification

Compound	RT	MS	MS^2	MS^3	Aglycone	Mass of sugar ring	Tentative identification
1	43.4	917	755	609, 343, 301[a]	Quercetin	162 × 2 + 146 × 2	2-Rhamnosyl–2-glucosyl quercetin
2	49.6	901	739	593, 285	Kaempferol	162 × 2 + 146 × 2	2-Rhamnosyl–2-glucosyl kaempferol
3	50.8	477	315	300	Isorhamnetin	162	Glucosyl isorhamnetin
4	51.0	931	769	605, 524	*m/z* 605	162 + 164	Unknown glucosyl flavonoid
5	52.4	751	605	–	*m/z* 605	146	Unknown rhamnosyl flavonoid
6	52.7	535	373	343,313	*m/z* 373	162	Unknown glucosyl flavonoid
7	53.6	771	609	301[a]	Quercetin	2 × 162 + 146	2-Glucosyl–rhamnosyl quercetin
8	54.7	949	787	625	*m/z* 625	162	Unknown glucosyl flavonoid
9	55.6	361	179	–	*m/z* 361	–	Unknown flavonoid aglycone
10	55.7	721	523	361	*m/z* 361	198 + 162	Unknown glucosyl flavonoid
11	55.8	521	359	329	*m/z* 359	162	Unknown glucosyl flavonoid
12	55.9	359	329	299, 178	*m/z* 359	_	Unknown flavonoid aglycone
13	56.2	449	287	259	*m/z* 287	162	Unknown glucosyl flavonoid
14	56.9	801	639	331	Methylmyricetin	2 × 162 + 146	2-Glucosyl–rhamnosyl methylmyricetin
15	58.0	1079	917	755, 609	Quercetin	3 × 162 + 2 × 146	3-Glucosyl–2-rhamnosyl quercetin
16	59.1	359	344	313	*m/z* 359	_	Unknown flavonoid aglycone
17	59.2	537	375	195, 179	*m/z* 375	162	Unknown glucosyl flavonoid
18	59.4	771	609	300[a]	Quercetin	2 × 162 + 146	2-Glucosyl–rhamnosyl quercetin
19	60.1	551	389	341, 193	*m/z* 389	162	Unknown glucosyl flavonoid
20	60.5	609	463, 447	301[a]	Quercetin	162 + 146	Rhamnosyl–glucosyl quercetin
21	60.6	755	593	285	Kaempferol	2 × 162 + 146	2-Glucosyl–rhamnosyl kaempferol
22	61.6	639	477	315	Isorhamnetin	2 × 162	2-Glucosyl isorhamnetin
23	61.8	553	391	343, 151	*m/z* 391	162	Unknown glucosyl flavonoid
24	62.0	785	623	315	Isorhamnetin	2 × 162 + 146	2-Glucosyl–rhamnosyl isorhamnetin
25	62.7	375	360, 327	–	*m/z* 375	_	Unknown flavonoid aglycone
26	63.2	449	287	259	*m/z* 287	162	Unknown glucosyl flavonoid
	63.4	1063	_	_	Quercetin	3 × 146 + 2 × 162	Coumaroyl 2-rhamnosyl–2-glucosyl quercetin
27	63.8	815	653	345	*m/z* 345	2 × 162 + 146	Unknown 2-glucosyl–rhamnosyl flavonoid
28	64.3	449	287, 269	–	*m/z* 287	162	Unknown glucosyl flavonoid
29	65.1	719	557	526	*m/z* 557	162	Unknown glucosyl flavonoid
30	65.4	755	593	285	Kaempferol	2 × 162 + 146	2-Glucosyl–rhamnosyl kaempferol
31	66.2	521	503, 491	341, 327	*m/z* 521	_	Unknown flavonoid aglycone
32	67.0	575	431	269	*m/z* 269	144 + 162	Unknown glucosyl flavonoid
33	67.1	593	431, 447	285	Kaempferol	162 + 146	Glucosyl–rhamnosyl kaempferol
34	67.8	477	315	300	Isorhamnetin	162	Glucosyl isorhamnetin
35	68.5	417	181	166, 151	*m/z* 417	_	Unknown flavonoid aglycone
36	69.1	447	387, 285	–	Kaempferol	162	Glucosyl kaempferol
37	69.8	913	751	733, 685, 555	*m/z* 751	162	Unknown glucosyl flavonoid
38	70.7	683	521	–	*m/z* 521	162	Unknown glucosyl flavonoid
39	71.5	755	609, 300[a]	271, 255, 179, 151	Quercetin	162 + 2 × 146	2-Rhamnosyl–glucosyl quercetin
40	73.8	917	755	609, 300[a]	Quercetin	162 × 2 + 146 × 2	2-Rhamnosyl–2-glucosyl quercetin
41	74.5	625	317	287, 271, 179, 151	Myricetin	162 + 146	Rhamnosyl–glucosyl myricetin
42	75.3	325	281, 251, 163	–	_	_	Bilobalide
43	77.2	847[b]	423	349	_	_	Ginkgolide J
44	76.1	739	593, 575, 285	–	Kaempferol	162 + 2 × 146	2-Rhamnosyl–glucosyl kaempferol
45	78.2	769	315	300	Isorhamnetin	162 + 2 × 146	Coumaroyl– rhamnosyl–glucosyl isorhamnetin
46	79.8	901	739	593, 285	Kaempferol	162 × 2 + 146 × 2	2-Glucosyl–2-rhamnosyl kaempferol
47	81.8	879[c]	439	395, 383, 365	_	_	Ginkgolide C
48	82.5	609	301[a]	271, 179, 151	Quercetin	162 + 146	Rutin
49	83.2	639	331	316, 315, 179	Methymyricetin	162 + 146	Rhamnosyl–glucosyl methymyricetin
50	86.2	463[c]	301	271,179,151	Qquercetin	162	Quercetin-3-β-D-glucoside
51	86.6	579	417	371, 181, 166, 151	*m/z* 417	162	Unknown glucosyl flavonoid
52	90.2	609	301[a]	271, 255, 179, 151	Quercetin	162 + 146	Glucosyl–rhamnosyl quercetin
53	92.6	593	285	267, 257, 229, 151	Kaempferol	162 + 146	Glucosyl–rhamnosyl kaempferol
54	93.7	623	315	300	Isorhamnetin	162 + 146	Glucosyl–rhamnosyl isorhamnetin
55	95.1	447	259, 151	–	Quercetin	146	Quercetin-3-rhamnoside
56	96.0	477	315	300, 285, 271, 243	Isorhamnetin	162	Glucosyl isorhamnetin
57	96.2	287	259	215, 173, 125	*m/z* 287	_	Unknown flavonoid aglycone
58	96.7	653	345	330, 315	*m/z* 345	162 + 146	Unknown glucosyl–rhamnosyl flavonoid
59	98.2	913	739	593, 285	Kaempferol	174 + 146 × 2 + 162	Glucosyl–rhamnosyl kaempferol with unknown sugar ring
60	99.0	593	285	267, 255, 229, 151	Kaempferol	162 + 146	Glucosyl–rhamnosyl kaempferol
61	101.7	447	285	–	Kaempferol	162	Glucosyl kaempferol

Table 1 (*continued*)

Compound	RT	MS	MS^2	MS^3	Aglycone	Mass of sugar ring	Tentative identification
62	102.3	785	609	301,300	Quercetin	176 + 162 + 146	Glucosyl–rhamnosyl quercetin with Unknown sugar ring
63	102.7	815[b]	407	379, 363, 351,	_	_	Ginkgolide A
64	103.5	755	609	300[a], 271, 255	Quercetin	2 × 146 + 162	2-Rhamnosyl–glucosyl quercetin
65	104.0	847[b]	423	395, 367, 305	_	_	Ginkgolide B
66	105.9	431	285	255, 163	Kaempferol	146	Rhamnosyl kaempferol
67	106.7	755	609	300[a], 271, 255	Quercetin	2 × 146 + 162	2-Rhamnosyl–glucosyl quercetin
68	107.2	739	593	285	Kaempferol	2 × 146 + 162	2-Rhamnosyl–glucosyl kaempferol
69	108.8	903	593	285	Kaempferol	174 + 2 × 146 + 162	Glucosyl–2-rhamnosyl kaempferol with unknown sugar
70	110.9	739	593	285	Kaempferol	2 × 146 + 162	2-Rhamnosyl–glucosyl kaempferol
71	111.3	755	609	300[a], 271, 255	Quercetin	2 × 146 + 162	Coumaroyl–glucosyl–rhamnosyl Quercetin
72	111.4	1047[c]	_	_	Kaempferol	3 × 146 + 2 × 162	Coumaroyl–2-rhamnosyl–2-glucosyl kaempferol
73	117.1	739	593	285	Kaempferol	2 × 146 + 162	Coumaroyl–glucosyl–rhamnosyl kaempferol
74	118.1	301	271, 255	–	Quercetin	_	Quercetin
75	120.6	739	593	285	Kaempferol	2 × 146 + 162	2-Rhamnosyl–glucosyl kaempferol
76	127.3	285	257	–	Kaempferol	_	Kaempferol
77	136.3	315	300	283, 255, 227, 151	Isorhamnetin	_	Isorhamnetin

[a] *m/z* 301 and 300 are both present, more abundant one are listed in the table.
[b] Dimer.
[c] No MS/MS obtained because mass spectrometer fragment the co-eluted high abundant flavonoid. Thus, the sample which had the similar HPLC chromatographic pattern and RRT value compared with the typical fingerprint chromatogram could be authenticated as genuine.

full scan mass spectra. Another terpene lactone ginkgolide J (GJ), can be observed at retention time 77.2 min and was detected with the dimer *m/z* 847 as the base peak, upon fragmentation it formed *m/z* 423, an isomer of ginkgolide B, however, MS^3 of *m/z* 423 shows ions with *m/z* 349, unlike ginkgolide B's *m/z* 423, which further fragment to *m/z* 395, 367, 305. The majority of components are identified as flavonoids, 72 flavonoids can be detected in this analysis, among these, 17 flavonoids originate from quercetin, eighteen flavonoids from kaempferol, and eight flavonoids from isorhamnetin. Two flavonoids, Compounds 14 and 49, are thought to originate from methymyrincetin (*m/z* 331) and one compound **41** from myrincetin (*m/z* 317). There are eighteen compounds that are suspected as being flavonoids linked to unknown aglycones with *m/z* of 605, 373, 625, 361, 359, 287, 751, 375, 389, 391, 345, 557, 417, 521, 269. Also Compounds **59**, **62** and **69** are thought to be flavonoid glycosides with product ions at *m/z* 285 or 301 from MS/MS representing a kaempferol or quercetin aglycone, however the identity of the sugar bound requires further analysis. In Hasler's HPLC fingerprint paper thirty three flavonoids were identified (Hasler and Sticher, 1992) and, in general, the flavonoids identified in this capillary LC/MS study agree with Hasler's in terms of elution sequence. However, some compounds identified differ compared with Hasler's data and this is thought to be due to the detector utilised, as UV is more sensitive to some flavonoids and mass spectrometry is more sensitive to others. From our results, it is clear that MS is more sensitive to flavonoid glycoside whilst being less sensitive to aglycones or bioflavones, of the 11 such peaks (Peaks 23–33) in Hasler's report (Hasler and Sticher, 1992), only three major aglycones were seen in our data. The HPLC/MS approach allows more information to be recorded in data-dependent analyses and can study the levels of the terpene lactones which are not readily detected by HPLC in combination with UV.

Fig. 6 shows the mass spectrometric base peak fingerprint profile of the five *G. biloba* commercial samples. The base peak in sample 3, Compound **70** at 111 min, can be used as reference peak (IS) to evaluate the other components by comparison of peak areas. For example the QD/IS ratio in sample 2 is much higher than other samples, also the RH/IS ratio in sample 1 is higher than other samples, which indicates the fortifying of rutin in sample 1 and quercetin in sample 2, respectively. A study of the overall profile of compounds present indicates that samples 4 and 5 are similar in composition and sample 2 is most similar to the "pure" sample except QD content is exceptionally high (3), whilst sample 1 readily indicates elevated RH levels. Despite these differences, all five samples satisfy the traditional quality control procedures, and this finding is consistent with our previous quantitative results (Ding et al., 2006a). This indicates that the HPLC/MS fingerprint is a useful technique in quality control of the plant extract compared to our previous study involving the accurate quantitation of a few known compounds (Ding et al., 2006b). Detection of both flavonoid glycosides and flavonoid aglycones allows the determination of spiked fortification which can not be detected by traditional quality control methods and also detect fortification even if reference standards are not easily available. It was considered that methanol extraction has the potential to extract many additional components from *G. biloba*, as well as the bioactive components flavonoids and terpene lactones. In order to study the bioactive components more specifically and more selectively, different data processing techniques can be utilised. According to the fragmentation pathways of

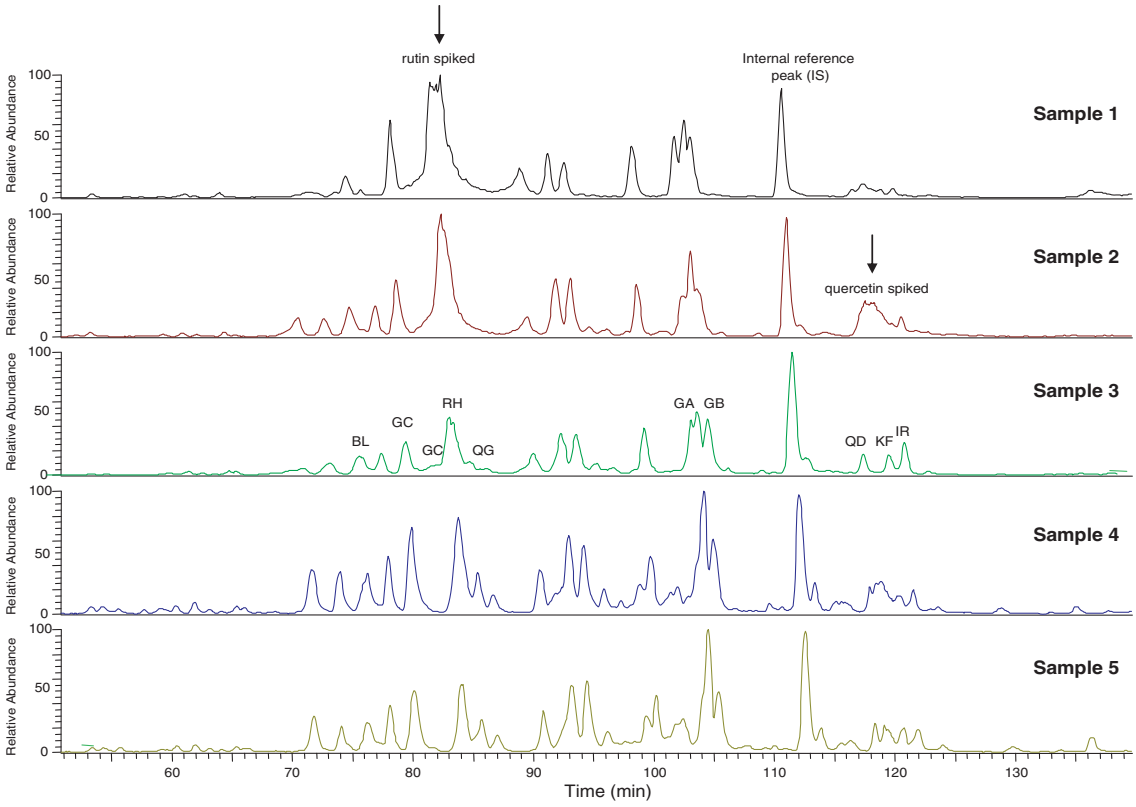

Fig. 6. Bask peak full scan of the five *Ginkgo biloba* commercial samples.

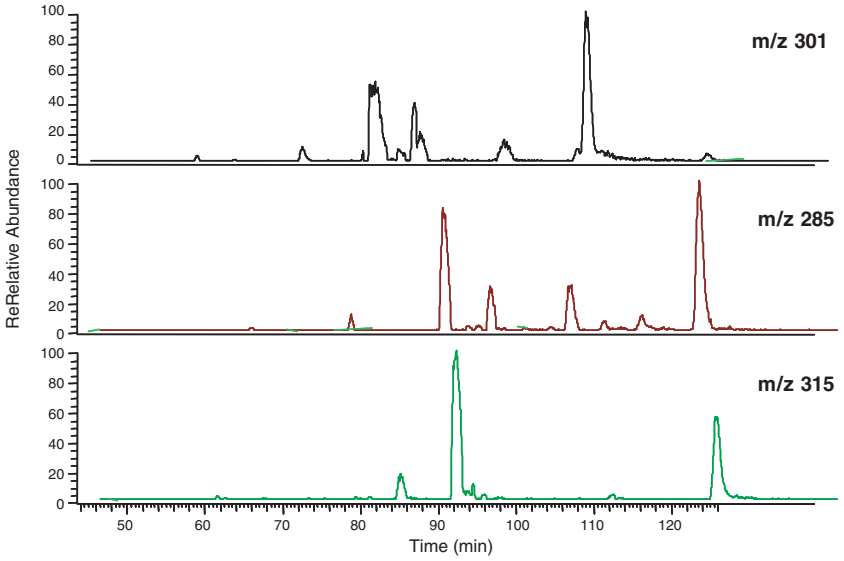

Fig. 7. Capillary LC/MS extracted ion chromatogram (XIC) of *Ginkgo biloba* extract at *m/z* 301, 285, 315.

flavonoid glycosides, all flavonoid glycosides will form their aglycones (and also radical aglycone ions) upon fragmentation (be it in-source or by CID) and so the extracted ion chromatography (XIC) of the flavonoid aglycones *m/z* 301, 285, 315 can be utilised in order to analyse the flavonoid glycoside components in the sample. Fig. 7 shows a profile of the flavonoids in *G. biloba* extract monitored for in this manner and how many flavonoids originate from quercetin, kaempferol and isorhamnetin, respectively. Many peaks can be seen in each *m/z* range which indicates that *G. biloba* is a complex mixture of various flavonoid glycosides.

Alternatively, since the detected flavonoids are mainly glucoside and rhamnoside, (or coumaroyl) conjugated (with additional masses of 162 and 146 Da, respectively), constant neutral loss analysis of the characteristic sugar

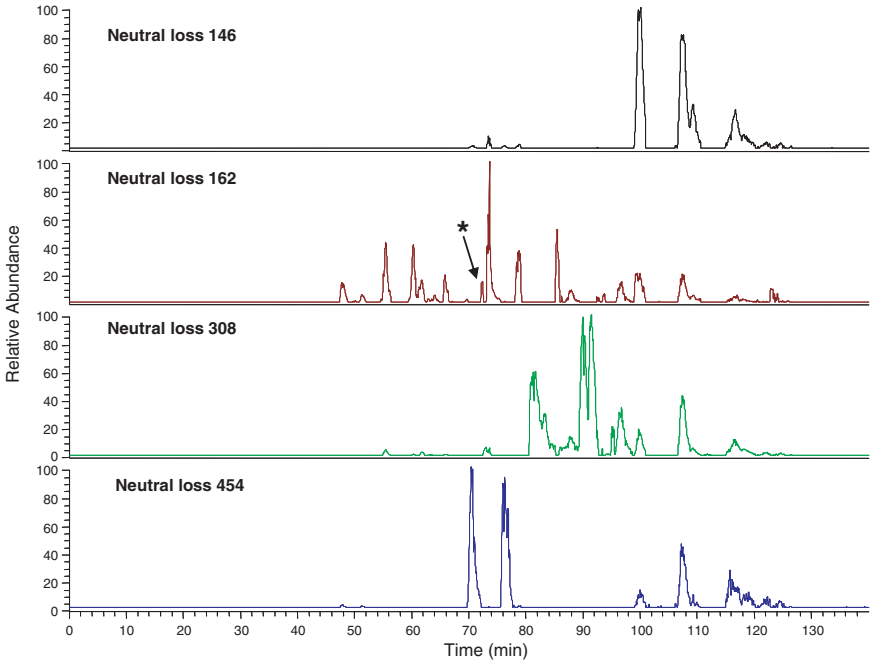

Fig. 8. Capillary LC/MS extracted ion chromatogram (XIC) of *Ginkgo biloba* extract by neutral loss of *m/z* 162, 146, 308, 454 Da.

ring can be used to identify the flavonoids from the methanol extract and obtain a profile of which flavonoid aglycone are conjugated to which sugar moieties. As shown in Fig. 8, using neutral loss of *m/z* 162, 146, 308, 454 (loss of a single glucoside, a single rhamnoside/coumaroyl, a combination of the two and a glucoside and two rhamnosides (or one rhamonoside one coumaroside each), respectively) most of the flavonoids in Table 1 are more selectively determined, and there is an additional peak, (highlight with * in Fig. 8) corresponding to *m/z* 523 → *m/z* 361 at 72.3 min which was not detected using full scan base peak analysis and only observed by constant neutral loss data processing. The data also indicates that there are more glucoside flavonoids than rhamnoside flavonoids in *G. biloba* extract. The neutral loss study of terpene lactones proved to be less successful, as the dimers of the terpene lactones were more abundant in the full scan mass spectra than the de-protonated molecule, and the charac-

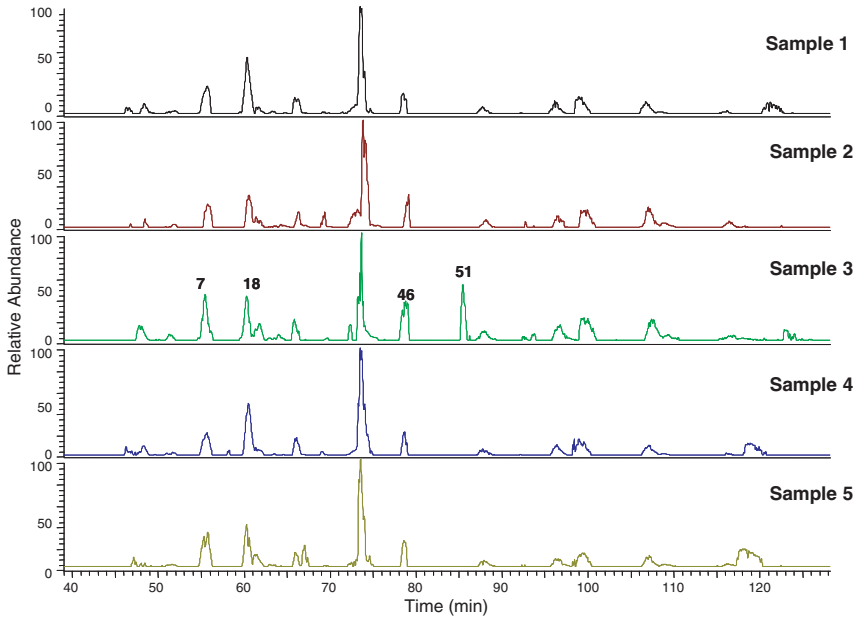

Fig. 9. Capillary LC/MS extracted ion chromatogram (XIC) of neutral loss of *m/z* 162 Da of five *Ginkgo biloba* commercial samples.

teristic fragmentation of the terpene lactones is only observed in the MS^3 scan in which the quality of the data and the signal to noise ratio of the observed losses is not as good as that observed in the flavonoid MS analysis. Reducing the MS scan range could solve this problem of dimer predominance, however the increased mass range is required so as not to miss many of the flavonoids and hence the simultaneous analysis of both compounds becomes more complicated. The flavonoid neutral losses profile can also be utilised to compare different samples, for example, by comparing neutral loss of *m*/*z* 308, it can be seen that sample 1 has more rutin than other samples, which indicates fortification (data not shown). Interestingly, by comparing neutral loss of *m*/*z* 162 as shown in Fig. 9 the compounds **7**, **18**, **46** and **51** (as listed in Table 1) show obvious variations between samples, this is thought to be because of the different origins of the extract or processing and storage conditions which are very important in the quality control of plant extracts. The compound labelled **51** appears to be unique to the proposed "pure" sample and lacking from all others, which also exhibit reduced amounts of component **46**, furthermore sample 5 indicates a unique component at retention time 67 min.

4. Conclusion

HPLC/MS is applicable to the chemical fingerprint quality control analysis of plant extracts due to its high sensitivity and specificity. In this study, the fingerprint profiles of *G. biloba* extracts were established by capillary column HPLC/MS and the capillary column was shown to give good resolution and sensitivity. By reprocessing the mass spectrometry data using tandem MS/MS, XIC and neutral loss information, considerably more information can be obtained to compare the similarity of *Ginkgo* extract from different origins. This is a major advantage over HPLC-UV and TLC methods which are currently the main techniques used for fingerprinting analysis. According to the fragment pathway of *Ginkgo* flavonoids, the XIC of *m*/*z* 301, 285 and 315 were constructed and it can be concluded that more than seventy flavonoids can be detected by capillary column HPLC/MS, constant neutral loss analysis also provides important information regarding identification of components and evaluation of the similarity and stability of the samples. This post-acquisition processing of the data proved useful in studying variations in the flavonoids, however, a similar approach to the terpene lactones was complicated due to prominent adduct and dimer formation of these compounds. Fingerprint analysis can generate an overview of all the components in the sample under investigation, by comparing the relative peak area with standard sample, semi-quantitative data can be obtained and artificial addition of rutin and quercetin can be detected without using the reference standards. Thus the fingerprint method is therefore especially useful when reference standards for all components are not available.

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