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# Quality Control Analyses for Ginkgo Extracts Require Analysis of Intact Flavonol Glycosides

B. DUFF SLOLEY\*, SOHEIR R. TAWFIK, KATHERINE A. SCHERBAN AND YUN K. TAM

Analytical Department, Kinetana Inc., 108 Advanced Technology Centre, 9650 – 20th Avenue N.W., Edmonton, Alberta, Canada, T6N 1G1.

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## ABSTRACT

Ten commercial standardized *Ginkgo biloba* extracts were examined for flavonol glycoside and terpene lactone content. All samples arrived with certificates of analysis stating that the extracts were standardized *Ginkgo biloba* leaf extract and that they contained a minimum of 24% total flavonol glycosides and 6% terpene lactones. Samples were analyzed for total flavonol glycosides using a method involving acid hydrolysis and subsequent quantification of the flavonol aglycones by HPLC coupled with UV diode array absorbance detection. Analysis of intact flavonol glycosides and terpene lactones was accomplished using HPLC coupled with UV diode array detection and electrospray mass spectrometric detection. Of the ten standardized extracts eight samples provided chromatograms and chemical values characteristic of previously published analyses of ginkgo leaf extracts. One sample provided analyses that suggested that the extract had been adulterated with rutin in order to elevate total flavonol glycoside levels. Another sample indicated that it was not an extract of *Ginkgo biloba* leaf at all. Adulteration with rutin was not strongly evident when the acid hydrolysis method was used to analyze the sample for total flavonol glycosides. However, this particular adulteration was easily evident when analyzing the samples for characteristic intact flavonol glycosides. This work demonstrates the need for extensive quality control analyses for herbal products and that the analysis of intact characteristic compounds provides a reliable method for detecting adulteration.

Key words: *Ginkgo biloba*, flavonol glycosides, terpene lactones, quality control analyses

## INTRODUCTION

During the course of our investigations of potential suppliers of consistent high quality *Ginkgo biloba* extracts, we felt it was necessary to expand our analyses to include an evaluation of the intact flavonol glycosides characteristic of this product. Previously almost all analyses have involved the determination of flavonol aglycones following acid hydrolysis<sup>(1)</sup> and terpene lactones<sup>(2)</sup> to provide data supporting the characteristic 24% flavonol glycoside and 6% terpene lactone content required for standardized *Ginkgo biloba* leaf extracts. The presence of the characteristic terpene lactones (bilobalide and ginkgolides A, B, C and J) provides evidence that the extract originates from *Ginkgo biloba* plant materials. However, the measurement of the characteristic flavonol aglycones (quercetin, kaempferol and isorhamnetin) does not necessarily preclude the possibility of adulteration of ginkgo extracts with pure flavonol aglycones (eg. quercetin, kaempferol, isorhamnetin), pure flavonol glycosides (eg. rutin, hyperoside, quercitrin) or other plant materials containing flavonols and flavonol glycosides. Such adulteration may occur when the producer has determined that the extract is too low in its flavonol glycoside content. Adulteration with inexpensive flavonols (eg. quercetin) or flavonol glycosides (eg. rutin) may permit an unscrupulous supplier to circumvent the tests performed by almost all purveyors of ginkgo products.

The analysis of intact flavonol glycosides has been extensively examined<sup>(3-5)</sup> and the equipment required for such analyses is routinely available. Unfortunately the standards required for quantitative analysis of intact flavonol glycosides are for the most part unavailable or very costly. This, however, does not preclude conducting studies designed to compare the flavonoid profile characteristic of standardized ginkgo extracts from various suppliers, particularly if the identities of the compounds can be confirmed with electrospray mass spectrometric detection. Detector responses, whether UV absorbance, fluorescence or electrospray mass spectrometric can be used to evaluate the comparative amounts of various identified flavonoids in different supplied extracts. A significant deviation from the expected profile can indicate whether the extract has been adulterated.

## MATERIALS AND METHODS

### I. Materials

#### (I) *Ginkgo extracts*

Ten standardized *Ginkgo biloba* leaf extracts were obtained from suppliers in Europe, Asia and North America. Each extract was accompanied by a manufacturer's certificate of analysis indicating that the extract had a minimum of 24% flavonol glycosides and 6% terpene lactones.

\* Author for correspondence. TEL:1-780-414-0214;  
Fax:1-780-432-2096; E-mail:duff@kinetana.com

## (II) Standards

Pure standards of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, quercetin dihydrate, kaempferol and isorhamnetin were purchased from ChromaDex Inc. (Laguna Hills, CA). Rutin trihydrate was obtained from Sigma Aldrich (St. Louis MO)

## (III) Solvents and chemicals

All solvents were HPLC grade. All water was purified using an in-house Milli-Q System (Millipore, Milford, MA). All chemicals were reagent grade or HPLC grade.

## II. Methods

### (I) Analysis of total flavonol glycosides

Analysis of total flavonol glycosides was based on the methods described in Sticher *et al.*<sup>(6)</sup>; Lin-Chin *et al.*<sup>(7)</sup>; Srinivasan<sup>(8)</sup> and the USP method described by Giancasporo<sup>(9)</sup>.

*Ginkgo biloba* samples were dissolved in 25% aqueous HCl:methanol (10:70 v/v) at a concentration of 4 mg/ml. The samples containing filler were clarified by centrifugation. 200  $\mu$ L of this solution was placed in a sealed glass container and heated at 100°C for 25 minutes. The samples were removed, cooled and 10  $\mu$ L was injected directly onto a HPLC system.

The amounts of quercetin, kaempferol and isorhamnetin in the samples were determined using HPLC equipped with diode array absorbance detection. The equipment used was a Hewlett Packard Series 1100 HPLC system. The column used was a Phenomenex Luna 3  $\mu$ C<sub>18</sub><sup>(2)</sup> (150  $\times$  4.6 mm) equipped with a guard column (Phenomenex Security Guard C<sub>8</sub> 4  $\times$  3 mm). Separation was achieved using isocratic elution (Mobile phase 28% acetonitrile, 72% 0.1% phosphoric acid with 0.01% triethylamine at a flow of 1 mL/min). Quantification was based on absorbance at 370 nm. Total flavone glycoside values were calculated by multiplying the sum of the quercetin, kaempferol and isorhamnetin concentrations by the suggested conversion factor of 2.51 which provided an estimate of the contribution of the sugars to the total mass according to Sticher *et al.*<sup>(6)</sup>.

### (II) Analysis of intact flavonol glycosides

*Ginkgo biloba* samples were dissolved at a concentration of 0.5 mg/mL in 20% methanol with sonication. The samples were clarified by centrifugation. The supernatant was collected and 10  $\mu$ L was injected directly onto the HPLC system.

The amounts and identity of a number of intact flavonol glycosides in the samples were determined using HPLC equipped with diode array and electrospray mass spectrometric detection. The equipment used was a

Hewlett Packard Series 1100 HPLC system. The column used was a Phenomenex Luna 3  $\mu$ C<sub>18</sub><sup>(2)</sup> (150  $\times$  4.6 mm) equipped with a guard column (Phenomenex Security Guard C<sub>8</sub> 4  $\times$  3 mm). Separation was achieved using gradient elution (mobile phase A, 5% acetonitrile 0.1% formic acid; mobile phase B, 85% acetonitrile, 0.1% formic acid at a flow rate of 0.75 mL/min). The gradient timetable was time = 0 min solvent B = 0%; time = 7 min solvent B = 15%; time = 20 min solvent B = 15%; time = 55 min solvent B = 30%; time = 75 min solvent B = 80%; time = 80 min solvent B = 0%. Elution of chemicals was monitored using diode array absorbance detection between 190 nm and 900 nm and by electrospray mass spectrometry with masses monitored between 200 amu and 1000 amu in positive mode. All other mass spectrometer conditions were the same as those described in Li *et al.*<sup>(10)</sup>. Peak identities were based on the retention characteristics previously published by Sticher *et al.*<sup>(6)</sup> and confirmed by mass spectral data. Concentrations of flavonoids could be estimated by comparison with pure chemical standards when available. Quantification can be accomplished using absorbance at 280 or 370 nm. Limits of detection (3 times noise) using quercetin and kaempferol as references were estimated to be between 10 and 20 pmol. Electrospray signals for flavonoids were rather weak and were not used for quantitative estimates. At present, values are reported as peak areas only because commercial standards are not available for many of the predominant intact flavonol glycosides found in ginkgo. UV absorbance detector response to the predominant flavonol glycosides is linear to concentrations of authentic standardized extracts of between 0.1 and 10 mg/mL when 20 to 100  $\mu$ L injections are applied to the column.

### (III) Analysis of terpene lactones

*Ginkgo biloba* samples were dissolved in 20% methanol at a concentration of 0.5 mg/mL with sonication. The samples were clarified by centrifugation. The supernatant was removed and 20 mL was injected directly onto a HPLC system.

The amounts of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C and ginkgolide J in the samples were determined using HPLC with electrospray mass spectrometric detection as described by Li *et al.*<sup>(10)</sup>. The equipment used was a Hewlett Packard Series 1100 HPLC system equipped with diode array and electrospray mass spectrometric detection. Monitoring in single ion positive mode (SIM) was used with the mass spectrometric detector to provide specific quantifiable signals. Mass spectra were monitored between 50 and 500 amu and in single ion mode (SIM) at 327, 344, 349, 409, 425, 426, 431, 441, 442, 447, 458 and 463 amu.

The column used was a Phenomenex Luna 3  $\mu$ C<sub>18</sub><sup>(2)</sup> (150  $\times$  4.6 mm) equipped with a guard column (Phenomenex Security Guard C<sub>8</sub> 4  $\times$  3 mm). Separation was achieved using gradient elution (mobile phase A, 20

mM ammonium acetate; mobile phase, B methanol at a flow of 0.75 mL/min). The gradient timetable was time = 0 min solvent B = 25%; time = 20 min solvent B = 75%; time = 23 min solvent B = 90%; time = 34 min solvent B = 90%; time = 34.8 min solvent B = 25%.

## RESULTS

Of the ten standardized extracts examined, eight provided terpene lactone and flavonoid profiles consistent with previously published data<sup>(1-4,6,11,12)</sup> and many other standardized ginkgo extracts previously analyzed by our group. One product contained concentrations of rutin that were up to 6 times the concentration observed in other products, while another product contained neither terpene lactones nor flavonol glycosides characteristic of standardized *Ginkgo biloba* extracts.

The concentrations of individual terpene lactones determined in the ten standardized extracts are presented in Table 1. In extracts described by Camponovo and Soldati<sup>(11)</sup>, the ratio of bilobalide to the other ginkgolides was about bilobalide:ginkgolide J (10:1); bilobalide:ginkgolide C (10:2); bilobalide:ginkgolide A (10:4); bilobalide:ginkgolide B (10:2), but this could vary somewhat between season and ginkgo genders. The nine extracts in the present study that were determined to contain ginkgo terpene lactones followed this general pattern with bilob-

alide being the most prominent terpene lactone followed by ginkgolide A. Ginkgolide B and ginkgolide C were almost equal in concentration and were less prominent than ginkgolide A. Ginkgolide J made up the least amount of the total terpene lactones. The ginkgo extract from supplier 5 contained almost no terpene lactones (less than 1% of the other extracts) and so was considered to be a fraud.

The total flavonol glycoside content evaluated after acid hydrolysis in the ten samples is presented in Table 2. The pattern of relative concentrations of quercetin, kaempferol and isorhamnetin followed the pattern described for flavonol aglycones by Camponovo and Soldati<sup>(11)</sup>. Quercetin was the most prominent flavonol followed closely by kaempferol. The concentration of isorhamnetin was about one fifth that of either quercetin or kaempferol. Prominent differences in the chromatographic profile were not readily evident in any of the extracts, although it might be observed that sample 1 from supplier 3 has relatively high quercetin to kaempferol and isorhamnetin ratios. Statistical analyses indicated that the quercetin concentration in sample 1 from supplier 3 was greater than 2 standard deviations from the mean of the nine authentic samples and was considered to be an outlier. The extract from supplier 5 contained little or none of the characteristic flavonols associated with ginkgo. It did however contain a peak that cochromatographed with luteolein.

The absorbance values at 280 nm for six individual flavonol glycosides in the ten standardized ginkgo extracts

**Table 1.** Concentrations of five terpene lactones in 10 commercial extracts.

Supplier	Lot #	Bilobalide % by weight	Ginkgolide J % by weight	Ginkgolide C % by weight	Ginkgolide B % by weight	Ginkgolide A % by weight	Total Terpene Lactones % by weight
1	1	4.36	0.67	1.49	0.83	1.57	8.92
2	1	3.16	0.61	1.36	1.34	2.27	8.75
	2	3.54	0.69	1.42	0.43	1.75	7.83
	3	2.56	0.63	1.19	1.37	2.17	7.93
3	1	4.54	0.70	1.75	1.75	2.45	11.19
	2	7.05	0.78	1.58	0.80	2.44	12.65
	3	8.64	0.63	1.26	0.80	2.90	14.23
	4	3.41	0.52	1.25	1.29	2.09	8.56
4	1	4.98	0.66	1.49	0.80	2.01	9.94
5	1	0.03	0.03	N.D.	N.D.	0.01	0.07

Values are the mean of samples analyzed in duplicate. N.D. < 0.005% by weight

**Table 2.** Concentrations of flavonol aglycones and total flavonol glycosides in 10 commercial extracts performed in duplicate.

Supplier	Lot #	Quercetin % by weight	Kaempferol % by weight	Isorhamnetin % by weight	Total Flavonol Glycosides Σ aglycones X 2.51
1	1	6.22	5.57	0.88	31.8
2	1	6.95	4.62	0.90	31.3
	2	6.18	4.82	0.83	29.7
	3	7.28	4.84	0.95	32.8
3	1	8.34*	3.64	0.82	32.1
	2	6.46	5.16	0.97	31.6
	3	6.23	5.03	0.93	30.6
	4	5.82	4.98	1.04	29.7
4	1	6.83	5.20	1.13	33.0
5	1	N.D.	0.02	0.05	0.18

Values are the mean of samples performed in duplicate. N.D. < 0.01 %

\*: Greater than two standard errors from the mean of authentic samples.

**Table 3.** Peak areas for six selected flavonol glycosides in 10 commercial extracts performed in duplicate.

Supplier	Lot #	RRGK	RGQ	RGK	RGI	CGRQ	CGRK
1	1	85	189	109	72	163	124
2	1	126	457	138	147	330	202
	2	134	373	142	142	285	194
	3	141	496	147	155	312	195
3	1	98	1322*	120	137	248	159
	2	152	323	161	163	334	214
	3	156	313	161	174	386	246
4	4	166	308	171	172	316	192
	1	169	335	163	177	370	221
5	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Values are peak areas per sample in mAU and are the mean of analyses performed in duplicate. N.D. < 5 mAU

\*: Greater than two standard errors from the mean of authentic samples.

RRGK = 3-O-[2-O, 6-O-Bis( $\alpha$ -L-rhamnosyl)- $\beta$ -D-glucosyl]kaempferol

RGQ = 3-O-[6-O-( $\alpha$ -L-Rhamnosyl)- $\beta$ -D-glucosyl]quercetin = rutin

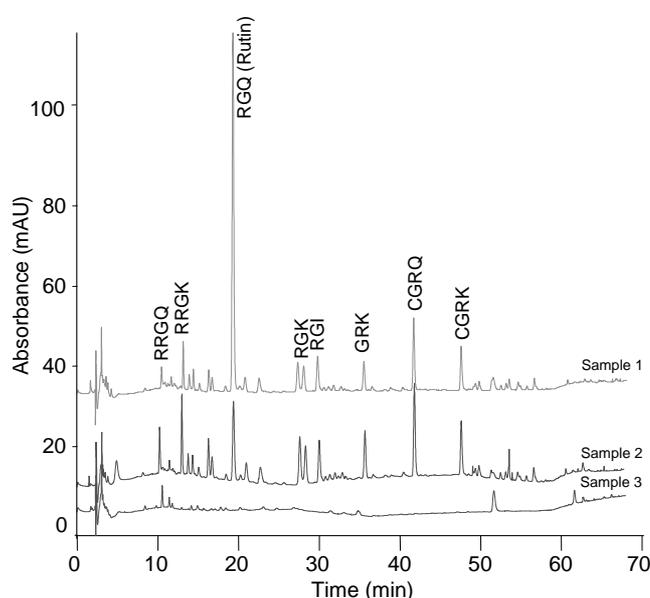
RGK = 3-O-[6-O-( $\alpha$ -L-Rhamnosyl)- $\beta$ -D-glucosyl]kaempferol

RGI = 3-O-[6-O-( $\alpha$ -L-Rhamnosyl)- $\beta$ -D-glucosyl]isorhamnetin

CGRQ = 3-O-[2-O-(6-O-{*p*-Coumaroyl})- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnosyl]quercetin

CGRK = 3-O-[2-O-(6-O-{*p*-Coumaroyl})- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnosyl]kaempferol

are presented in Table 3. Within most analyzed extracts the relative ratios of absorbance of the individual flavonol glycosides did not vary much (usually less than 10%). For example, the ratio of 3-O-[2-O-(6-O-{*p*-coumaroyl})- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnosyl] quercetin (CGRQ) to 3-O-[2-O-(6-O-{*p*-coumaroyl})- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnosyl] kaempferol (CGRK) for the nine samples containing these compounds was  $1.56 \pm 0.11$  (mean  $\pm$  S.D.,  $n = 9$ ). The exceptions noted were the absorbance value obtained for rutin (RGQ) in extract 1 from supplier 3 and the absence of characteristic flavonol glycosides from the sample obtained from supplier 5. Due to the complete absence of characteristic ginkgo flavonol glycosides, the sample obtained from supplier 5 was again considered a fraud. In all other samples except sample 1 from supplier 3, the absorbance values for the coumaroyl conjugates of quercetin and kaempferol (CGRQ and CGRK) were about equivalent to that of rutin (RGQ). In the case of sample 1 from supplier 3, the rutin absorbance was at least 5 to 6 times that of the coumaroyl conjugates. The fact that rutin is a quercetin glycoside explains why the ratios of quercetin to kaempferol and isorhamnetin were so high when hydrolyzed flavonoids were analyzed (see Table 2). Comparing the differences among chromatograms of the intact flavonoids derived from the eight typical extracts and sample 1 from supplier 3 and the sample from supplier 5, it becomes very evident that substantial differences exist (Figure 1). Certainly the sample from supplier 5 contains no substantial amount of any chemicals characteristic of standardized *Ginkgo biloba* extracts. The rutin concentration in sample 1 from supplier 3 far exceeds any other rutin concentration determined in this group of extracts or any other *Ginkgo biloba* extract that we have encountered. Furthermore, the absorbance values for the other important flavonol glycosides were lowest in sample 1 from supplier 3 (the exception being the sample from supplier 1 which had rather high aglycone levels in the intact flavonoid analysis,



**Figure 1.** Chromatograms of three standardized ginkgo extracts obtained from commercial suppliers. Sample 2 (Supplier 3, Lot 2) is representative of the majority of authentic extracts encountered. Sample 1 (Supplier 3, Lot 1) is considered to be adulterated with rutin as the relative absorbance for this peak exceeds the mean of the rutin absorbance value in authentic samples (sample 1 included) by more than two standard deviations. Sample 3 (Supplier 5) contains no flavonol glycosides characteristic of ginkgo.

RRGQ = 3-O-[2-O, 6-O-Bis( $\alpha$ -L-rhamnosyl)- $\beta$ -D-glucosyl] quercetin

RRGK = 3-O-[2-O, 6-O-Bis( $\alpha$ -L-rhamnosyl)- $\beta$ -D-glucosyl] kaempferol

RGQ = 3-O-[6-O-( $\alpha$ -L-Rhamnosyl)- $\beta$ -D-glucosyl] quercetin = rutin

RGK = 3-O-[6-O-( $\alpha$ -L-Rhamnosyl)- $\beta$ -D-glucosyl] kaempferol

RGI = 3-O-[6-O-( $\alpha$ -L-Rhamnosyl)- $\beta$ -D-glucosyl] isorhamnetin

GRK = 3-O-[2-O-( $\beta$ -D-Glucosyl)- $\alpha$ -L-rhamnosyl] kaempferol

CGRQ = 3-O-[2-O-(6-O-{*p*-Coumaroyl})- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnosyl] quercetin

CGRK = 3-O-[2-O-(6-O-{*p*-Coumaroyl})- $\beta$ -D-glucosyl]- $\alpha$ -L-rhamnosyl]kaempferol

data not presented). This suggests that the manufacturer decided to increase the levels of flavonoids in a product that provided relatively low total flavonol glycoside concentrations. Interestingly, the total flavonol glycoside values determined for sample 1 from supplier 3 were among the highest, indicating that the adulteration may have been unnecessary, possibly because of an inaccurately low estimate of total flavonol glycosides due to poor hydrolysis of the original sample. In our hands, hydrolysis under these conditions is usually greater than 90% complete, but a slight reduction in temperature, time or acid concentration can greatly influence the degree of hydrolysis. We can verify the degree of hydrolysis by running samples of the hydrolyzed extract through the intact flavonoid analysis to determine how complete the hydrolysis has been.

## DISCUSSION

It is evident from this work that relying on a producer's or distributor's certificate of analysis for standardized ginkgo extracts is not sufficient to ensure genuine and high quality products. Furthermore, quality control based on the measurement of flavonol aglycones derived from acid hydrolysis of standardized extracts is probably not sufficient to discover adulteration of products with inexpensive flavonol glycosides such as rutin. High purity rutin can be obtained from Sigma Chemical Co. at a cost of about US \$240.00 per kilogram. This is often less than the wholesale value of standardized ginkgo extract. Therefore it seems feasible that rutin of a quality that could be used for adulteration of standardized ginkgo extracts might be obtained for a price considerably less than that of the final standardized ginkgo extract product. Thus, adulteration is not only economically viable, but also indeed profitable.

Adulteration of ginkgo extracts with a mixture of quercetin and kaempferol and possibly isorhamnetin is very difficult to detect using the established hydrolysis method for measuring total flavonol glycosides. In fact, adulteration with a balanced mixture of aglycones would be undetectable. In contrast, as standardized ginkgo extracts contain very low concentrations of flavonol aglycones, adulteration with these compounds is obvious with the use of the intact flavonol glycoside analysis. High levels of aglycones would be evident as these compounds elute at 50 minutes (quercetin), 57 minutes (kaempferol) and 57.5 minutes (isorhamnetin) in the chromatogram illustrated in Figure 1 and their peak areas are usually less than 5% those of the characteristic coumaryl conjugated flavonol glycosides. Adulteration with rutin or any single selected flavonol glycoside is also obvious when the intact flavonol glycoside analysis is used. In contrast, if the hydrolysis method is used, the adulteration becomes less evident due to the contribution of quercetin from the other endogenous quercetin glycosides present which mutes the difference.

Of the commercial extracts that meet the quality

criteria that we have examined, most have total flavonol concentrations relatively close to the 24% (29% to 33%) value, whereas the terpene lactone contents often exceed the 6% level to a much greater extent (8% to 14%). This observation has been noted elsewhere<sup>(5)</sup>. Thus it appears that most often it is the total flavonol glycosides that are the limiting factor when establishing quality based on the 24% flavonol glycosides and 6% terpene lactones criteria. Furthermore, since sources of flavonols and flavonol glycosides are much less expensive than ginkgolides and bilobalide, it is far more likely that if adulteration occurs, it would be with some form of flavonol and adulteration candidates are probably quercetin and rutin.

In conclusion, it is evident that certificates of analysis provided by manufacturers or suppliers of standardized ginkgo extracts are not sufficient to ensure a quality product. Conscientious marketers may wish to subcontract impartial third parties for quality assurance. Furthermore, an analysis of the intact characteristic flavonoids is the best as this provides the highest assurance that the product has not been adulterated with either flavonol aglycones or flavonol glycosides.

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