An effective identification and quantification method for *Ginkgo biloba* flavonol glycosides with targeted evaluation of adulterated products

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A B S T R A C T

Background: *Ginkgo biloba* L. (*Ginkgoaceae*) leaf extract is one of the most popular herbal products on the market, as it contains flavone glycosides (≥ 24%) and terpene lactones (≥ 6%), which are proposed to have significant physiological effects. Unfortunately, the challenging financial climate has resulted in a natural health product market containing adulterated ginkgo products.

Purpose: 42 ginkgo samples were analyzed to establish an HPLC profile for authentic ginkgo and common ginkgo adulterants, and to develop a method capable of easily detecting adulteration in ginkgo commercial products.

Method: In this study an efficient and targeted HPLC analysis method was established that is capable of distinguishing flavonol glycosides and aglycones simultaneously for the evaluation of ginkgo powdered extracts (PFEs) and finished products in a single, 13 min run. Thirteen ginkgo leaf samples, fifteen standardized powdered extracts, and fourteen commercially available ginkgo products have been analyzed using this new HPLC method. Chromatograms were compared to six standard reference materials: one flavonol glycoside (rutin), three aglycones (quercetin, kaempferol and isorhamnetin), and two isoflavones (genestin and genistein). The quantitative chromatographic data was interpreted by principal component analysis (PCA), which assisted in the detection of unexpected chromatographic features in various adulterated botanical products.

Results: Only three of the commercially available ginkgo products tested in this study were determined to be authentic, with flavonol glycoside rutin, and aglycones quercetin, kaempferol, and isorhamnetin found to be common adulterants in the ginkgo powdered extract and finished product samples.

Conclusion: Despite evidence of adulteration in most of the samples, each of the samples discussed herein met most of the current pharmacopeial standards. It is therefore critical that a preliminary evaluation be utilized to detect adulteration in commercial ginkgo products, prior to the acid hydroysis procedure utilized in the current testing methods.

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Introduction

In the last 2000 years, the *Ginkgo biloba* L. plant has been of great interest due to its use in improving the mental capacities of patients with regular use. (Zhang et al. 2011) Ginkgo leaf extract is one of the most popular herbal products on the market, as it contains well-studied active ingredients that are proposed to
have significant physiological effects. (Lin et al. 2008; Song et al. 2010; van Beek and Montoro 2009) In fact, extensive clinical research has found that standardized ginkgo extract may reduce patients’ risk of developing a number of mental diseases, including Alzheimer’s. (Sierpina et al. 2003) These findings have the potential to make a great impact on mental health worldwide, as the occurrence of Alzheimer’s disease is expected to quadruple by 2050. (Vellas et al. 2012)

The natural health product market is constantly expanding as it provides natural remedies and promotes a healthy lifestyle. Unfortunately, the challenging financial climate is resulting in a market containing adulterated products. North America is a major provider of herbal products, a commodity that has recently come under considerable scrutiny in the media. In February 2015, the New York State Attorney General released a statement indicating that an investigation of a number of well-known herbal supplements, including G. biloba, revealed that many of the products tested (using DNA barcoding technique) contain no DNA of the herbal ingredient. (Kaplan 2015) It has since been shown that this technology is not appropriate for routine analysis of herbal extracts and their products, due to the temperatures and solvents involved in processing; however, this event did bring to light concerns about the quality of today’s herbal products. Not long after, the China Food and Drug Administration (CFDA) ordered over 200 Chinese pharmaceutical manufacturers to recall their ginkgo products due to quality issues. (China-Food-and-Drug-Administration May 19 2015) These investigations have incited a need for more effective quality control in general, including commercial ginkgo products.

Extensive research has revealed that the active compounds of ginkgo are flavonol glycosides and terpene lactones. (Kakigi et al. 2011; Kakigi et al. 2010) These compounds are typically found in standardized ginkgo extracts at ≥ 24% and ≥ 6% for flavonol glycosides and terpene lactones respectively. The current analytical methods available to test ginkgo require an initial acid hydrolysis step. This acid hydrolysis step results in the cleaving of the flavonol glycosides to form aglycones, a series of compounds which are often not found in the original raw ginkgo herb. It is, however, these aglycones – quercetin, kaempferol, and isorhamnetin – that are analyzed in the quality control monographs of ginkgo products.

Monographs are comprehensive testing methods developed by pharmacopeias for the purpose of ensuring the standardization of herbal materials including raw leaves, powdered extracts (PE), and commercial products. The ginkgo monographs published by the United State Pharmacopeia (USP) (United-States-Pharmacopeial-Convention 2015), British Pharmacopoeia (BP) (British-Pharmacopoeia-Commission 2012), European Pharmacopoeia (EP) (European-Pharmacopoeia 2015), and Chinese Pharmacopoeia Commission (CP) (Chinese-Pharmacopoeia-Commission 2010) are generally in agreement with respect to their testing methods and required contents of 22.0–27.0% flavonol glycosides. There are, however, slight variations with respect to the high performance liquid chromatography (HPLC) peak ratios of the aglycones in hydrolyzed ginkgo samples. USP (United-States-Pharmacopeial-Convention 2015) states that the peak ratios of kaempferol/quercetin and isorhamnetin/quercetin should be ≥ 0.7 and ≥ 0.1 respectively, while values of 0.8–1.2 and ≥ 0.15 are required by the Chinese Pharmacopoeia Commission (Chinese-Pharmacopoeia-Commission 2010) for the same respective ratios. The BP and EP monographs do not specify peak ratios, they simply require the standard 22.0–27.0% ginkgo flavone glycosides, 2.6–3.2% bilobalide and 2.8–3.4% ginkgolides A, B and C, and not more than (NMT) 5 ppm of ginkgolic acids. (British-Pharmacopoeia-Commission 2012; European-Pharmacopoeia 2015) Although the monographs provided by USP, BP, EP, and CP play an essential role in the quality control of ginkgo products, these monographs do not provide methods for the analysis of samples prior to acid hydrolysis. (British-Pharmacopoeia-Commission 2012; European-Pharmacopoeia 2015; United-States-Pharmacopeial-Convention 2015) One exception, the China Chamber of Commerce for Import & Export of Medicines & Health Products (CCCMHP-IE) does provide analysis specifications for ginkgo prior to acid hydrolysis: rutin ≤4%; quercetin ≤0.5%; kaempferol ≤0.5%; and isorhamnetin ≤0.2%. (CCCMHP-IE 2015) They also list the peak ratio of kaempferol/quercetin ≥ 0.7 for post acid hydrolysis samples. USP (United-States-Pharmacopeial-Convention 2015) recently added a new test entitled “Limit Criteria of Rutin and Quercetin”, in the second supplement of USP 37-25 (Bzhehansky et al. 2014; United-States-Pharmacopoeial-Convention 2015) however, it does not cover the limits for kaempferol or isorhamnetin. Although the above certified values are vital in providing a standard for ginkgo products in the marketplace, in almost all cases these values do not allow for the detection of aglycones or other constituents in ginkgo products prior to sample acid hydrolysis. This leaves an opening for the undetected adulteration of ginkgo products.

There are a number ways that a ginkgo product can be adulterated. The most common form of adulteration is to spike original plant extracts or product formulations with flavonol glycosides or aglycones. (Ko et al. 2013) This allows manufacturers to use compounds that are significantly less expensive than ginkgo leaf extract to achieve the typical 24% flavonol glycoside concentration. (van Beek and Montoro 2009) Rutin, a flavonol glycoside, and aglycones quercetin, kaempferol, and isorhamnetin, are currently the most popular ingredients used to spike products, as they are highly effective in inflating the flavonol glycosides assay values in ginkgo products. (Sloley et al. 2003) Another form of adulteration is the use of other G. biloba plant parts (roots, bark, and seeds) to reduce costs. (Nguyen et al. 2012) It is reported in the literature that the other parts of the G. biloba plant contain different sets of active components. (Liu et al. 2014) The consumption of extracts manufactured from these parts could therefore have significantly different physiological effects, which could be harmful to consumers. (Nguyen et al. 2012) A third method, unapproved manufacturing procedures, involves the use of inappropriate extraction solvents (3% hydrochloric acid in the extraction solvent instead of ethanol). This extraction procedure results the hydrolysis of flavonol glycosides, forming aglycones. (China-Food-and-Drug-Administration May 19 2015) This procedure therefore produces a product which contains compounds consistent with adulteration. In fact, a recent announcement from the Chinese government stated that the use of HCl can “decompose the effective constituents of medicine and affect the curative effect of medicine” regarding ginkgo extract. (China-Food-and-Drug-Administration May 19 2015) Though the aglycones produced through unapproved manufacturing practices are not formally defined as adulterants, they will be referred to as such throughout the paper for simplification.

In addition to the above mentioned adulterants, compounds made from other flavonoid-rich materials have a high potential for use in spiking original plant extracts and product formulations. (Cheng et al. 2000; Crupi et al. 2014) Researchers have reported the presence of ginkgo native flavonol glycosides in other plant species; these plant species could therefore be used for the adulteration of ginkgo products. (Riihinen et al. 2014) The fructus and flos Styphnolobium japonicum (L.) Schott species (syn: Sophora japonica L., Fabaceae) are natural sources of the flavonol glycoside rutin and aglycone quercetin, (Chandra et al. 2011) however, in addition to the compounds found in hydrolyzed ginkgo, they also contain two other active compounds, genestin and genistein. (Avula et al. 2015; Wohlmuth et al. 2014) If used as ginkgo adulterant, these additional compounds might also be present.

With such a high potential for adulteration using flavonol glycosides, aglycones, and different plant species containing additional active compounds, it is critical to establish new methods
to test ginkgo samples prior to acid hydrolysis: one to test authentic ginkgo including all necessary flavonol glycosides, and one with the ability to simultaneously detect and analyze flavonol glycosides, aglycones, and other potential adulterants as mentioned above.

HPLC analysis is an effective method for the quality control of herbal preparations and samples. (Kakigi et al. 2012) An analytical method, which can simultaneously analyze a broad range of chemical constituents, and the relative content levels of the major compounds (peak ratios and the total peak area), that will help in the identification of adulterated botanical products. (Ma et al. 2011a; Ma et al. 2011b; Ma et al. 2012; Ma et al. 2011c; Ma et al. 2011d; Ma et al. 2011e) The goal of this study is to establish the HPLC profile of ginkgo flavonol glycosides, aglycones, and other potential adulterants in a single run to help verify the authenticity of ginkgo extracts and commercial products.

Herein we discuss the comparison of raw ginkgo leaves, ginkgo extracts, commercially available ginkgo supplements, and standard reference compounds of potential adulterants.

Material and methods

Reagents and materials

HPLC grade methanol and acetonitrile were purchased from Anachemica (Canada). Rutin, quercetin, kaempferol, isorhamnetin, genistein, and genistin were used as reference compounds and purchased from ChromaDex (Irvine, USA). Ginkgo leaves were collected from a variety of areas in China (nine provinces/cities including: Shandong, Chong qing, Hubei and Henan). These samples were harvested in the same year, from a variety of sources and conditions, including: various authentic plants, different agricultural soils, and various cultivating environments. Powdered extracts were received from various Canadian suppliers. Seven of the commercial products used for the analyses were purchased from local pharmacies and seven were purchased from pharmacies in China.

Preparation of standards and samples

Reference standard solutions of rutin, quercetin, kaempferol, isorhamnetin, genistein, and genistin were prepared to the desired concentration with methanol (99.96%, HPLC grade) and used as reference standards for quantitative purposes only. Ginkgo samples were prepared at a concentration of 12 mg/ml in methanol (99.96%, HPLC grade) for extracts, and at 100 mg/ml for leaves. Dried ginkgo leaves, powdered extracts, and commercially formulated capsules and tablets were kept under identical conditions. Approximately 1 g of dried raw herb of G. biloba was milled with a grinder into fine powder, then suspended and sonicated in methanol for one hour. 120 mg of the powdered extracts and equivalent weight of each commercial product were accurately calculated. For the commercially formulated capsules and tablets, the contents equivalent to 80 mg of flavonol glycoside were weighed into a 10 ml volumetric flask and ultrasonic extracted with pure MeOH as stated above. The mixture was then mixed with 10 ml 99.96% MeOH (HPLC grade), and sonicated for an additional 20 min. After sonication, the volume was adjusted to 10 ml with 99.96% MeOH. Prior to injection, all liquid samples were filtered through a Phenex RC 0.2 μm syringe filter. Hydrolyzed ginkgo samples were prepared according to USP 37-NF 32, and were evaluated with the same method used to analyze the pre-hydrolysis samples of this study. Ginkgo PE (0.300 g) was accurately weighed into a 250 ml round-bottom flask and 78 ml of methanol: water: HCl (50: 20: 8, v/v/v) was added. The solution was refluxed at a moderate heat for 135 min (deep red color), cooled to room temperature, and transferred to a 100 ml volumetric flask. The solution was diluted to volume with water and mixed thoroughly. Aliquots were filtered through a Phenex RC 0.2 μm syringe filter into vials and analyzed using the HPLC conditions described below.

Instrument conditions

HPLC

Analysis was carried out using an Agilent series 1200 HPLC instrument (Agilent, CA, US) equipped with a binary pump, a micro vacuum degasser, a multi-wavelength (MW) detector, an auto-sampler, and a thermostated column. Input data (signals and integrations) was applied using ChemStation revision B.04.02 SP1 software. Optimum resolution and peak shape were obtained on a Luna C18-HST (High Speed Technology) column (2.5 μm, 3 x 100 mm) from Phenomenex (Torrance, CA, U.S.A.). The mobile phase consisted of ultrapure water (18.3 MΩ·cm) (phase A) and acetonitrile (phase B). At a flow rate of 1.0 ml/min, the linear gradient was as follows: 0–1.5 min, 15–15% B; 3–4 min, 17–17% B; 7–14 min, 20–35% B. UV detection wavelengths of 260 and 360 nm, a column temperature of 34°C, and an injection volume of 1 μl were applied. Each run was followed by a 5 min post run and an equilibration period of 14 min. The relative retention times for quercetin, kaempferol, and isorhamnetin are approximately 1.00 min, 1.17 min, and 1.20 min, respectively.

LC-MS

The LC-MS system consisted of a Thermo Accela 1250 pump coupled with a Thermo Exactive high resolution mass spectrometer (HRMS, Thermo Fisher Scientific, Waltham, MA, USA). The separation was carried out on an AMT Halo C18 (2.7 μm, 3 x 100 mm) column, with a gradient elution using acetonitrile (with 0.1% formic acid) and water (with 0.1% formic acid) as the mobile phase. The flow rate was 1 ml/min. Negative polarity scan was acquired at alternating MS scan of 0.25 s (4 Hz) across a mass range of m/z 190–1500, and higher energy dissociation scan of 0.1 s (10 Hz) at 60 eV. Ion source conditions consisted of a spray voltage of 3 kV, sheath gas of 50, auxiliary gas of 15, and capillary and heater temperatures of 250°C and 300°C, respectively. Acquisition was carried out using Xcalibur 2.2.

Calculation of flavonol glycoside content

The flavonol glycoside content of hydrolyzed ginkgo samples was calculated according to USP 37-NF 32.

Total flavonol glycosides = (r_{ij}/r_s) x (C_s/W) x F x 10

Where r_{ij} is the peak area of the relevant aglycone in the sample solution, r_s is the peak area of the aglycone in the corresponding standard solution, C_s is the concentration (mg/ml) of the aglycone in the standard solution, W is weight of the ginkgo sample (e.g. PE) taken to prepare the sample solution (g), and F is the factor used to convert each aglycone into a flavonol glycoside with a mean molecular mass of 756.7 (2.504 for quercetin, 2.588 for kaempferol, and 2.437 for isorhamnetin).

Data analysis

Multivariate statistical analysis (MVSA) was carried out using OriginLab version 9 software. The data matrix was constructed using HPLC responses of each peak as variables, with the observations/samples in columns and the peaks in rows. Principal component analysis (PCA) was used to calculate a basic model and overview the data. Consequently, only the first three components were selected for the description of the data, corresponding to 67%
of their variability, with the remaining components each contributing 5% or less.

**Results and discussion**

A chromatographic method was optimized to establish a reliable HPLC fingerprint for the standardization of ginkgo products. The method development included the optimization of the mobile phase, column type, column temperature, and UV detector wavelength. Method reproducibility and repeatability are discussed herein.

Initial investigations detected thirteen signals in ginkgo leaf (sample no. 7) and ginkgo PE (sample no. 28; CANPHY® standard reference material) (Fig. 1). Twelve of those signals were identified as flavonol glycosides using LC-MS (Table 1) with analysis of [M+H]^+ and [M − H]^− ions; the identity of peak 10 remains unknown. The identity of the most prominent flavonol glycoside – rutin (peak 5R) – was confirmed by direct comparison with an analytical standard. These identifications are in complete agreement with the flavonol glycoside compounds identified in previous reports. (Kakigi et al. 2012; Lin et al. 2008; van Beek and Montoro 2009) (Ding et al. 2008)

Although quercetin, kaempferol, and isorhamnetin have comparatively low detectable levels in leaves, these three aglycones have been proposed as potential adulterants along with isoflavones genistin and genistein (active components from the fructus and flos Styphnolobium japonicum species). The simultaneous analysis of flavon glycosides, aglycones, and isoflavones from various ginkgo products would therefore allow for the detection of adulterants in adulterated botanical products. Thus, the five potential adulterants were also investigated by HPLC. Genistin, quercetin, genistein, kaempferol, and isorhamnetin were identified using LC-MS (Table 1), and confirmed by comparison with their respective analytical standards. The six reference standards – rutin, genistin, quercetin, genistein, kaempferol, and isorhamnetin – and the ginkgo PE standard reference material, were used for the qualitative and quantitative analysis in this study.

The HPLC method developed was capable of separating signals from twelve flavonol glycosides, three aglycones, and two isoflavones in ginkgo products within 13 min, with high peak resolution (Fig. 1). This is a significant improvement over previous reports where the HPLC methods for the analysis of adulterated ginkgo products were noted to have run times of over 60 min, with poor separation of the ginkgo flavonol glycosides. (Harnly et al. 2012) The results described herein are in agreement with previous work (Kakigi et al. 2012), however, the previous study did not establish the simultaneous analysis of ginkgo flavonol glycosides and aglycones.
Calibration curves were obtained for each of the six reference standards: rutin, genistin, quercetin, genistein, kaempferol, and isorhamtinit. The calibration curves showed good linear relationships ($R^2 \geq 0.9995$) for each of the analytes over the concentration range 0.0003–5.5190 μg/μl (Table 2). The applied calibration model for all curves was $y = ax + b$, where $y$ is the peak area, $x$ is the concentration, $a$ is the slope, and $b$ is the y-intercept.

Several analytical performance validations were performed on the method developed in this study: precision, accuracy, recovery rate, limit of detection (Krauze-Baranowska et al. 2004), and limit of quantification (LOQ). (Krauze-Baranowska et al. 2004) The samples analyzed in this study were examined over four consecutive weeks. For accuracy, the method reproducibility and repeatability were evaluated by the analysis of three injections for each sample solution, and five injections for each standard solution. Cycle time per injection was 3–5 min.

The recovery values for the six standard constituents (in the specified concentration range) were determined to be 96.5–101.3%, with relative standard deviation (RSD) values ≤2.0% ($n = 5$). The LOD values were 170, 11.5, 8.0, 2.5, 4.8, and 12.0 μg/ml for rutin, genistin, quercetin, genistein, kaempferol, and isorhamtinit, respectively, and the LOQ values were 57.0, 38.0, 26.0, 8.4, 16.0, and 40.0 μg/ml for rutin, genistin, quercetin, genistein, kaempferol, and isorhamtinit, respectively. The RSD % for different concentration levels was also determined by analysis in triplicate and shows good precision with RSD values ≤5.21%, verifying the HPLC method utilized.

Using the method developed for the analysis of a combination of flavonol glycosides, aglycones, and isoflavones, 42 samples were analyzed including: thirteen dried leaf samples (nos. 1–13), fifteen standardized powdered extracts (nos. 14–28), and fourteen commercially available ginkgo products (nos. 29–42) (Table 3). Fig. 1 shows the HPLC profiles of selected adulterated ginkgo samples, with the major peaks (1–13) indicated on the chromatogram.

The thirteen dried leaf samples contained levels of flavonol glycosides that varied significantly between samples. However, the authentic ginkgo leaf samples gave a relatively consistent chromatographic fingerprint to use in the qualitative analysis of the ginkgo powdered extracts and commercial samples. Predictably, none of the leaf samples contained detectable levels of aglycones quercetin, kaempferol, or isorhamtinit, which is in agreement with previous reports. (Harly et al. 2012; Liu et al. 2005; Sloley et al. 2003) Most of the powdered extract samples (nos. 14–18, 21, 22, and 25–28) contained signals which were qualitatively consistent with those of authentic ginkgo leaves, however, in the chromatograms of sample nos. 19, 20, 23, and 24 (Fig. 1), those characteristic signals were either significantly minimized, or absent altogether. The majority of the commercially prepared ginkgo supplements have HPLC profiles that differ significantly from those of the authentic leaves and the leaf-based extracts.

The right hand side of Fig. 1 includes five commercial ginkgo products (sample nos. 38–42) as representative examples. The signals and relative signal heights present in the chromatograms of the samples were directly compared with those of the authentic ginkgo leaves (Fig. 1). This comparison showed large discrepancies, including the addition of extra signals in the commercial samples. These discrepancies indicated that the samples had been spiked with rutin, quercetin, kaempferol, or isorhamtinit, or combinations therein.

Evidence for the presence of the potential adulterant genistein was found in only three ginkgo finished products (sample nos. 23, 30, and 35) and at extremely low concentrations. This was
### Table 3
Flavonol glycoside and aglycones in unhydrolyzed ginkgo products. The concentrations are in percentage (%, w/w).

<table>
<thead>
<tr>
<th>S. #</th>
<th>Sample type</th>
<th>Total 12-peak area</th>
<th>Rutin %</th>
<th>Quercetin %</th>
<th>Kaempferol %</th>
<th>Isohamnetin %</th>
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<tr>
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The total 12-peak area for the main flavonol glycoside peaks was calculated to be not less than 500 ± 20 mAU·s (sample no. 27) in ginkgo PE standard reference material (calculated to be 24% flavonoids). This can be considered as a preliminary estimate for the total of flavonol glycoside content in the 42 ginkgo samples, shown in Table 3. The 12-peak area value may be used to simply pre-evaluate the concentration of flavonoids in a ginkgo product before proceeding with the acid hydrolysis step.

Fig. 2 displays the normalized peak area data of the 13 major signals for all 42 samples to allow for visual comparison, as was used in a previous study. (Harnly et al. 2012) The samples are arranged in order from authentic ginkgo samples on the left to adulterated ginkgo product samples on the right. The distribution of peak areas for the same constituents clearly varies greatly among the samples. The area of peak nos. 14–16 representing quercetin, kaempferol, and isohamnetin respectively, are typically < 0.1% of the total area for authentic leaf samples. Analysis of the chromatographic peak areas showed that many of the samples contained significant amounts of aglycones with 36–62% quercetin in sample nos. 16, 17, 19, 20, 21, 23, 24, and 25; 32% kaempferol in sample no. 19; and 4.2% isohamnetin in sample no. 41. In fact, an average of surprising, as genistein has previously been identified as an adulterant of ginkgo extracts. (Avula et al. 2015; Chandra et al. 2011; Feng et al. 2015; Wohlmut et al. 2014; Zhi et al. 2015) The second isoflavone tested, genistin, was not detected in any of the powdered extracts or commercial products.

In addition to the qualitative analysis, the ginkgo samples were analyzed quantitatively using the calibration curves discussed above. The amounts of rutin, quercetin, kaempferol, and isohamnetin present in all 42 samples are reported in Table 3 as weight percentages of the original samples. These values were used to evaluate the ginkgo product samples against the CCCMHP requirements of rutin ≤ 4%, quercetin ≤ 0.5%, kaempferol ≤ 0.5%, and isohamnetin ≤ 0.2%. (CCCMHP 2015) A previous report suggested that these new standard levels are suitable maximum levels for the aglycones content of ginkgo leaf extract. (Wohlmut et al. 2014)

Eleven of the ginkgo extracts contained either negligible amounts of aglycones, or none (sample nos. of 14–18, 21–23 and 25–28). The remaining three ginkgo extracts (sample nos. of 19, 20, and 24 in Fig. 1) contained significant levels of quercetin and kaempferol. The values in Table 3 also shows that for the commercial samples, quercetin and kaempferol varied significantly, up to seven-fold, and isohamnetin was only minimally detected. As a result, sample nos. 19, 20, 23, and 24 were found to be adulterated extracts, and only three of the commercial products met the levels associated with the authentic samples (sample nos. 32, 36, and 37).

...
36% aglycone was obtained for the majority of the finished products. This average excludes sample nos. 32, 36, and 37, as they are the commercial samples that have distributions consistent with the authentic samples. This analysis also showed that the total peak area of the chromatogram for sample no. 23 consisted of 92.5% rutin. Although all of the samples listed above have been determined to be adulterated, they are still allowed under the current standards.

Fig. 3 shows the chromatograms of sample nos. 10, 17, 23, 24, 35, and 42, both pre- and post-hydrolysis (blue). This selection, including one authentic ginkgo leaf, three powdered extracts, and two commercial products, were selected to represent the range of adulteration indicated by the above results. These samples are categorized into six specific groups: an authentic leaf sample (no. 10), an authentic powdered extract sample (no. 17), a sample consisting almost entirely of aglycones (quercetin, kaempferol, isorhamnatin, no. 35), and two samples displaying authentic leaf-like flavonol glycoside profiles but were partially spiked with either aglycones (sample no. 24), or a flavonol glycoside (sample no. 42). As is seen in the chromatograms in Fig. 3, each hydrolyzed sample contains the three aglycones: quercetin, kaempferol, and isorhamnatin. Sample no. 23 is an exception, as it contains only quercetin, the aglycone resulting from the hydrolysis of flavonol glycoside rutin.

Once the quantities of quercetin, kaempferol, and isorhamnatin were determined (using peak surface area extracted from the HPLC plots in Fig. 3), the total flavonol glycoside content was calculated using the formula proposed by EP (European-Pharmacopoeia 2015) and USP (United-States-Pharmacopeial-Convention 2015). The factor $F$ equals 2.51, and is used to convert each aglycone into a flavonol glycoside. The total quantity of flavonol glycosides in the sample can then be calculated by summing the values for the quercetin, kaempferol, and isorhamnatin glycosides, as described in the literature. (Wohlmuth et al. 2014)

Fig. 4 shows the ratios of kaempferol/quercetin and isorhamnatin/quercetin (on the left y-axis - see arrows on the leftmost white and black bars) and the total flavonol glycosides percentage (on the right y-axis - see the arrow on the rightmost gray bar) for sample nos. 10, 17, 23, 24, 35, and 42 post-hydrolysis. As mentioned above, the minimum requirement for the total flavonol glycosides percentage of ginkgo products in North America is 24% (United-States-Pharmacopeial-Convention 2015). The three powdered extracts, sample nos. 17, 23, and 24, all meet this requirement; however, neither of the commercial samples do (nos. 35 and 42). Sample no. 10, the authentic leaf sample, is not required to meet the $\geq 24\%$ value, as it is not a concentrated extract. The fact that sample no. 23 meets the required value is misleading. This result implies that the sample is mixture of flavonol glycosides, when it was determined in Fig. 3 to consist almost entirely of rutin.

The minimum values required in North America for the ratios of kaempferol/quercetin and ginkgo PEisorhamnatin/quercetin, shown as horizontal white and black lines in Fig. 4, are $\geq 0.7$, and $\geq 0.1$ (United-States-Pharmacopeial-Convention 2015), respectively. The authentic leaf sample, two of the powdered extracts, and both commercial products (nos. 10, 17, 24, 35, and 42) meet those values established for ginkgo products post-hydrolysis. The single peak found in the chromatogram for sample 23 (post-hydrolysis) led to peak area ratios (kaempferol/quercetin and isorhamnatin/quercetin) of zero. These results are consistent with a previous report, where the aglycone ratios (kaempferol/quercetin and isorhamnatin/quercetin) of three adulterated ginkgo commercial products also complied with the relevant USP-NF monographs. (Wohlmuth et al. 2014)

Each of the adulterated products (sample nos. 23, 24, 35, and 42) met at least one of the current requirements for standardized ginkgo products. These findings indicate that the use of the pharmacopoeia provided formula (Formula 1), can lead to results that are misleading, and that the current protocols for the analysis and standardization of ginkgo products do not fully cover the detection of adulterants. The rapid method developed in this study can simply resolve this issue with respect to all of the types of adulterations categorized above and shown in Fig. 3.

OriginLab 9 was used to perform PCA on the chromatographic data of the 42 ginkgo samples to calculate a basic model and...
summarize the data. This type of approach was used in a previous study for the comparison of flavonol compositions of ginkgo products on the Japanese market. (Kakigi et al. 2012)

The principal components were calculated using the entire set of chromatographic signals as variables, including both sets of flavonol glycoside and aglycone signals. The principal values calculated for each sample, called factor scores, were interpreted as the projections of the originally observed variables onto the principal components. Plotting the principal components resulted in score plots where each data point represented a single ginkgo sample. The separations observed for the ginkgo samples resulted from variations in the chromatographic signals, as the original variables were the flavonol glycoside and aglycone signals. Statistically different peaks were calculated with a confidence interval of 95% and significance level of 0.05.

The 2-D projection score plot of principal components PC1 and PC2, for the 42 samples, can be classified into four groups: A, B, C and D (Fig. 5a). The authentic ginkgo leaf (nos. 1–13) and

*Fig. 3. The chromatograms of hydrolyzed (blue) and unhydrolyzed (black) sample nos. of 10, 17, 23, 24, 35 and 42, including one ginkgo leaf, three powdered extracts, and two commercial products. All were identified by the retention time and UV spectra (360 nm wavelength) of corresponding standards (rutin, quercetin, kaempferol, and isorhamnetin). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)*
extract (nos. 14–18, 21, 22, 25–28) samples are focused in two areas marked with solid circles (Groups A and B). This indicates that those samples have very similar flavonol glycoside distributions (Fig. 5a). Three of the finished ginkgo products (sample nos. 32, 36, and 37) are also clustered in group B, indicating that they have similar flavonol glycoside distributions to the authentic samples. This is consistent with the qualitative and quantitative results above, which indicate that those commercial samples contain authentic unadulterated ginkgo. The remaining finished products are focused on two different positions marked with dashed-line circles, Groups C (nos. 24, 29 and 35) and Group D (nos. 19, 20, 30, 31, 33, 34, 38–41). These clusters are separated from their authentic counterparts based on their quercetin, kaempferol, and isorhamnetin contents. Within Group D, four of the commercial samples (nos. 30, 31, 33 and 34) are separated from the remaining adulterated samples, due to the high quercetin and high kaempferol combination found in each of the samples, see Table 3. The significant separation of Groups A and B from Groups C and D indicates that the chemical composition of the majority of the commercial samples are substantially different from authentic ginkgo and thus confirms the adulteration results discussed above regarding the 42 ginkgo samples. These findings are clearly in agreement with the flavonol glycoside and aglycone distributions and percentages shown in Fig. 2 and Table 3.
Using the original variables of the HPLC chromatographic signals as the input data, a three-dimensional (3-D) projection plot of three principal components was used to further interpret the relationships between the 42 ginkgo samples. The three principal components, PC1, PC2, and PC3, describe the chromatographic feature variations related to the samples and were used to clarify the differentiation in a 3-D configuration, shown in Fig. 5b. As in Fig. 5a, the authentic commercial ginkgo samples (nos. 32, 36, and 37) are gathered in two areas of the 3-D projection plot with the authentic leaf and extract samples. This is also the case for the adulterated ginkgo samples which remained focused in a completely different area of the plot. This further confirms that many of the ginkgo extracts and commercial samples analyzed in this study are not consistent with authentic ginkgo leaves and extracts, regardless of satisfying the testing requirements.

The data points representing sample no. 23 on the 2-D (Fig. 5a) and 3-D (Fig. 5b) projection plots were not positioned near any of the data point clusters. This is due to the fact that the only signal present in the chromatogram of sample no. 23 was identified as rutin, and the lack of additional flavonol glycoside or aglycone signals for this sample. Including sample 23 in the PCA is beneficial in locating samples containing larger quantities of rutin when compared to the other samples.

The results presented herein effectively demonstrate the necessity for a preliminary evaluation of ginkgo products, prior to acid hydrolysis. This evaluation must include the identification of adulterants in ginkgo products using the HPLC method described above. Following the identification, the ginkgo products need to be evaluated by comparing the aglycone contents to the limits reported for products prior to hydrolysis: rutin ≤4%, quercetin ≤0.5%, kaempferol ≤0.5% and isorhamnetin ≤0.2%.

Conclusion

For the first time, a feasible and systematic HPLC method was developed for the simultaneous analysis of a broad range of chemical constituents, including ginkgo flavonol glycosides, aglycones, and potential adulterants in authentic and spiked ginkgo samples, in a single run, which is practical for all types of ginkgo materials and products. The initial approach determined the HPLC separation of twelve major flavonol glycosides, three aglycones, and two isoflavones within 13 min and with high resolution. Subsequent testing was performed on numerous ginkgo samples including: thirteen raw ginkgo leaves, fifteen ginkgo powdered extracts, and fourteen commercially available ginkgo supplements.

Of the 29 extracts and commercial products tested, 90% were found to have chromatograms that were inconsistent with that of authentic ginkgo leaves. However, this widespread adulteration is almost entirely undetectable using the current testing methods as most of the samples were found to meet pharmacopoeial standards.

The current pharmacopoeial standards only contain specifications for the total flavonol glycosides content and relative quantities of quercetin, kaempferol, and isorhamnetin, determined post hydrolysis. These methodologies are not sufficient to evaluate the authenticity of ginkgo products and to detect adulteration with aglycones.

The HPLC analysis method established in this study is both effective and efficient, providing a qualification that is more thorough than those previously reported, including the direct comparison of commercial ginkgo HPLC profiles with an authentic ginkgo HPLC fingerprint. It is useful not only for the authentication of samples prior to acid hydrolysis, but also for the quantification of flavonol glycosides as required by the CC-CIEMHP, and the pharmacopoeial monographs of USP (United-States-Pharmacopeial-Convention 2015), Chinese Pharmacopoeia (Chinese-Pharmacopoeia-Commission 2010), British Pharmacopoeia (British-Pharmacopoeia-Commission 2012), and European Pharmacopoeia. (European-Pharmacopoeia 2015)

This will improve the quality control and standardization procedures for ginkgo leaves, extracts, and commercial products, and thus greatly improve the quality of products available in today’s natural health product market. It is critical that ginkgo analysis methods incorporate a preliminary evaluation, prior to the acid hydrolysis procedure, with recommended maximum values of 4% rutin, 0.5% quercetin, 0.5% kaempferol and 0.2% isorhamnetin. This additional analytical step and these new standard values will help ensure the exposure of adulterated ginkgo products.

Conflict of interest

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Acknowledgment

This work is financially supported by Canadian Phytopharmaceuticals Corporation.

References


