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

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ABSTRACT

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 (PEs) 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 finished 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 hydrolysis procedure utilized in the current testing methods.

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

Abbreviations: CCCIEMHP, China Chamber of Commerce for Import & Export of Medicines & Health Products; CP, Chinese Pharmacopeia Commission; CPC, Canadian Phytopharmaceuticals Corp.; CFDA, China Food and Drug Administration; EP, European Pharmacopeia; *G. biloba*, *Ginkgo biloba*; HPLC, high performance liquid chromatography; I, isorhamnetin; K, kaempferol; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; NMT, not more than; PCA, principal component analysis; PE, powdered extract; Q, Quercetin; R, Rutin; RSD, relative standard deviation; SRM, standard reference material; USP, united state pharmacopeia.

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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 finding 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 $\geq$ 24% and $\geq$ 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 Pharmacopeia (BP) (British-Pharmacopoeia-Commission 2012), European Pharmacopeia (EP) (European-Parmacopoeia 2015), and Chinese Pharmacopeia 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 $\geq$ 0.7 and $\geq$ 0.1 respectively, while values of 0.8–1.2 and $\geq$ 0.15 are required by the Chinese Pharmacopeia 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-Parmacopoeia 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-Parmacopoeia 2015; United-States-Pharmacopeial-Convention 2015) One exception, the China Chamber of Commerce for Import & Export of Medicines & Health Products (CCCMHPIE) does provided analysis specifications for ginkgo prior to acid hydrolysis: rutin $\leq$4%; quercetin $\leq$0.5%; kaempferol $\leq$0.5%; and isorhamnetin $\leq$0.2%. (CCCMHPIE 2015) They also list the peak ratio of kaempferol/quercetin $\geq$ 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 37-2S (Bzhelyansky et al. 2014; United-States-Pharmacopeial-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 extractions 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 components, 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