HPLC-UV/HRMS methods for the unambiguous detection of adulterations of Ginkgo biloba leaves with Sophora japonica fruits on an extract level

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\section*{Introduction}

Ginkgo biloba L. (Ginkgoaceae) is one of the oldest living tree species existing on earth for 200 million years. Extracts prepared from its leaves are among the top-selling herbal products in the world and are sold as active ingredients of numerous dietary supplements, botanicals, herbal medicinal products, and complementary medicines (Liu et al. 2014; Wohlmuth et al. 2014). The majority of ginkgo extracts on the market are made from leaves cultivated at plantations in China, the USA, and France. After extraction, the samples were analyzed using two high-performance liquid chromatography (HPLC) coupled with UV/HRMS methods for the detection of genistein and sophoricoside, respectively. Chromatograms were compared to standard reference materials.

There have been reports on poor quality and adulteration of ginkgo leaf extracts or the powdered plant material with extracts or powder of Styphnolobium japonicum (L.) Schott (Fabaceae) (syn. Sophora japonica L.) fruits, which is rich in flavone glycosides.

Materials and methods: A total of 33 samples of dried ginkgo leaves were sourced from controlled plantations in China, the USA, and France. After extraction, the samples were analyzed using two high-performance liquid chromatography (HPLC) coupled with UV/HRMS methods for the detection of genistein and sophoricoside, respectively. Chromatograms were compared to standard reference materials.

Results: In none of the tested ginkgo samples, neither genistein nor sophoricoside could be detected. The applied method was designed to separate genistein from apigenin. The latter is a genuine compound of ginkgo leaves, and its peak may have been previously misidentified as genistein because of the same molecular mass. The method for the detection of sophoricoside allows identification of the adulteration with sophora fruit without prior hydrolysis. By both HPLC methods, it was possible to detect adulterations of $\geq 2\%$ sophora fruits in the investigated ginkgo extract.

Conclusion: The methods allow unambiguous detection of adulterations of ginkgo leaves with sophora fruits, using genistein and sophoricoside as marker compounds.
of adulterations of plant material is not always feasible, since samples may already be in powdered form when purchased from vendors on the global market.

Sophorae fructus, the dried ripe fruit of *S. japonicum*, is used in Traditional Chinese Medicine (TCM) for its haemostatic properties. Based on the chemical analysis, Sophorae fructus contains flavonoids, alkaloids, terpenoids, amino acids, saccharides, and phospholipids. Isoflavones, such as sophoricoside, genistein, and genistin, and flavonols, like rutin, quercetin, and kaempferol, are the main components (Chang et al. 2012; Chinese Medicine and Healthcare, Council of Europe 2019). It has also been reported, that the amount of genistein in *G. biloba* leaf extracts (Frommenwiler et al. 2019) along with the wrong assignment of apigenin as genistein because of the same molecular mass, revealed the need for elaborating a more efficient high-performance liquid chromatography (HPLC) method. In this study, mass, revealed the need for elaborating a more efficient high-performance thin-layer chromatography (HPTLC) method for the detection of genistein in ginkgo leaf extracts (Yao et al. 2017), or only in leaves, stems, and fruits of male ginkgo trees and not of the leaves was dependent on the season (Pandey et al. 2014). Moreover, in 2015, a limit of 1% sophoricoside (genistein-4′-O-glucoside) in ginkgo extracts was set by the China Food and Drug Administration (China Food and Drug Administration 2015).

Intentional or accidental adulteration of *G. biloba* extract is an ongoing problem. To detect possible adulterations with sophora fruit, the objective of this study was to investigate whether genistein and sophoricoside are genuine constituents of ginkgo leaves. Difficulties in developing a high-performance thin-layer chromatography (HPTLC) method for the detection of genistein in ginkgo leaf extracts (Frommenwiler et al. 2019) along with the wrong assignment of apigenin as genistein because of the same molecular mass, revealed the need for elaborating a more efficient high-performance liquid chromatography (HPLC) method. In this study, HPLC combined with ultraviolet (UV) photodiode array detection and high-resolution mass spectrometry (HRMS) was used to develop methods for unambiguous detection of genistein and sophoricoside in ginkgo leaf and *S. japonica* fruit extracts.

**Materials and methods**

**Test samples**

Thirty-three dried *G. biloba* leaf samples were obtained from plantations in China (controlled contract cultivation) (12 samples), the USA (South Carolina) (13 samples), and France (Département Gironde) (8 samples), by Dr. Willmar Schwabe GmbH & Co. KG. The herbal drug complies with the requirements described in the monograph of *G. biloba* in the European Pharmacopoeia (European Directorate for the Quality of Medicines and Healthcare, Council of Europe 2019) and this material is used for the production of quantified EGb 761®. The various samples were harvested in different years (2013, 2014, 2015, and 2016) from plantations in China, (2013, 2014, 2015, and 2016) from the USA and (2014, 2015, and 2016) from France. Voucher specimens are deposited at Dr. Willmar Schwabe (Karlsruhe, Germany). *Sophora japonica* fruits were obtained from Kräuter Schulte (Gernsbach, Germany) and a voucher specimen is deposited at Dr. Willmar Schwabe (Karlsruhe, Germany).

**Solvents, reagents and chemicals**

Ethanol (analytical grade), methanol hypergrade for liquid chromatography (LC)-mass spectrometry (MS), and acetonitrile hypergrade for LC-MS, for extraction and HPLC analysis, were purchased from Merck, Germany. The deionized water was obtained by a water purification system (Evoqua, Water Technologies, Günzburg, Germany). Isopropanol (2-propanol) and hydrochloric acid 32%, for the preparation of the hydrolysis solution, were both purchased from Merck, Germany. For the preparation of the hydrolysis solution, 300 mL isopropanol was added to a 1 L volumetric cylinder and filled up with hydrochloric acid 3 M to the final volume.

**Reference standards**

Genistein was purchased from HWI Group (Rüllzheim, Germany), sophoricoside from Sigma-Aldrich (St. Louis, USA), and apigenin from Fluka (Buchs, Switzerland).

**Extraction and hydrolysis**

Dried *G. biloba* leaves or *S. japonica* fruits were introduced into a flask, and extracted twice (Büchi Rotavapor R-124 and Büchi water bath B-480, both from Büchi, Switzerland) with 60% (v/v) ethanol (1:7, drug to solvent ratio) at 60 °C for 1 h. After cooling at room temperature (25 °C), the two extraction suspensions were filtered (T 1500 filter paper, Pall Corporation, Germany) and mixed. The obtained extract solution was evaporated under vacuum and subsequently lyophilised (Alpha 2–4, Christ, Bühl, Germany). All the produced ginkgo extracts are not commercially available and were only used for these investigations.

In order to test the detectability of adulteration, *G. biloba* leaves were intentionally adulterated with 2% (w/w) *S. japonica* fruits and an extract was prepared according to the procedures described above.

Hydrolysed *G. biloba* and *S. japonica* extract samples were prepared by transferring 20.0 mg of the dry extract into hydrolysis vials and the addition of 1 mL of hydrolysis solution. Subsequently, the solutions were heated at 100 °C in a water bath (Labortechnik HB4 basic, IKA, Staufen im Breisgau, Germany) for 45 min.
Sample preparation

Reference standards (genistein, sophoricoside, and apigenin) were dissolved in the initial gradient solution of each of the appropriate HPLC methods, at a concentration of 0.5 mg/mL. The samples were sonicated for 10 min at room temperature, then filtered through a filter with pore size 0.45 μm (Rotilabo® PTFE, Carl Roth, Germany) and transferred into individual vials which were subjected to HPLC-UV/HRMS analysis.

For the analysis of genistein, the hydrolyzed mixtures of the 33 ginkgo samples were transferred into individual vials for HPLC-UV/HRMS after filtration through a syringe filter with pore size 0.45 μm (Rotilabo® PTFE, Carl Roth, Germany).

For the analysis of sophoricoside, the 33 samples of lyophilized ginkgo extracts were dissolved at a concentration of 10 mg/mL in the initial gradient solution. The solutions were sonicated for 10 min at room temperature and then transferred into individual vials for HPLC-UV/HRMS analysis after filtration using a 0.45 μm filter (Rotilabo® PTFE, Carl Roth, Germany).

Apparatus and chromatographic conditions for genistein analysis

HPLC-UV/HRMS was performed using a Thermo Orbitrap Fusion system coupled with a Thermo Vanquish UHPLC using a Waters Cortecs UPLC C18 1.6 M (2.1 × 150 mm) column. The mobile phase consisted of 0.4% aqueous (deionized water) formic acid (phase A) and acetonitrile: methanol (50:50 v/v) LC-MS grade with 0.4% formic acid (phase B). At a flow rate of 0.2 mL/min, the linear gradient was as follows: 0.00–40.00 min, 70–30% (A–B%) to 15–85% (A–B%) followed by a 5 min column wash with 15–85% (A–B%) and 5 min equilibration period with 70–30% (A–B%). UV detection wavelength of 254 nm, a column temperature of 40 °C, and an injection volume of 2 μL were applied.

MS parameters in positive ionization mode were: ionization voltage 3500 V, electrospray ionization (ESI), ion transfer tube temperature 350 °C, vaporizer temperature 350 °C, 3 scans, resolution of 30000, HCD collision energy (%) 50. System control and data evaluation were performed with Thermo® Xcalibur for LC-MS.

Apparatus and chromatographic conditions for sophoricoside analysis

The analysis of sophoricoside was performed by a modified HPLC method described in the monograph of Sophorae Fructus in the Hong Kong Chinese Materia Medica Standards (Chinese Medicine Division, Department of Health 2012).

HPLC-UV/HRMS was performed with the same instrument configuration as described above. The mobile phase consisted of 0.4% aqueous (ultra-power water) formic acid (phase A) and acetonitrile: methanol (50:50 v/v) LC-MS grade with 0.4% formic acid (phase B). At a flow rate of 0.2 mL/min, the linear gradient was as follows: 0.00–40.00 min, 70–30% (A–B%) to 15–85% (A–B%) followed by a 5 min column wash with 15–85% (A–B%) and 5 min equilibration period with 70–30% (A–B%). UV detection wavelength of 254 nm, a column temperature of 40 °C, and an injection volume of 2 μL were applied.

MS parameters in positive ionization mode were: ionization voltage 3500 V, electrospray ionization (ESI), ion transfer tube temperature 350 °C, vaporizer temperature 350 °C, 3 scans, resolution of 30000, HCD collision energy (%) 50. System control and data evaluation were performed with Thermo® Xcalibur for LC-MS.
acid (phase B). At a flow rate of 0.2 mL/min, the linear gradient was as follows: 0.00–40.00 min, 85–15% (A–B%) to 70–30% (A–B%) followed by a 5 min column wash with 15–85% (A–B%) and 5 min equilibration period with 85–15% (A–B%). UV detection wavelength of 254 nm, a column temperature of 40 °C, and an injection volume of 2 µL were applied.

MS parameters in the positive ionization mode were: ionization voltage 3500 V, ESI, ion transfer tube temperature 350 °C, vaporizer temperature 350 °C, 3 scans, resolution of 30000, HCD collision energy (%) 50. System control and data evaluation were performed with Thermo Excalibur for LC-MS.

Data analysis
ACD/Spectrus Processor (v2017.2.1) software was used to process and analyze all data files.

Results
Analysis of genistein
A new HPLC-UV/HRMS method was developed to investigate whether genistein is a genuine constituent of *G. biloba* leaf extract. This method features slightly different retention times (Rt) of apigenin and genistein (11.14 min and 10.38 min, respectively) (Figure 2). Both compounds have the same molecular mass, which may have led to misinterpretations in studies claiming that genistein is a genuine constituent of *G. biloba* leaves. The new UHPLC method can unambiguously distinguish these two compounds due to different retention times, UV spectra, and MS fragmentation patterns, and thus, misinterpretations can be avoided (Table 1 and Figure 2).

All 33 ginkgo samples described above were analyzed for the presence of genistein by LC-DAD-HRMS using positive selective ion monitoring. The chromatograms of the hydrolyzed *G. biloba* leaf and *S. japonica* fruit extracts are shown in Figure 3. Processing of the UHPLC-DAD-HRMS data of the ginkgo leaf extracts, *S. japonica* fruit extract, and of the reference compounds apigenin and genistein showed that genistein could not be detected in any of the tested ginkgo samples, whereas traces of apigenin were detected instead (Figure 3).

Analysis of sophoricoside
Besides the method for the specific detection of genistein, one additional method was developed for the detection of sophoricoside, in which hydrolysis of the extract is not necessary. Due to the reduced number of manual steps, this method is more

| Table 1. Fragmentation pattern of apigenin and genistein. |
|-----------------|-----------------|-------|-----------------|-----------------|-----------------|
| | Compound | Ion mode | Rt (min) | Monoisotopic mass m/z | MS2 fragments + | |
| | Apigenin | Positive | 11.14 | 270.0528 | 271.060 | 271.060; 243.065; 229.050; 225.055; 171.029; 163.039; 153.018; 145.028 |
| | Genistein | Positive | 10.38 | 270.0528 | 271.060 | 271.060; 253.050; 215.070; 197.059; 153.018; 149.023; 141.070 |

![Figure 3. The total absorbance chromatograms (TAC) and total ion chromatograms (TIC) of *G. biloba* leaf extract (A) and *S. japonica* fruit extract (B) (top) as detected with DAD and the identification of apigenin and genistein respectively, with their MS (middle) and MS2 (bottom) spectra in the positive ion mode.](image-url)
suitable for the screening of a high number of samples. Since sophoricoside is among the major constituents of *S. japonica* fruits (He et al. 2016), adulteration of *G. biloba* leaves with *S. japonica* fruits can be detected using sophoricoside as a marker compound and not only genistein. To find out whether sophoricoside is a native constituent of *G. biloba* leaves, all 33 ginkgo samples described above were analyzed with the corresponding method.

The UV-detected chromatograms at 254 nm of *G. biloba* extract, an extract of *G. biloba* leaves with an intentional 2% adulteration of *S. japonica* fruits, *S. japonica* fruit extract and the reference compound sophoricoside were compared (Figure 4). Sophoricoside with a retention time of 20.12 min could not be detected in any of the 33 ginkgo extracts, whereas it could be detected in the sample intentionally adulterated with *S. japonica* fruit extract.

**Discussion**

When reproducing the method described by López-Gutiérrez et al. (2016), the coelution of genistein and apigenin became evident by using LC-HRMS-MS. A trace peak with the same retention time and monoisotopic mass as the genistein reference substance, but with different MS fragmentation patterns was observed in the chromatogram of ginkgo extracts (Table 1 and Figure 2). This peak could eventually be identified as apigenin by its fragmentation pattern and by comparison with a reference compound. Moreover, the UV spectrum of apigenin is different from genistein, with maxima at 336 nm and 261 nm, respectively (Mabry et al. 1970). Thus, apigenin was identified as a genuine constituent of *G. biloba* leaves, while genistein could not be detected in any of the tested samples. This confirms findings by Avula et al. (2015), that *G. biloba* leaf and its extracts do not contain genistein and that it is a marker of adulteration. Also, Wohlmuth et al. (2014) stated that the presence of genistein in ginkgo leaf extract products is considered as evidence of adulteration. However, so far, it was not clear why other authors described genistein as a native constituent in ginkgo leaf extracts (Yao et al. 2017), why some even claimed to have isolated and identified genistein from ginkgo leaf extract (Wang et al. 2007). The lack of data such as the fragmentation patterns or considerations of coeluting isomers could be the reason for the misidentification of apigenin as genistein. Also, for chemotaxonomic reasons, it is quite unlikely that genistein is contained in *G. biloba*, because isoflavonoids are typical for Fabaceae species. It is also possible that adulterated material has been used in the described studies. This clearly shows the importance of documentation of the botanical authenticity of the investigated raw materials.

Sophoricoside, the glucoside of genistein can be used as another marker compound to detect adulterations of *G. biloba* leaves with *S. japonica* fruits without prior hydrolysis of the extracts, making it a suitable method for screening a high number of samples. Using the suggested HPLC method for the analysis of sophoricoside, we were able to detect a ≥2% adulteration of *G. biloba* leaves with *S. japonica* fruit in the investigated extract as shown in Figure 4.
Conclusions

Our results have shown that genistein and sophoricoside, which are constituents of *S. japonica* fruits, could not be detected in *G. biloba* leaves using HPLC with UV and HRMS detection. Thus, both genistein and sophoricoside are suitable marker compounds for detecting adulterations of *G. biloba* leaves with *S. japonica* fruits on an extract level, and HPLC analysis can be an important tool for monitoring the authenticity and purity of *G. biloba* leaf extracts.

Disclosure statement

Stefan Germer and Žarko Kulic are employees, whereas Evangelia Bampali had an internship at Dr. Willmar Schwabe GmbH & Co. KG, Germany.

References

Avula B, Sagl S, Gafner S, Upton R, Wang YH, Wang M, Khan IA. 2015. Identification of *Ginkgo biloba* supplements adulteration using high performance thin layer chromatography and ultra high performance liquid chromatography-diode array detector-quadrupole time of flight-mass spectrometry. Anal Bioanal Chem. 407(25):7733–7746.

Basta D. 2017. Schwindel im Alter und vaskuläre Formen diagnostizieren. Die Medizinische Welt. 68:46–52.

Booker A, Frommenwiler D, Reich E, Horsfield S, Heinrich M. 2016. Detection and quality assurance of *Ginkgo biloba* supplements. J Herb Med. 6(2):79–87.

Chandra A, Li Y, Rana J, Persons K, Hyun C, Shen S, Mulder T. 2011. Quality categorization of supplement grade *Ginkgo biloba* leaf extracts for authenticity. J Funct Foods. 3(2):107–114.

Chang L, Ren Y, Cao L, Sun Y, Sun Q, Sheng N, Yuan L, Zhi X, Zhang L. 2012. Simultaneous determination and pharmacokinetic study of six flavonoids from *Fructus Sophorae* extract in rat plasma by LC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 904:59–64.

China Food and Drug Administration. 2015. Announcement of the China Food and Drug Administration on the supplemental test method for *Ginkgo* leaves soft capsule and so on: Attachment 6. The supplemental test method for Sophoricoside in the *Ginkgo* extracts, *Ginkgo* leaves tablets and capsules. No. 142. Chinese Medicine Division, Department of Health. 2012. Hong Kong Chinese Materia Medica Standards. Vol. 5. Hong Kong (China): Chinese Medicine Division, Department of Health; p. 379–389.

Committee on Herbal Medicinal Products, European Medicines Agency. 2015. European Union herbal monograph on *Ginkgo biloba* L. folium. Amsterdam (The Netherlands): EMA; [accessed 2021 Feb 01]. https://www.ema.europa.eu/en/documents/herbal-monograph/final-european-union-herbal-monograph-ginkgo-biloba-l-folium_en.pdf.

European Directorate for the Quality of Medicines and Healthcare, Council of Europe. 2019. European Pharmacopoeia. 10th ed. Supplement 10.0. Stuttgart (Germany): Deutscher Apotheker Verlag; p. 1451–1453.

Frommenwiler DA, Booker A, Vila R, Heinrich M, Reich E, Canigueral S. 2019. Comprehensive HPTLC fingerprinting as a tool for a simplified analysis of purity of ginkgo products. J Ethnopharmacol. DOI:10.1016/j.jep.2019.112084.

Gaefner S. 2018. Adulteration of Ginkgo biloba leaf extract. Botanical Adulterants Bulletin. [accessed 2021 Feb 01]:[8 p.]. http://cms.healthgram.org/BAP/BAB/GinkgoBulletin.html.

Gauthier S, Schlaefke S. 2014. Efficacy and tolerability of *Ginkgo biloba* extract EGb 761 in dementia: a systematic review and meta-analysis of randomized placebo-controlled trials. Clin Interv Aging. 9:2065–2077.

Harnby JM, Luthria D, Chen P. 2012. Detection of adulterated *Ginkgo biloba* supplements using chromatographic and spectral fingerprints. J AOAC Int. 95(6):1579–1587.

He X, Bai Y, Zhao Z, Wang X, Fang J, Huang L, Zeng M, Zhang Q, Zhang Y, Zheng X. 2016. Local and traditional uses, phytochemistry, and pharmacology of *Sophora japonica* L.: a review. J Ethnopharmacol. 187:160–182.

Horsch S, Walther C. 2004. *Ginkgo biloba* special extract EB761 in the treatment of peripheral arterial occlusive disease (PAOD)–a review based on randomized, controlled studies. Int J Clin Pharmacol Ther. 42(2):63–72.

Liu XG, Yang H, Cheng XL, Liu L, Qin Y, Wang Q, Qi LW, Li P. 2014. Direct analysis of 18 flavonol glycosides, aglycones and terpene trilactones in *Ginkgo biloba* tablets by matrix solid phase dispersion coupled with ultra-high performance liquid chromatography tandem triple quadrupole mass spectrometry. J Pharm Biomed Anal. 97:123–128.

López-Gutíérrez N, Romero-González R, Vidal JLM, French AG. 2016. Quality control evaluation of nutraceutical products from *Ginkgo biloba* using liquid chromatography coupled to high resolution mass spectrometry. J Pharm Biomed Anal. 121:7–11.

Mabry TJ, Markham KR, Thomas MB. 1970. The Systematic Identification of Flavonoids. Berlin, Heidelberg: Springer.

Pandey R, Chandra P, Arya KR, Kumar B. 2014. Development and validation of an ultra high performance liquid chromatography electrospray ionization tandem mass spectrometry method for the simultaneous determination of selected flavonoids in *Ginkgo biloba*. J Sep Sci. 37(24):3610–3618.

von Boetticher A. 2011. *Ginkgo biloba* extract in the treatment of tinnitus: a systematic review. Neuropsychiatr Dis Treat. 7:441–447.

Wang F, Jiang K, Li Z. 2007. Purification and identification of genistein in *Ginkgo biloba* leaf extract. Chin J Chromatogr. 25(4):509–513.

Wohlmut H, Savage K, Dowell A, Mouatt P. 2014. Adulteration of *Ginkgo biloba* products and a simple method to improve its detection. Phytomedicine. 21(6):912–918.

Yao JB, Du X, Jin HH, Fang L, Min H, Qiao HX, Wang RW, Kuchta K. 2017. Seasonal variability of genistein and 6-hydroxykynurenic acid contents in *Ginkgo biloba* leaves from different areas of China. Nat Prod Commun. 12:1241–1244.