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Quality control evaluation of nutraceutical products from Ginkgo biloba using liquid chromatography coupled to high resolution mass spectrometry

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ABSTRACT

Analysis of 11 commercial nutraceutical products obtained from ginkgo has been performed using ultra-high performance liquid chromatography coupled to single-stage Orbitrap high resolution mass spectrometry (UHPLC-Orbitrap-MS). The main phytochemicals present in these samples were detected and quantified, utilizing a database containing 65 compounds. Phytochemicals were extracted using a mixture of an aqueous solution of methanol:water (80:20, *v*/*v*) in two sequential solid-liquid extractions. Adequate validation parameters were obtained. The validated compounds exhibited suitable linearity with determination coefficients (R^2) higher than 0.99, and intra and inter-day precision were lower than 17 and 22%, respectively. Limits of detection (LODs) and quantification (LOQs) were calculated, ranging from 2 to 10 $\mu g\,L^{-1}$, except for myricetin (LOD, 150 $\mu g\,L^{-1}$ and LOQ, 300 $\mu g\,L^{-1}$). Results indicate that the amount of terpenoids greatly varies among samples, ranging from 1133 (C7) to 12706 $mg\,kg^{-1}$ (C11). This emphasizes the importance of improve quality control in ginkgo-based products. Moreover, retrospective analysis allowed the detection of some undesirable substances as ginkgolic acid in the samples evaluated.

1. Introduction

Ginkgo biloba L. (*Salisburia adiantifolia, Salisburia biloba*), a "living fossil", is one of the oldest living tree species [1] existing on earth since 200 million years [2]. It was introduced into Japan about 800 years ago, and later into Europe (1730), and North America (1784) [3]. Nowadays it is mainly present in China, Korea and Japan [2].

The most important constituents present in ginkgo are the terpene trilactones, many flavonols glycosides, biflavones, proanthocyanidins, alkylphenols, simple phenolic acids, 6-hydroxykynurenic acid, 4-*O*-methylpyridoxine and polyprenols [4,5]. Moreover, three different classes of alkylphenols (ginkgolic acids, ginkgols and bilobols) could also be detected, and they can occur in various parts of ginkgo biloba [6]. Terpene trilactones have received the most attention [4], and the ginkgolides A–C are usually found at higher concentrations [7], whereas the minor compounds are ginkgolide M and J, being M only isolated from roots [7].

Ginkgo biloba leaf extracts have been used in nutraceutical products due to potential benefit in alleviating symptoms associated with cognitive impairment, dementia, Alzheimer's disease [8], hypertension [9], asthma [10] and tinnitus [11].

All these compounds are responsible of biological activity of ginkgo biloba, so therefore, their quantitative analysis is relevant. Nevertheless, the quality and content of bioactive components are significantly influenced by age [5], cultivation type, climate, time of harvesting and processing methods [12].

Therefore, it is important to develop suitable screening methods that allow the determination of bioactive components in this kind of products, in order to clarify the therapeutic material basis and the discovery of leading compounds, as well as to supply suitable chemical markers that can be used during the quality control of these products [13]. Up to now, numerous analytical methods, based on liquid chromatography (LC) [5,14,15], gas chromatography (GC) [16], and nuclear magnetic resonance (NMR) [6], have been used to analyse different kind of bioactive components in ginkgo biloba extracts.

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Most of these methods are generally coupled to UV–vis [17], diode-array detection (DAD) [15,18] or mass spectrometry (MS) [19,20], although MS and DAD can also be coupled during the same analysis [21,22]. However, high resolution mass spectrometry analysers as Orbitrap have not been applied for this purpose yet, although they have been effectively used for the determination of bioactive compounds in natural products [23,24].

For the great majority of studies the most important issue is the determination of ginkgolic acids [6,25], flavonoids [15,17,18], or the simultaneous determination of flavonoids and terpenes [5,26], due to these compounds are usually included in the current criteria utilized during quality control of ginkgo products. Up to now, many papers have been described the analysis of bioactive compounds from ginkgo biloba leaves [20,21,25], but dietary supplements have been scarcely analysed [17,27,28] in relation to possible adulterations [26].

In this paper, an efficient and sensitive analytical method was developed and validated to achieve a simultaneous and fast separation of bioactive components from ginkgo biloba nutraceutical products by ultra high performance liquid chromatography (UHPLC) coupled to Orbitrap-MS. High resolution offers the possibility of a restrospective analysis without further injections, which can be a very useful tool to improve the knowledge of the composition of these products, due to it works in full scan acquisition mode without limitations in the number of monitored compounds. All this information would be helpful for the quality control of these products and for the detection of product adulteration in order to assure their safety.

2. Experimental

2.1. Chemicals and reagents

Phytochemical standards were obtained from four different suppliers: Extrasynthese (Genay, France), Sigma-Aldrich (Madrid, Spain), ChromaDEX (Irvine, CA, USA) and Fluka (Steinheim, Germany). Purity was higher than 95% for all standards, except for some of them as chrysin, daidzin, kaempferol-7-*O*-glucoside, quercetin-3-*O*-glucoside and theaflavin with a purity ≥90%.

Standard solution was prepared in methanol, ethanol, dimethyl sulfoxide or in a mixture of methanol:water (50:50, *v*/*v*), ranging from 90 to 750 mg L^{-1}. Three working solutions at 5000 μg L^{-1}, 500 μg L^{-1} and 50 μg L^{-1} were prepared in methanol by appropriate dilution of aliquots of each individual stock standard solution. All solutions were stored in amber bottles at −18 °C in the dark and they were renewed every 6 months.

Acetonitrile (ACN) (LC–MS grade) was purchased by Fisher Scientific (Fair Lawn, NJ, USA). Dimethyl sulfoxide (HPLC grade) and methanol (MeOH) (LC–MS grade) were obtained from Sigma–Aldrich.

Ammonium acetate (purity 97%) and ethanol (HPLC grade) were supplied by Panreac (Barcelona, Spain). Formic acid (LC–MS grade) and water (LC–MS grade) were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was supplied by J.T. Baker (Deventer, The Netherlands).

The Orbitrap analyser used a mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrat and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Thermo-Fisher (Waltham, MA, USA) in order to accurate mass calibration.

2.2. Apparatus

A Centronic BL II centrifuge (J.P. Selecta, Barcelona, Spain), a Reax 2 rotatory agitator from Heidolph (Schwabach, Germany), a vortex mixer WX from Velp Scientifica (Usmate, Italy) and a coffee grinder (Orbit, Hong Kong, China) were used to process all samples.

2.3. UHPLC-Orbitrap-MS

Chromatographic apparatus consisted of an UHPLC system Transcend (Transcend 600 LC, Thermo Fisher Scientific, San Jose, CA, USA). Analyses were carried out using a Waters (Milford, MA, USA) Acquity C18 column (2.1 × 100 mm, 1.7 μm particle size).

For detection purposes, a single-stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) was used. The mass spectrometer was operated using a heated electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in positive (ESI+) and negative ionization (ESI−) modes.

Optimal ionization conditions were as follows: sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); spray voltage, 4 kV (−4 kV in ESI−); skimmer voltage, 18 V (−18 V in ESI−); capillary voltage, 35 V (−35 V in ESI−); tube lens voltage, 95 V (−95 V in ESI−); heater temperature, 305 °C; capillary temperature, 300 °C. The automatic gain control (AGC) was set at a target value of 1×10^6.

The system was operated employing four alternating acquisition functions: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (2) All-ion fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s; (3) full MS, ESI− without fragmentation (HCD collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (4) AIF, ESI−, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s. Considering the scan time of the four acquisition functions, and the polarity switching (approx. 0.27 s), an overall scan rate of 0.56 Hz was achieved. Mass range in the full scan experiments was set at *m*/*z* 100–1000, whereas for MS/MS, it was set from *m*/*z* 70–700.

All the analyses were performed without lock mass, using external calibration mode. Mass accuracy was checked with multicompound standards every day and the analyser was calibrated every two weeks with mass accuracy standards (see Section 2.1). The instrument was controlled and the data were analysed on a computer equipped with Xcalibur™ version 2.2.1 (Thermo Fisher Scientific, Les Ulis, France) with Qual and Quan browser. ICIS peak detection was applied. For screening ToxID™ 2.1.1 (automated compound screening software, Thermo Scientific) was used.

2.4. Samples

A total of eleven samples were analysed. Six of them were capsules and the others were acquired as tablets. Samples were purchased from local stores and commercial companies located in Almería (Spain), and Krakow (Poland). Homogeneous powders were obtained through coffee grinder and were kept together in a desiccant at 5 °C in a refrigerator.

2.5. Phytochemical extraction from nutraceutical products

An accurately weighed sample (150 mg) was placed into a 50-mL polypropylene tube. After that, the sample was extracted with 30 mL of a mixture of methanol:water (80:20, *v*/*v*), followed by vigorously mixing for 1 min in a vortex. Phytochemicals extraction was accomplished via continuous shaking for 2 h at room