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Detection of adulterants in dietary supplements with *Ginkgo biloba* extract by attenuated total reflectance Fourier transform infrared spectroscopy and multivariate methods PLS-DA and PCA

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ABSTRACT

The infrared spectroscopy with attenuated total reflectance (ATR) sampling coupled with chemometric methods has been applied to non-destructive detection of adulterants in dietary supplements containing *Ginkgo biloba* extract. The sample set comprised the spectra of six drugs and sixteen dietary supplements with ginkgo leaf extract. Spectral data (900–1800 cm^{-1}) were analyzed using multivariate partial least squares regression combined with a discriminant analysis (PLS-DA). The second derivative of spectra followed by mean centering was used as preprocessing method. Three models were constructed and validated for detection of potential adulterants: kaempferol, quercetin, and rutin. The iPLS-DA classification models achieved about 87.5%, 93,7%, and 87,5% of correct classification for adulteration with kaempferol, quercetin and rutin, respectively. The results obtained from classification models were verified by chromatographic fingerprints of unhydrolyzed sample extracts. Two-trace two-dimensional asynchronous correlation maps were constructed from pairs of spectra (each dietary supplement spectrum vs. averaged spectrum of drugs) and then analyzed by multiway PCA which revealed good discrimination between samples.

1. Introduction

Growing consumption of herbs, over-the-counter herbal drugs, and dietary supplements is primarily driven by increased consumer health-consciousness, a rising tendency for self-medication and changing lifestyle [1,2]. Because of the frequent use of dietary supplements, there has been tremendous growth in sales of these products and new products are continuously launched into the market and can be easily purchased in pharmacies, health food stores, and supermarkets. Many dietary supplements of botanical origin such as ginseng, *Ginkgo biloba*, and black cohosh are sold as "100% natural" products, but natural not always mean better and, moreover, natural may not always be considered safe. One of the essential factors affecting the safety of dietary supplements is their adulteration with undeclared substances.

Many adulteration issues were reported for the dietary supplements containing *Ginkgo biloba* extract [3]. Ginkgo leaf extract is effective in the improvement of memory, treatment of multiple sclerosis and can help prevent Alzheimer disease and other types of dementia [4,5] and is used worldwide in herbal medicinal products and dietary supplements. The typical way of ginkgo extract adulteration is the use of free flavonol aglycones: quercetin and kaempferol or glycoside rutin. Recently, *Sophora japonica* fruits or flowers extract has been used as a natural adulterant for ginkgo extracts [6–8]. To guarantee the quality of dietary supplements containing *Ginkgo biloba* extract, there is a need for reliable adulteration detection method.

The most common methods applied for determination of plant dietary supplements adulteration are chromatographic methods [8–11]. An alternative to chromatographic techniques are vibrational spectroscopic methods [12]. Conventional FTIR spectroscopy, which generates 1D linear spectra, is routinely used as an analytical tool in chemistry. However, for complex samples, 1D spectra are often too overlapped and broaden to get a detailed analysis. A beneficial alternative to standard infrared spectroscopy has become the two-dimensional techniques such as two-dimensional infrared spectroscopy (2D IR) or generalized two-dimensional correlation infrared spectroscopy (2D-COS) [13–15]. In one dimensional spectroscopy, signals are recorded vs. a time or a single frequency whereas in multidimensional techniques the signals are measured as a function of several parameters. 1D FTIR spectroscopy yields valuable information on energy levels, transition dipole moments, and electronic and nuclear motions [16,17]. In 2D IR, infrared spectra are spread into a second dimension providing information on vibrational couplings. It is a powerful tool for studying and visualizing the structural changes in complex molecules and dynamics of hydrogen bonding [13].

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Two-dimensional (2D) correlation spectroscopy has become a useful tool for analysis of adulterated samples. Generalized 2D correlation spectroscopy needs to record a series of spectra of sample exposed to some external perturbation such as temperature, pH, or concentration. Construction of meaningful generalized 2D correlation spectra requires a minimum of three or more spectra of each sample subjected to an external perturbation. However, recently [18] has published a beneficial modification of correlation spectroscopy intended for the comparison of a pair of spectra, called two-trace two-dimensional (2T2D). 2D correlation infrared spectroscopy with or without perturbation has already been successfully applied in various adulteration issues such as detection of milk adulteration with melamine [19–21], detection of *Aquilariae Lignum Resinatum* adulteration [22,23] and analysis of crystallized lactose in milk powder [24].

This work presents potential application of mid-infrared spectroscopy with ATR sampling to identifying *Ginkgo biloba* dietary supplements adulterated with the flavonols: rutin, quercetin, and kaempferol. We have applied the ATR-FTIR spectra combined with interval partial least squares regression and discriminant analysis (iPLS-DA) to detect the three adulterants in *Ginkgo biloba* extracts. Comparison with chromatographic fingerprints verified results obtained using iPLS-DA models. Moreover, we have constructed the asynchronous 2T2D correlation spectra for each dietary supplement and average spectrum of ginkgo drugs. The multiway principal components analysis (MPCA) for discrimination of samples adulterated with different flavonols has been provided.

2. Experimental

2.1. Standards and Samples

Rutin, quercetin, and kaempferol were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Solvents (acetonitrile, water, ethanol) used in this study were all analytical and HPLC grade. In the study, 22 herbal products containing *Ginkgo biloba* extract were analyzed. Six of them were drugs with standardized (24/6) ginkgo extracts (two in the form of capsules and four in the form of tablets): Bilobil® (Krka, Novo Mesto, Slovenia), Bilobil forte® (Krka, Novo Mesto, Slovenia), Tanakan® (Ipsen, Paris, France), Tebokan® (Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany), Gingio® (Salutas Pharma GmbH, Barleben, Germany) and Ginkofar® (Biofarm, Poznań, Poland). The other samples were dietary supplements (8 in the form of capsules and 8 in the form of tablets). The commercial products were bought from local pharmacies and markets (Bydgoszcz, Poland) and online pharmacies. All products have been coded as L1 to L6 (for drugs) and S1 to S16 (for supplements) to maintain the confidentiality of the supplier's identity.

2.2. ATR-FTIR Spectroscopy

The ATR-FTIR experiments were carried out with a Ge based ATR accessory (Pike Technologies, Madison, WI, USA) and a Shimadzu 8400 s spectrometer (Shimadzu Corp., Kyoto, Japan). The solid samples in form tablets and capsules were ground to a fine powder in a mortar, and a small amount of each sample was applied onto the surface of the ATR crystal and pressed by the clamp with constant pressure. The spectra (20 scans each) were measured in absorbance mode, within 750–4000 cm^{-1} wavenumber's range, at a resolution of 4 cm^{-1}. The ATR crystal was cleaned with ethanol after each measurement. A new background spectrum was acquired before applying a sample to the crystal. Each sample was measured five times. The 1D FTIR spectra were exported in *.dx format from the instrument software IRSolution to MATLAB.

2.3. Multivariate Analysis

In this study, a partial least squares regression (PLS) combine with discriminant analysis (DA) was used. The training (calibration) set for classification contains spectra of drugs with ginkgo extract (class 0) and spectra of the averaged drug sample mathematically fortified by adulterant (class 1). To obtain the first part of calibration set (class 0), a combination of 2- and 3-element subsets of the 6-element set of drug spectra were calculated. The following combinations $C(2,6) = 15$ and $C(3,6) = 20$ gave a total of 35 subsets. Finally, the training set consisted of 35 averaged spectra of six drugs. The class 1 of calibration set contained the drugs spectra fortified by adulterants. The normalized spectrum of each potential adulterant (rutin, quercetin and kaempferol) was mixed with the averaged normalized drug spectrum in various proportions. As a result, the X block for training (calibration) consisted of 35 spectra of averaged drug samples and spectra of fortified samples (15 for each adulterant). In turn, the test set contained spectra of 16 dietary supplements.

PLS discriminant analysis (PLS-DA) applies extended PLS algorithm to classification. In PLS-DA, the categorical variable Y is a vector holding the class information. In this case, we used notation 0/1 to indicate class membership of each sample. A vector with values of 0 represents the sample belonging to the class of non-adulterated samples (drugs), and 1 represents each adulterated sample. To determine the threshold of discrimination the Bayesian statistics were applied. It provides the best class separation with minimal probability of false classification for test samples. For simplicity, within each class, the distribution of predictions is assumed to be Gaussian. The most efficient pre-processing method of correlation spectra, before multivariate analysis, was found to be second derivative followed by mean centering [25]. For optimization of PLS modeling the interval selection of variables were applied as follows: forward mode, step size 10–40 variables and automatic selection of number of intervals.

All the calculations were performed using the PLS-Toolbox 7.5 (Eigenvector Research, Inc., Manson, WA, USA) in Matlab software version R2018a (The Matworks, Inc., Natick, MA, USA).

2.4. Two-Dimensional Correlation Spectroscopy

According to [18] a pair of two spectra may be compared by constructing the synchronous $\Phi(\nu_1,\nu_2)$ and asynchronous $\Psi(\nu_1,\nu_2)$ maps.

$$\Phi(\nu_1,\nu_2) = \frac{1}{2}[s(\nu_1)\cdot s(\nu_2) + r(\nu_1)\cdot r(\nu_2)] \quad (1)$$

$$\Psi(\nu_1,\nu_2) = \frac{1}{2}[s(\nu_1)\cdot r(\nu_2) + r(\nu_1)\cdot s(\nu_2)] \quad (2)$$

were $s(\nu_1)$ is the sample spectrum and $r(\nu_1)$ - reference spectrum.

2D correlation spectra obtained in this way were composed into three-dimensional matrix used further in a chemometric analysis. Sample spectrum means ATR-FTIR spectrum (900–1800 cm^{-1}) of each dietary supplement. The averaged spectrum of drugs containing ginkgo extract was used as a reference spectrum.

2.5. HPLC Analysis

The reverse-phase high performance liquid chromatography (RP-HPLC) was applied as a reference method. To obtain *fingerprint* chromatograms of commercial products with ginkgo extract a Grace Smart® column (150 mm × 4,6 mm, 5 μm) was used. The analysis was carried out using high-performance liquid chromatograph with photodiode array detector (HPLC-DAD, (Shimadzu Corp., Kyoto, Japan)). The mobile phase consisted of water with 0,2% formic acid (phase A) and acetonitrile with 0,2% formic acid (phase B). At a flow rate of 0,6 ml/min, the gradient was as follows: 0–4 min 17% B,