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Fluorescence, electrophoretic and chromatographic fingerprints of herbal medicines and their comparative chemometric analysis

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ABSTRACT

The aim of the present study was to compare the polyphenolic compositions of 47 medicinal herbs (HM) and four herbal tea mixtures from Central Estonia by rapid, reliable and sensitive Spectral Fluorescence Signature (SFS) method in a front face mode. The SFS method was validated for the main identified HM representatives including detection limits (0.037 mg L^{-1} for catechin, 0.052 mg L^{-1} for protocatechuic acid, 0.136 mg L^{-1} for chlorogenic acid, 0.058 mg L^{-1} for syringic acid and 0.256 mg L^{-1} for ferulic acid), linearity (up to 5.0–15 mg L^{-1}), intra-day precision (RSDs = 6.6–10.6%), inter-day precision (RSDs = 6.4–13.8%), matrix effect (−15.8 to +5.5) and recovery (85–107%). The phytochemical fingerprints were differentiated by parallel factor analysis (PARAFAC) combined with hierarchical cluster analysis (CA) and principal component analysis (PCA). HM were clustered into four main clusters (catechin-like, hydroxycinnamic acid-like, dihydrobenzoic acid-like derivatives containing HM and HM with low/very low content of fluorescent constituents) and 14 subclusters (rich, medium, low/very low contents). The average accuracy and precision of CA for validation HM set were 97.4% (within 85.2–100%) and 89.6%, (within 66.7–100%), respectively. PARAFAC-PCA/CA has improved the analysis of HM by the SFS method. The results were verified by two separation methods CE-DAD and HPLC-DAD-MS also combined with PARAFAC-PCA/CA. The SFS-PARAFAC-PCA/CA method has potential as a rapid and reliable tool for investigating the fingerprints and predicting the composition of HM or evaluating the quality and authenticity of different standardised formulas. Moreover, SFS-PARAFAC-PCA/CA can be implemented as a laboratory and/or an onsite method.

1. Introduction

The use of herbal medicines (HM) for health promotion purposes has been known for ages. Unlike synthetic drugs, HM are complex mixtures of substances that might be responsible for their therapeutic effects. Indeed, how many constituents and which of them, in complexes with other constituent(s), are responsible for the therapeutic mechanism has not been well defined for any HM. Therefore, it is important to define as many constituents in HM as possible to understand and explain their bioactivity.

Various techniques have been proposed for identification of the authenticity of HM. These include high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) [1,2] or UV–vis spectrophotometric detection [3,4], gas chromatography (GC) [5,6], capillary electrophoresis (CE) [7–9], paper-based colorimetric assays [10,11], and infrared [12] and fluorescence spectroscopy [13]. Nowadays, there is an increasing need to exploit methods that are quick, reliable and efficient and, moreover, can be easily automated for on-site analysis. Indeed, one of these methods is fluorescence spectroscopy. It has been shown to be a rapid and accurate method for identifying plant materials [14,15], oil in water [16], illegal drugs [17] and polyphenols in wines [18].

Recently, an innovative approach was proposed by Babichenko et al. [19], which is known as the spectral fluorescence signature method (SFS). SFS could be described as a 2D coloured pattern where colours represent the intensity of fluorescence or a 3D fluorescence matrix, where fluorescence is a function of excitation and emission wavelengths. SFS is a sum of all profiles of intrinsic fluorophores and, therefore, could also be suggested as unique fingerprints of the sample under investigation. SFS does not interfere with Rayleigh scattering due to a special measuring window, where the Rayleigh scattering is outside the measuring range. Therefore, the fluorescent fingerprints do not interfere with Rayleigh scattering that is a frequent issue for the conventional

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excitation emission matrix (EEM) spectroscopy, where the signal of compounds can be totally hidden in the intensive scattering.

Actually, fluorescence spectroscopy may be divided into two types: classical angle and front-face fluorescence analysis. The first one has a crucial disadvantage over the last approach. The classical angle fluorescence requires the dilution of samples with an appropriate solvent. Indeed, it is necessary to measure those samples whose absorbance is not greater than 0.05 [20]. Unfortunately, the dilution affects the composition of herbal samples. As a result, the fluorescence signal of one constituent can start to predominate over others in the SFS image. In the worst case, other constituents can disappear in the SFS image. Therefore, the authentic SFS fingerprinting of HM can be totally lost.

As proposed by Genot et al. [21], this problem can be overcome using the front-face fluorescence spectroscopy, which enables the analysis of turbid untreated samples such as herbal medicines extracts. Therefore, utilisation of SFS method in the front-face mode for the analysis of undiluted HM extracts is of utmost importance. Unfortunately, there are more fluorescence-affecting factors such as quenching, concentration, and molecular environment. Therefore, the correct experimental conditions must be found for analysis of HM samples.

Undoubtedly, herbal medicines are very complex sample matrices, containing up to thousands of different compounds, e.g. polyphenols such as flavonoids, and non-flavonoid compounds. Although many compounds present in herbs have been thoroughly studied and quantified, there are still a lot of unknown chemical constituents in plants that need to be identified and investigated. The well-known compounds found in herbs can be grouped as simple polyphenols, catechins, anthocyanins, flavonoid glycosides and aglycones, theaflavins, chalcones and anthraquinone derivatives [16]. However, many chemical standards are unavailable or/and are too expensive to be applied to the identification of components present in HM. In this case, it is impossible or economically unreasonable to conduct the identification of every HM constituent. Therefore, the spectral data obtained by several detection methods such as UV–vis absorbance spectroscopy, the excitation emission matrix spectroscopy and the MS detection challenges the benefit making it possible to predict the substance group and/or identify the unknown substances in HM.

Despite the optimised experimental conditions, data analysis is of utmost importance for the final result. The application of chemometric techniques such as principal components analysis (PCA), hierarchical cluster analysis (CA) is very popular for interpretation of chromatographic and electrophoretic HM fingerprints. Several studies have shown good results to be obtained for differentiation of salad vegetables by different polyphenols [22], Oolong tea *Camellia sinensis* from different sources by different polyphenols and alkaloids [23], hops chemical screening by proanthocyanidins [24] and others studies [25–28]. In case of multi-way data such as diode array detector or SFS data, multi-way chemometric techniques must be used. One commonly used approach for EEM data analysis is Parallel Factor Analysis (PARAFAC) [29]. It has proven to be effective for analysis of complex food matrices [18].

The aim of the present study was to evaluate the capability of the SFS method in the front face mode combined with chemometric techniques such as PARAFAC, PCA and hierarchical cluster analysis for the authentication of HM available in the local Estonian market. The results were compared with two independent well-known reference methods, CE-DAD and HPLC-DAD-MS. The reference methods were also combined with PARAFAC-PCA/CA. The comparative study of HM discrimination by three independent methods was conducted. The performance characteristics of SFS method were evaluated and detection capabilities of three methods were compared. Additionally, some of the main chemical constituents in plant extracts were identified by MS/MS.

2. Experimental

2.1. Reagents and samples

The air dried samples of HM (moisture content 7–8%) were obtained from OÜ Kubja Ürt (Central Estonia, 73302 Karinu, Järvamaa, GPS: 59.0360289, 25.9611146) harvested in 2011 (three samples of each HM). HM grow in a natural environment. The mineral and chemical fertilizers are not used. The list of HM with their respective identification number is presented in Table 1.

Vanillic acid, protocatechuic acid, gallic acid, tannic acid, ferulic acid, caffeic acid, *p*-coumaric acid, chlorogenic acid, sinapic acid, syringic acid, *trans*-resveratrol, catechin, quercetin, myricetin, kaempferol, apigenin, luteolin, quercitrin, and rutin were purchased from Sigma-Aldrich (Germany). MS grade acetonitrile and formic acid (Sigma-Aldrich, Germany) were used for the HPLC-DAD-MS analysis. Deionised water was purified by the Milli-Q system (Millipore, Bedford, MA, USA). Sodium tetraborate decahydrate, sodium hydroxide and methanol were of analytical grade from Sigma-Aldrich (Germany).

2.2. Sample preparation

The herbal extracts of 51 samples were prepared using 80% (v/v) methanol. The plant material was ground into powder. The extracts were prepared by weighing 0.5 g of a plant sample, leaching with 10 mL methanol for 2 h at room temperature and extracting in an ultrasonic bath at a temperature of 35–40 °C for 0.5 h. The extract was centrifuged for 10 min at 5000 rpm and stored at −18 °C.

2.3. Fluorescence spectroscopy

All fluorescence measurements were carried out on a portable NarTest NTX2000® Drug Analyser (Nartest AS, Estonia), which generates special excitation emission matrixes (EEMs) or spectral fluorescence signatures (SFSs). This is a compact spectrofluorometer equipped with a 5 W pulsed Xenon lamp and a special 10 mL optical cell. SFSs were measured in a front-face optical layout (35°) from the surface at room temperature. The following experimental parameters were set: λ_{ex}=230–350 nm and λ_{em}=250–565 nm with 5 nm intervals in both directions, gain=500. One scanning took 2.3 min.

2.4. CE-DAD

All CE experiments were carried out using an Agilent 3D capillary electrophoresis instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) according to the method by Helmja et al. [30]. The separation of polyphenols was performed in a fused silica capillary (60 × 0.005 cm^2, Polymicro Technology, Phoenix, AZ, USA) with an effective length of 51.5 cm. Prior to use, the capillary was rinsed with a 1.0 M NaOH solution, water and a background electrolyte (BGE) for 5 min of each. A 50 mM sodium tetraborate solution (pH 9.3) was used as a BGE. The applied voltage for the separation was +25 kV. The diode array detector range was set to 200–400 nm.

The sample solutions were introduced at the anodic end of the capillary with 50 mbar pressure for 5 s. The peaks of polyphenols (peaks in the electropherogram) were identified by the standard addition method and by comparing UV spectra.

2.5. HPLC-DAD-MS

Analysis of methanolic extracts was performed on HPLC equipment of the Agilent 1200 series with a diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). The samples (10 μL) were separated on an Agilent Zorbax SB C-18 column (150 mm × 4.6 mm i.d., 5 μm particle size). The mobile phase