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Differentiation of tannin-containing herbal drugs by HPLC fingerprints

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A new HPLC system coupled with multiple detectors — Diode array detector (DAD), fluorescence detector (FLD), electrochemical amperometric detector (ADC) and mass spectrometry detector (MSD) was developed for the characterization and differentiation of tannin-containing herbal drugs included in *The European Pharmacopoeia*. The HPLC separation system consisted of an Agilent ZORBAX Eclipse XDB C_{18} column and a gradient water and methanol as the mobile phase which was kept at a flow rate of 0.3 mL• min^{-1}. Four kinds of detectors were connected by a micro-splitter valve and simultaneously recorded the response of each analytical sample. Thirty-one samples from eight kinds of tannin-containing drugs were measured using this HPLC system and their signals from all detectors were comprehensively processed via principal component analysis (PCA). The statistic result demonstrates that thirty-one batches from different herbal drugs can be reasonably identified and systematically classified by their chemical fingerprints. The proposed multi-detector HPLC method aided by chemometrics not only offers a new pattern for the study of tannin-containing herbs, but also provides a useful foundation for quality control of herbal medicines.

1. Introduction

Plant materials are used throughout developed and developing countries as home remedies, over-the-counter drug products and raw materials for the pharmaceutical industry, and represent a substantial proportion of the global drug market (World Health Organization, 1998). But the potent quality assessment of medicinal plant materials is still a conundrum even though much progress has been made in the pharmaceutical field. The complex ingredients of natural drugs make it difficult to assay the quality as a whole, and just one or more instead of all ingredients in drugs have been identified or quantified for a long time.

Fingerprint, a technique emphasizing on the systemic and comprehensive characterization of the analyzed samples, has been accepted as an effective approach for the species differentiation and quality control of the complex plant medicines (Alaerts et al. 2010; World Health Organization, 1991). However, almost all previous HPLC fingerprints were established at some specific ultraviolet wavelength where the maximum amount of chromatographic peaks appeared (Jiang et al. 2009; Lv et al. 2012; Yang et al. 2011; Zhang et al. 2008). This mode has its limitation that not all compounds in the plants have ideal ultraviolet absorbance. Even in mass spectrometry, not every compound shows response.

Tannin, a widely distributed polyphenolic compound, has shown noticeable biological and pharmacological activities such as inhibition of carcinogenesis, host-mediated antitumor activity, antiviral activity, and inhibition of active oxygen. Starting with the isolation of a crystalline tannin (geraniin) of mild property from a popular herb medicine (Geranii herba), various polyphenolic compounds including those belonging to new classes of tannins (oligomeric hydrolyzable tannins, complex tannins, and other metabolites and condensates) have been isolated from various medicinal plants (Harinder et al. 2007; Okuda et al. 1992). But up to now, quality of tannin-contained plants is still assessed by determining the total tannin using the old Folin-Ciocalteu reagent which reacts with any reducing substance (Suresh and Harinath 2010; Silva et al. 2011). In the monographs on eight kinds of tannin-containing herbs described in *The European Pharmacopoeia* (European Directorate of the Quality of Medicine 2010), only minimum contents of tannin quantified by this unspecific colour reaction are prescribed besides microscopy.

In the present paper, Agrimony, Alchemilla, Bilberry fruit, Hamamelis leaf, Loosestrife, Oak bark, Rhatany root and Tormentil, eight tannin-containing herbal drugs in *Ph. Eur.,* have been chosen to explore the feasibility of identifying and characterizing of herbal drugs by multi-detector chromatographic fingerprints and chemometric method.

2. Investigations, results and discussion

2.1. *Optimization of HPLC separation*

The HPLC analytical conditions were optimized to obtain favorable chemical fingerprints. Gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid (Fig. 1) which are the typical ingredients of tannin-containing drugs were used to optimize separation conditions (Møller et al. 2009; Yang et al. 2011). Meanwhile, in order to eliminate the drift interference of

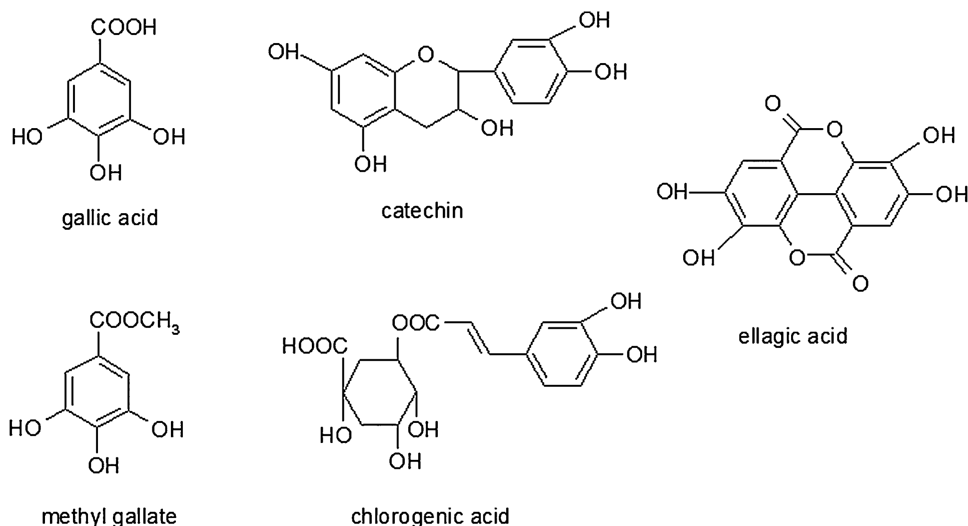


Fig. 1: The chemical structures of gallic acid, catechin, methyl gallate, chlorogenic acid and ellagic acid

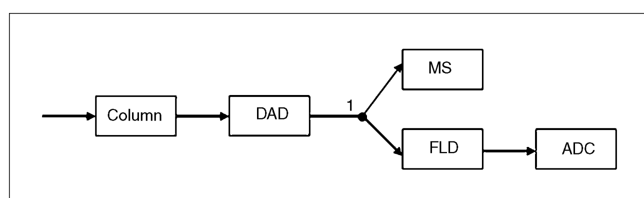


Fig. 2: Schematic representation of the detectors connection. (1) micro-splitter valve

retention time, 2,5-dihydroxybenzoic acid was added into the sample solution and used as a reference standard for its good separation with other compounds in tannin-containing drugs and good response in all detectors.

To obtain comprehensive information of samples, four kinds of detectors, DAD, FLD, ADC and MSD, were assembled by a micro-splitter valve (Fig. 2). ADC was located at the end of the set-up considering its high backpressure. An Agilent ZORBAX Eclipse XDB C_{18} column with a diameter of 3.0 mm was selected for higher separation efficiency and lower system pressure with lower flow rate.

Under the optimized conditions, the main constituents in tannin-containing samples were well separated, and their peaks displayed good peak resolution and symmetry in all detectors, as shown in the representative chromatograms in Fig. 3.

2.2. *Validation of methodology*

The method was validated with samples prepared from *Alchemilla vulgaris* L. (Batch No. 81606006). All detectors, DAD, FLD, ADC and MSD, were used during validation experiments.

Method precision was evaluated by the analysis of six injections of one sample, while repeatability was assessed by the injections of six samples prepared independently from the same batch. In all four detectors, the relative standard deviation (RSD) of retention times (t_R) and peak areas (PA) of four compounds — gallic acid, catechin, chlorogenic acid and ellagic acid (methyl gallate was not detected in *Alchemilla*) for precision were found in the range of 0.11∼0.39% and 0.26∼0.77%, respectively. And in the repeatability experiment, the values were also below 0.24% and 1.69%, respectively. Moreover, the stability test of the sample was performed after 0 h, 2 h, 4 h, 8 h, 12 h, 24 h at room temperature, the RSD of t_R and PA were less than 0.15% and 1.35%, respectively. All results indicated that the HPLC method for the fingerprint analysis was valid and satisfactory.

2.3. *PCA of the chromatographic fingerprints*

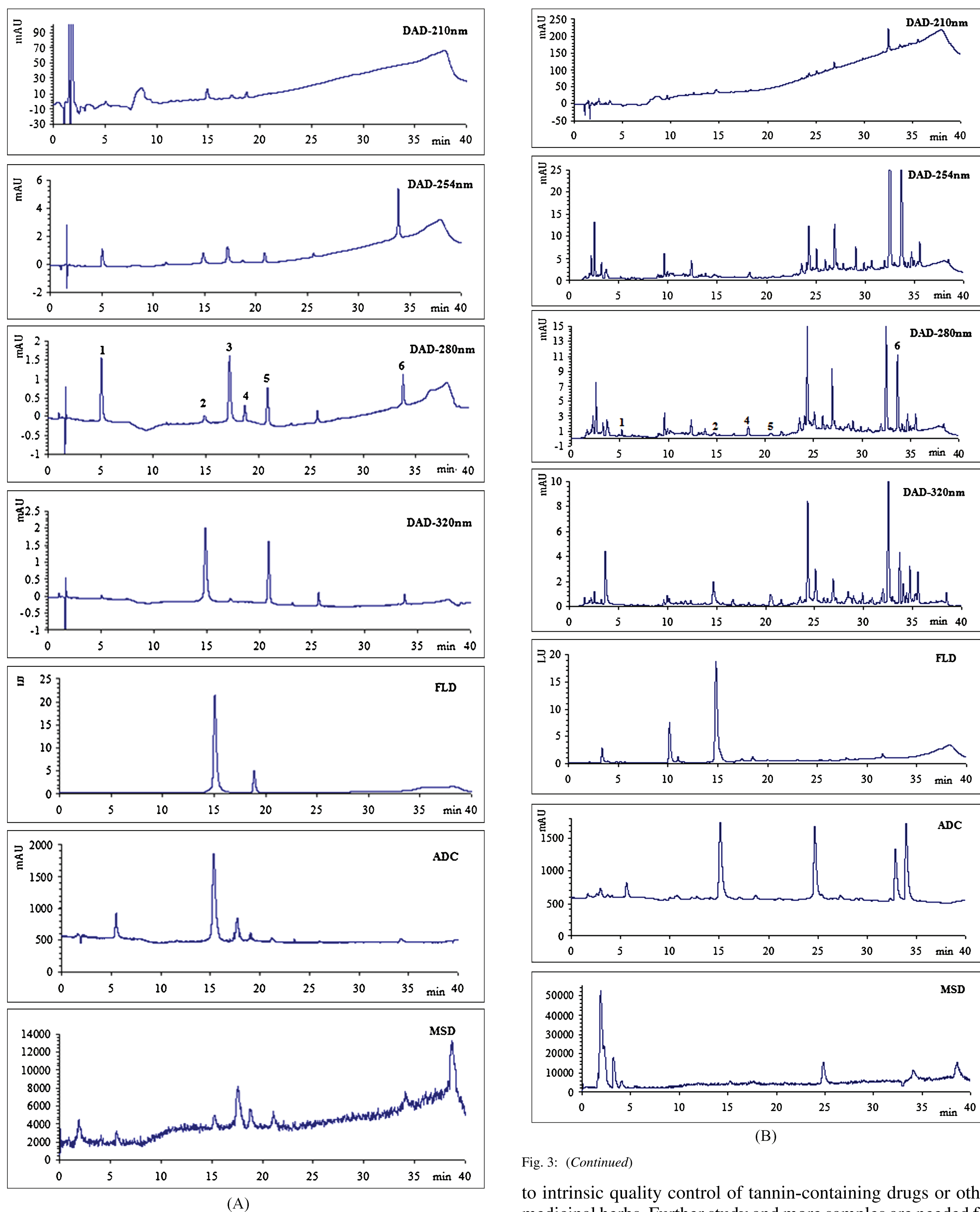
Multivariate analysis is the area of statistics that deals with observations made on many variables. The main objective was to study how the variables are related to one another, and how they work in combination to distinguish between the cases on which the observations are made (Izenman 2008). Multivariate analysis comprises a set of techniques dedicated to the analysis of data sets with more than two variables. Among them, principal component analysis (PCA) is the oldest and most versatile method which reduces complex data sets to a two- or three-dimensional scores map that indicates inherent relationship such as clusters and groupings among observations (Lindon et al. 2007; Yan et al. 2009; Yang et al. 2007).

For this reason, PCA was employed to assess the similarity and dissimilarity of the fingerprint profiles from different plants and different origins in our study. All signals from four detectors were utilized completely with 2,5-dihydroxybenzoic acid as the calibration reference. The high response values of MSD were scaled down to meet the analysis requirement.

The generated PCA scores plot revealed that the fingerprint profiles of eight kinds of tannin-containing herbal drugs deviated from each other, while only minor differences in the chromatographic profiles within samples from a certain herb (Fig. 4). At the same time, the distance on the plot largely suggested the diversity among species. Just like *Alchemilla* and Bilberry fruit, the visualized long distance indicated the obvious difference between them. This result effectively implied the feasibility of differentiating tannin-containing herbal drugs by multi-detector HPLC fingerprints. As for the variation of the data in the scoreplot within each type of herb, it was due to difference in harvesting time, drying methods, storage, etc., which also was the reason for some differences in the fingerprints of samples of a given herb obtained from different sources.

2.4. *Conclusions*

The developed HPLC system coupled with multiple detectors was used successfully for acquiring the extensive information of tannin-containing herbal samples, which also provided



(A)

(B)

Fig. 3: (*Continued*)

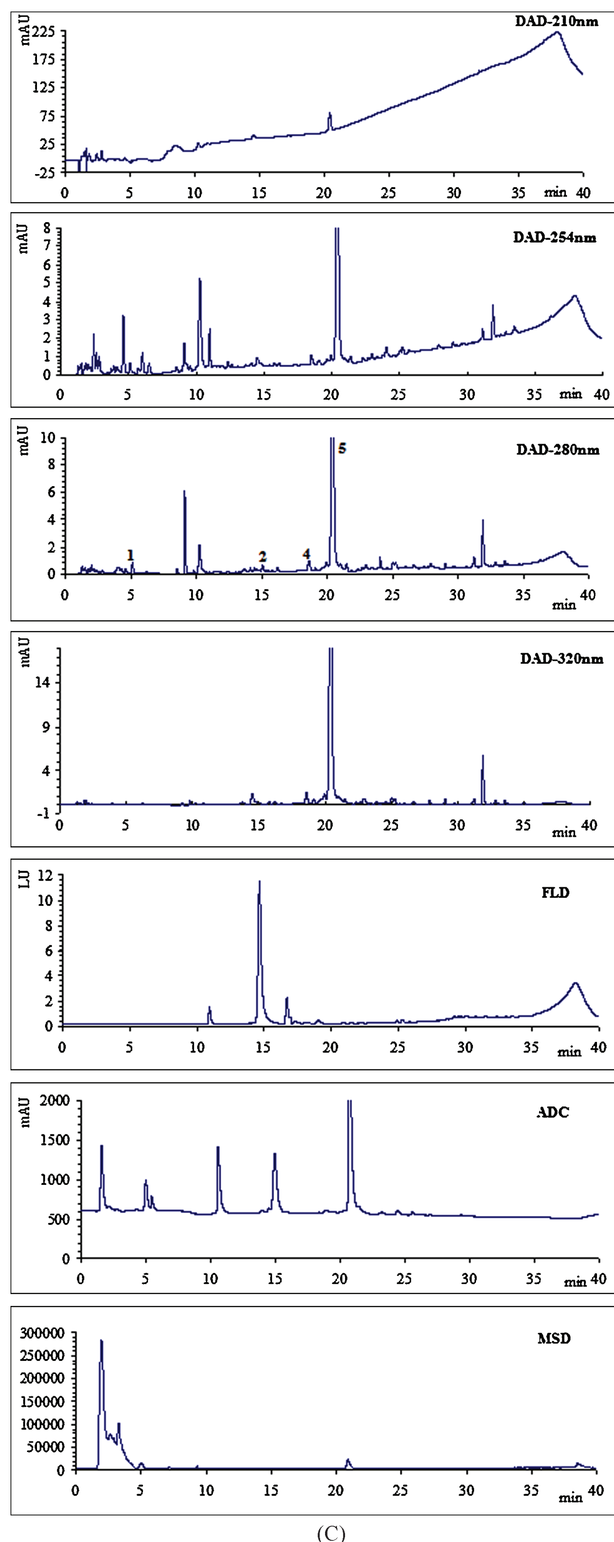
Fig. 3: Fig. 3: The chromatograms of (A) mix standard compounds, (B) Alchemilla sample with Batch No. 81606006 and (C) Bilberry fruit sample with Batch No. 82209006: (1) gallic acid; (2) 2,5-dihydroxybenzoic acid; (3) methyl gallate; (4) catechin; (5) chlorogenic acid; (6) ellagic acid

multiplex chromatographic fingerprints to identify and differentiate these herbal drugs. With the aids of this system and chemometrics, chromatographic fingerprinting of the constituents in herbs would prove to be an important part of identification and characterization, which herein to be well applied to intrinsic quality control of tannin-containing drugs or other medicinal herbs. Further study and more samples are needed for thoroughly exploiting this system with a good prospect.

3. Experimental

3.1. Plant materials and chemicals

Thirty-one batches of tannin-containing herbal drugs were purchased from local Danish pharmacies, or donated by NaturDrogeriet A/S (Hørning, Denmark) or Finzelberg GmbH and Co (Andernach, Germany), then authenticated by Prof. Steen Honoré Hansen, Department of Pharmaceutics and Analytical Chemistry, University of Copenhagen where the voucher



(C)

Fig. 3: (*Continued*)

specimens were deposited. The various herbal drugs investigated, with details of Latin plant name and batches were summarized in Table 1.

Gallic acid, (±)- catechin hydrate, methyl gallate, chlorogenic acid, ellagic acid were purchased from Sigma-Aldrich (Steinhem, Germany). 2,5-Dihydroxybenzoic acid was home-made. HPLC-grade methanol was purchased from VWR (Leicester, England). Formic acid was obtained from Merck (Darmstadt, Germany). And water was purified by using a Milli Q-water system (Millipore, Billerica, MA, USA)

3.2. Instrumentation and chromatographic conditions

Experiments were carried out using an Agilent 1100 HPLC system consisted of a G1379A on-line degasser, a G1312A binary pump, a G1316A column oven, a G1313A autosampler. Four kinds of detectors were connected by a micro-splitter valve (Upchurch Scientific®, a Unit of INDEX Corporation, Oak Harbor, WA) as shown in Fig. 2. The G1315C diode array detector (DAD) was operated at 210 nm, 254 nm, 280 nm and 320 nm. The G1312A fluorescence detector (FLD) was set at the excitation wavelength 325 nm and emission wavelength 450 nm in the first 17 min, then was transferred to the excitation wavelength 280 nm and emission wavelength 315 nm after 17 min. The digital electrochemical amperometric detector (Antec Leyden, Zoeterwoude, Holand, ADC) was operated at 0.55 V. The G1978A quadrupole MSD was operated with electrospray ionization in the negative mode using the following parameters: capillary voltage 2000 V, nebulizer pressure 60 psig, drying gas flow 8 L•min^{-1}, drying gas temperature 350 °C, vaporize temperature 150 °C. The MS spectra were acquired in full scan mode with a mass scan range from 100 to 400 amu. The split ratio of the eluant was about 30% to MSD and 70% to FLD and ADC.

The separation was achieved using an Agilent ZORBAX Eclipse XDB C_{18} column (100 mm × 3.0 mm, 5 μm) at 35 °C with a binary gradient elution mode of mobile phase A (formic acid and water, 0.1: 100, v/v) and mobile phase B (formic acid and methanol, 0.1: 100, v/v) The program was: 0 min 2% B, 4 min 2% B, 6 min 10% B, 16 min 15% B, 32 min 50% B, 35 min 60% B, 40 min 2% B. They were pumped at a flow rate of 0.3 mL• min^{-1}.

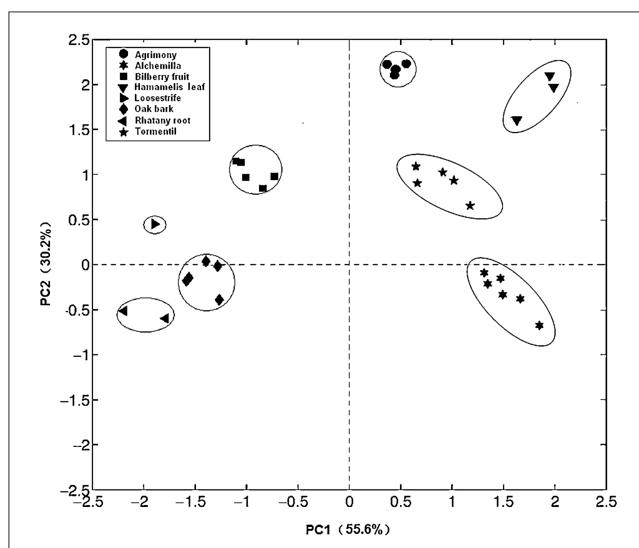


Fig. 4: PCA scores plot of different kinds of tannin-containing herbal drugs

3.3. Preparation of standard solution

Stock solution was prepared in a concentration of 0.5 mg•mL^{-1} in methanol and stored at 4 °C. For the slightly solubility of ellagic acid, it was first dissolved in DMSO, then mixed with other standard substances in methanol. 50% methanol-water solution was used to dilute the stock solution.

3.4. Preparation of sample solution

The plant extraction protocol was modified from the method recorded by the *Ph. Eur.* assay. A certain amount of samples was weighed into a glass tube (Pyrex®, France) and 15 mL water was added together with 150 μL of 0.5 mg•mL^{-1} 2,5-dihydroxybenzoic acid in methanol used as an internal standard. The tube was heated on a water-bath for 30 min, then the aqueous extract was centrifuged at 3000 rpm for 10 min (Hettich-centrifuge EBA-20, Germany). The supernatant was filtered through 0.45 μm syringe filter (Millex®-HV Syringe Driven Filter Unit, Millipore, Ireland) and injected 2 μL.

3.5. Sample analysis and data processing

Altogether, four batches of Agrimony, six batches of *Alchemilla*, five batches of Bilberry fruit, three batches of Hamamelis leaf, one batch of Loosestrife, five batches of Oak bark, two batches of Rhatany root and five batches of Tormentil from various locations were analyzed respectively.

All signals from DAD, FLD, ADC and MS were recorded by Agilent Chemstation (Rev. B.04.01). The data of thirty-one tannin-containing herbal drugs were processed using Unscrambler 9.7 (CAMO Software India Pvt. Ltd., India) by means of PCA.

Table 1: Raw materials used in the experiment

Herbal drug	Source	Batch
Agrimony	*Agrimonia eupatoria* L.	60102026, 60609005, Finzelberg, 81612001
Alchemilla	*Alchemilla vulgaris* L.	51108013, 62607007, Fcc, Fex, 70611008, 81606006
Bilberry fruit	*Vaccinium myrtillus* L.	52111021, 62002020, 60111010, Finzelberg, 82209006
Hamamelis leaf	*Hamamelis virginiana* L.	51705016, 60307021, Finzelberg
Loosestrife	*Lythrum salicaria* L.	01512011
Oak bark	*Quercus robur* L.	60808007, Finzelberg, 32710017, 60404014, 82801005
Rhatany root	*Krameria triandra* Ruiz et Pavon	22809046, Finzelberg
Tormentil	*Potentilla erecta* Raeusch	Rcc, 40912006, 61907010, 52406013, 82807014

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