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Quality control of *Citri reticulatae* pericarpium: Exploratory analysis and discrimination

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ABSTRACT

Extracts of *Citri reticulatae* pericarpium (PCR) are commonly used in the Traditional Chinese Medicine. The quality control of PCR is currently performed by single marker analysis, which can hardly describe the complexity of such natural samples. In this study, a fingerprint methodology for PCR based on high-performance liquid chromatography (HPLC) was developed and validated. A total of 69 fingerprints of authenticated PCR samples, commercial PCR samples, mixed peel samples, and other *Citrus* peels were recorded. Exploratory data analysis allowed optimizing the extraction procedure and detecting mixed peel samples. Once the optimizations were performed and the method validated, discrimination between the authentic PCR samples and all other samples was performed by *p*-Discriminant Partial Least Squares. The established model was able to differentiate between classes with a high reliability for each sample. Furthermore, evaluation of the score and loading plots of the model indicated nobiletin, tangeretin, naringin and hesperidin as important markers for the quality control of PCR.

1. Introduction

Traditional medicine is an ancient medical practice actually still applied. Derived from herbal, animal or mineral sources, traditional medicines still serve up to 80% of the population in several African, Asian and Latin-American countries to meet their primary health care needs. Even in today's Western Society, interest in traditional medicine has increased over the last decades. The population's increased interest and growing economic importance of the industry combined with the lack of legislation, concerned governmental agencies and scientists worldwide. The development of proper identification and quality control criteria for any traditional medicine is of primordial importance to ensure the efficacy of the treatment and the safety of the patient [1–4].

Contrasted to the rational of allopathic medicine which makes use of few well-studied and highly purified chemical compounds, the traditional medicines are complex biological samples containing a high number of mostly unknown substances. Especially amongst the herbal traditional medicines, formulations of several plant species are commonly used, increasing their complexity [5–7]. Moreover, numerous factors, including the climate, harvest time, drying, storage, extraction procedure and adulterations may cause a large variability in the active ingredients and influence the efficacy and safety of the medicine [8–10]. Therefore, proper quality control criteria should be developed.

The identification and quality control of herbal medicines is often performed by qualitative and quantitative analysis of a number of marker compounds [11,12]. However, the use of markers might not always be suitable for the quality control of herbal samples as many herbal species lack unique chemical compounds. Additionally, studying just a few markers does not reflect the complexity of the biological samples and ignores synergic effects between compounds.

To counter the uprising problem of marker identification, the World Health Organization (WHO), the European Medicine Agency (EMA), the American Food and Drug Administration (FDA), and the Chinese State Food and Drug Administration (SFDA) have accepted fingerprint technology as a suitable methodology for the assessment of herbal products [4,13–15]. Analyzing herbal samples with fingerprint technology allows obtaining a characteristic profile, the fingerprint, of the analyzed sample. Nowadays, chromatographic fingerprints have become widely spread and the most commonly used techniques to develop fingerprint profiles include established

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analytical separation techniques, such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) hyphenated to a suitable detector, as well as spectroscopy [16–18].

This paper focuses on the quality control of *Citri reticulatae* pericarpium (PCR), also referred to as '*Chen Pi*' in the Chinese '*pin-yin*' terminology. The samples consist of the dried peel of the mature tangerines of *C. reticulatae* Blanco. So far, PCR has been exploited to normalize the function of the stomach and spleen and to resolve phlegm. The dried peel is officially listed in the Chinese Pharmacopoeia in which hesperidin is chosen as the only marker compound for quality control [19]. This approach may be inadequate for the quality control of PCR as hesperidin occurs in most other *Citrus* species. Over time, many papers on PCR have been published focusing on its biological activities or on the differentiation between species using one or several marker compounds. None considered the use of entire fingerprint profiles for quality control [20–22].

To achieve adequate quality control and discrimination of PCR based on entire fingerprint analysis, a validated HPLC-DAD methodology for PCR fingerprints was developed and combined with data handling techniques. In a first step, the quality and consistency of the extraction procedure was ensured based on the results of Principal Component Analysis (PCA) [23–25]. Additionally, an exploratory data analysis of the authenticated PCR samples was performed and visualized by PCA, Hierarchical Clustering Analysis (HCA) [23,26] and Projection Pursuit (PP) [27]. In the final step, *p*-Discriminant Partial Least Squares (*p*-DPLS) [23–25,28,29] was performed on the entire data set to discriminate the authentic PCR samples from other *Citrus* species. The established model was then used to classify commercially bought PCR samples.

2. Theory

2.1. Data preprocessing

Prior to setting up the data matrix, all fingerprints are corrected for differences in weighted sample mass and subtracted with a blank chromatogram to remove baseline shift. Then, the chromatographic data was organized in an $n \times p$ data matrix **X**, where the n objects (number of herbal samples) constitute the rows and the p variables (measuring time points) the columns. Furthermore, several data preprocessing techniques were applied to the data matrix **X** to enhance the performance, interpretability and visualization of the data handling techniques.

In a first step, the fingerprints were aligned because of retention time shifts occurring between injections caused by instrument variability, column aging and small variations in the mobile phase composition, flow rate and temperature. Correlation Optimized Warping (COW) [30,31] is an available data treatment technique ensuring the alignment of corresponding peaks. By piecewise linear stretching and compression of the fingerprints, each fingerprint is individually aligned with a user-defined target fingerprint. The fingerprints are divided into a number of predefined sections of equal length, which are compressed or stretched by warping, i.e. moving, the position of each section's end point by a limited number of data points. The warped section with the highest correlation coefficient to the corresponding section of the target fingerprint is saved and the aligned fingerprint is constructed based on the combination of all optimally warped sections.

Additional to the alignment, column centering was applied to the data. By averaging the data over each individual column, the mean value over the columns is set to zero. The information concerning the origin is lost, but the distances between the data points remain the same, simplifying the model as the useful information resides in the between-sample variation [23–25].

2.2. Exploratory data analysis

In exploratory data analysis, the user aims to find patterns in the data that could not be derived from the a priori available knowledge of the data, in this case, the fingerprints. Several tools for exploring the data are available. Principal Component Analysis (PCA) [23–25] and Projection Pursuit [27] are variable reduction techniques defining a number of latent variables by making linear combinations of the original variables following a given criterion. In PCA the latent variables are called Principal Components (PCs), while in PP it are Projection Pursuit Features (PPFs). PCs are constructed orthogonally maximizing the description of the largest remaining variation in **X** [23–25]. In PP, informative low dimension spaces are created by optimizing an objective function, the projection index. For this study, the clustering tendency of the samples was evaluated based on the Yenyukov index describing the ratio Q of the mean of all inter-object distances, D, and the average nearest neighbour distance, d. Clustered data will result in a large value for Q [27]. For both methods, the projections of the n objects from the original data space on a latent variable are called the scores on this latent variable. They provide information on the (dis)similarity of the objects. The contribution of each original variable to the score is reflected by its loading. Both score plots and loading plots are useful features for exploratory data analysis visualizing the information residing in **X**.

Hierarchical Clustering Analysis (HCA) utilizes two major strategies for comparing samples. In the agglomerative strategy each observation starts in its own individual cluster and is merged while moving up in the hierarchy, while the divisive strategy starts with all samples in one cluster being split moving down the hierarchy. In order to decide when clusters should be merged or split, a measure of (dis)similarity between the samples is required as well as a linkage criterion specifying the (dis)similarity between the clusters. The (dis)similarity between samples was calculated by the Euclidean distance, which is best at detecting differences, while linkage was performed based on the single linkage method describing the distance between the two closest objects of the clusters [23].

2.3. Discriminant Partial Least Squares

In Discriminant Partial Least Squares (DPLS), a PLS regression model is used to classify samples. It studies the relationship between an $n \times p$ data matrix **X** and an $n \times 1$ response vector **y** indicating if a sample belongs to a particular class ω_c (class 1, ω_1 or class 0, ω_0). Classification of an unknown sample u is derived from its predicted value, $\hat{y}_u$, by the PLS model. As PLS is a regression method, the predicted value will be a real number instead of an integer. Based on $\hat{y}_u$ and a defined cut-off value between 0 and 1, a class is assigned to the unknown sample [23–25]. Usually, the cut-off value is defined arbitrarily. Another approach is assuming that the samples of the training set follow a Gaussian distribution. Hereby a probability density function (PDF) is estimated for each class based on the mean and standard deviation of their prediction [32]. The cut-off value is defined as the value of $\hat{y}$ at which the probability of both classes is equal. Even though the latter method improves the assignment of a cut-off value, it still ignores the uncertainty of PLS predictions and the possible non-Gaussian distribution of the predicted samples within a class.

In this study, the DPLS algorithm used (probabilistic DPLS or *p*-DPLS) does not assume a Gaussian distribution of within-class predictions, takes into account the uncertainty of the predictions, and additionally provides a measure of reliability of the classification [28]. The *p*-DPLS methodology is as follows: