ORIGINAL ARTICLE

Optimization of Extraction Solvent for High-performance Thin-layer Chromatography for detection of adulterants in *Valerianae radix*

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ABSTRACT

Background: Quality control of valerian represents a major concern in the pharmaceutical industry due to the huge variety of these species and their almost daily new scientific findings. **Objective:** The aim of the study was to investigate the sensitivity of FTIR spectroscopy for the detection of adulterated *Valerianae radix* and the optimization of a solvent extraction method for high-performance thin-layer chromatography (HPTLC) fingerprinting. **Material and Methods:** *Radix valerian* medicinal plant adulterant was measured by FTIR spectroscopy. The obtained data were evaluated using SIMCA (soft independent modeling of class analogies) to determine the detection limits for the undesired adulterants when systematically mixing their spectra with a data set comprising 50 batches of valerian root samples and the HPTLC system was utilized to evaluate a series of ultrasound extraction experiments. Optimal solvent composition consisting of methanol, water, and dichloromethane is used for which the flavonoid fingerprint is determined. **Results:** With regard to the chemometric analysis of the FTIR spectra, we found that for this particular dataset, the overall detection rates computed from leave-one-out cross-validation were 56.2%, 85.9%, and 98.3% at 5%, 10%, and 20%contamination levels, respectively. Solvent extraction optimization, we found that a mixture of 40–50% methanol, 30–50% dichloromethane, and 10–20% water was most efficient for the analysis of the flavonoid patterns in the six plant systems under investigation. **Conclusion:** Concerning the extraction optimization, a universal solvent could not be defined exactly by response surface methodologies as reported in similar studies involving different analytical platforms (mostly high-performance liquid chromatography) but suggestions for near-optimal solvent compositions with competitive performances compared to pure and 70% methanol could be made.

Key words: Chemometric, DModX, FTIR, high-performance thin layer chromatography, SIMCA, *Valerianae radix*

INTRODUCTION

Fingerprint searches are becoming very important for the classification and authentication of botanical species.[1] The fingerprint of a herbal medicine is considered a unique chemical signature for its secondary metabolites.[2] Herbal drugs, unlike synthetic drugs, contain usually hundreds of complex secondary metabolites, which are in most cases unknown.[3] Infrared fingerprints of herbal medicines become a point of interest in scientific research; big emphasis has been put on developing an IR fingerprint database and convenient chemometrics software.[4] Many studies have discussed the use of IR in combination with chemometrics in QC of herbal medicine and their adulteration as well as in the food industry.[5] An effective extraction method and a chromatogram with clear separation of the detected secondary metabolites are required for obtaining an informative fingerprint of herbal drugs that are useful, to assess their quality.[6] According to several

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publications, a mixture of three solvents methanol/chloroform and/or acetone/water in different ratios are most effective in extracting both polar and nonpolar metabolites.[7] It is a fact that extraction protocols in the pharmacopeia monographs are very heterogeneous and consequently leads us to the question, of whether one solvent combination can be optimized that is useful to analyze a large number of herbal plants in terms of identity and quality.[8] Nowadays, high-performance thin-layer chromatography (HPTLC) has been involved in many important fields such as QC of pharmaceuticals, cosmetics, food, and the detection of adulteration in herbal drugs.[9] HPTLC is the optimum unification of technology, science, and standardized methodology which offers many advantages such as high sensitivity, visual presentation of the results, equivalent analysis of samples, fast and simple operation, and cost-effectiveness.[10] The continuous development of innovative methods of adulteration is aggravating this dilemma in the quality control processes further. To identify a fingerprint for each plant, different analytical methods have been suggested such as infrared spectroscopy (IR) and HPTLC.[11] Hence, we investigated the detection of adulterated herbal medicines by FTIR spectroscopy and optimization of a solvent extraction method toward the wide coverage of secondary metabolites for HPTLC fingerprinting.[12]

MATERIALS AND METHODS

Preparation of samples

Herbal plants were collected from the K L University campus, Vaddeswaram, Guntur District, Andhra Pradesh, India, and or procured from the local market of Vijayawada, Andhra Pradesh, India. Samples of ground stocks were collected in small-labeled sachets and or made into smoothies without any further preparation and they were directly placed on the crystal surface of the FTIR spectrometer and measured as triplicates.[13]

Spectra acquisition

Spectra were collected using a Bruker Tensor 27 FTIR spectrometer (Bruker, Germany), equipped with a deuterated L-alanine doped triglycine sulfate (TE-DLATGS) detector.[14] Spectra were measured at room temperature, at a resolution of 4cm^{-1}, 16 scans for each sample in a wave number range of 4000 cm^{-1} to 600 cm^{-1}. A background spectrum was scanned before the analysis of each sample to cancel the effect on fair components in the spectrum. After each measurement, the crystal was wiped off with lab soft-tissue wetted by alcohol. Collected spectral data were subjected to a baseline correction (rubber band method) and vector normalization by software named Opus 5.5.[15] The final analysis was accomplished by in-house software Soft Independent Modeling of Class analogy using Matlab 2014b (Mathworks, Natick MA, USA).

Statistical data analysis

Spectra (n = 50) were imported to MATLAB and then divided into 49 training samples and one testing sample for leave-one-out cross-validation. PCA was used to build a model for the training samples (n = 50). The test sample or left-out spectrum was then synthetically adulterated with each of the 10 adulterant spectra in different concentrations ranging from 0% to 50% to detect which concentration is associated with a distance to the model (DModX) beyond the confidence region (critical distance). This procedure was replicated for every adulterant and concentration until every valerian spectrum had been left out from the modeling process once. Fifty valerian roots spectra were subsequently adulterated with each of the ten spectra in eight different concentrations (0–50%) one after the other.[16]

Extraction solvent optimization for HPTLC fingerprinting

Samples were freshly ground and 150 mg of the powdered samples were transferred to a 96-well plate and then mixed

Table 1: Pipetting scheme of a 96-well plate of solvent combinations

R/C	1	2	3	4	5	6	7	8	9	10	11	12
A	1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	1	2	3	4	5	6	7	8	9	10	11	12
D	13	14	15	16	17	18	19	20	21	22	23	24
E	1	2	3	4	5	6	7	8	9	10	11	12
F	13	14	15	16	17	18	19	20	21	22	23	24
G	1	2	3	4	5	6	7	8	9	10	11	12
H	13	14	15	16	17	18	19	20	21	22	23	24

Table 2: List of solvents and their composition in percentage

Solvent ID	Methanol	Water	Dichloromethane
1	100	0	0
2	0	100	0
3	0	0	100
4	40	10	50
5	10	40	50
6	50	40	10
7	60	10	30
8	10	30	60
9	30	60	10
10	80	10	10
11	10	10	80
12	10	80	10

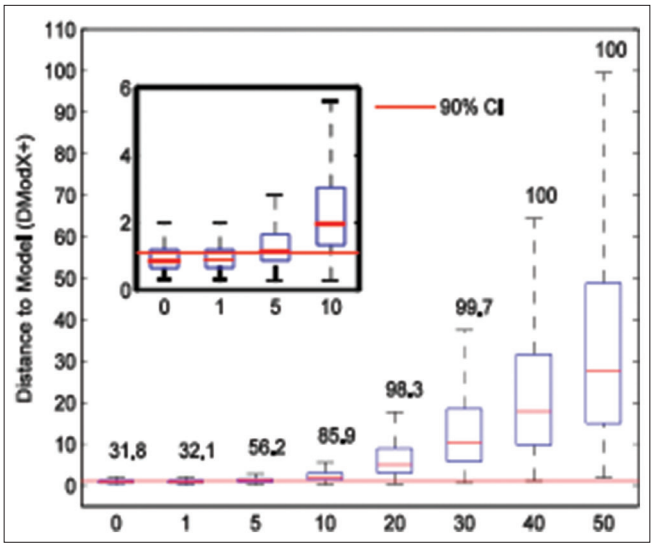

Figure 1: Detection frequency of adulterant samples. Adulterant level on the X-axis versus distance of model plane taking into consideration the orthogonal distance and score space distance on the Y-axis

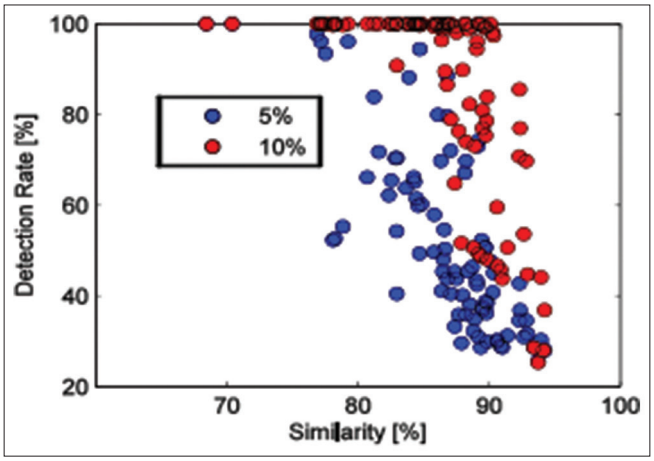

Figure 2: Relation between detection rate (%) and similarity between sample and adulterants at 5% (blue) and 10% (red) contamination level for all ten putative adulterants used in the study

with 1.2 mL solvent according to the Table 1. Samples were well mixed and sonicated for 10 min, then mixed again and sonicated for an additional 10 min. The extracts were then centrifuged at a maximum speed of 4400 rpm at room temperature for 10 min. Supernatants were transferred into a clean well plate and centrifuged again (maximum speed 4400 rpm at room temperature). Finally, supernatants were pipette off in HPLC vials with inlet and subjected to HPTLC analysis.[17]

Extract solvent preparation

We prepared combinations of three solvents of methanol, water, and dichloromethane in different ratios as listed in the Table 2 as modern methods of sample preparation

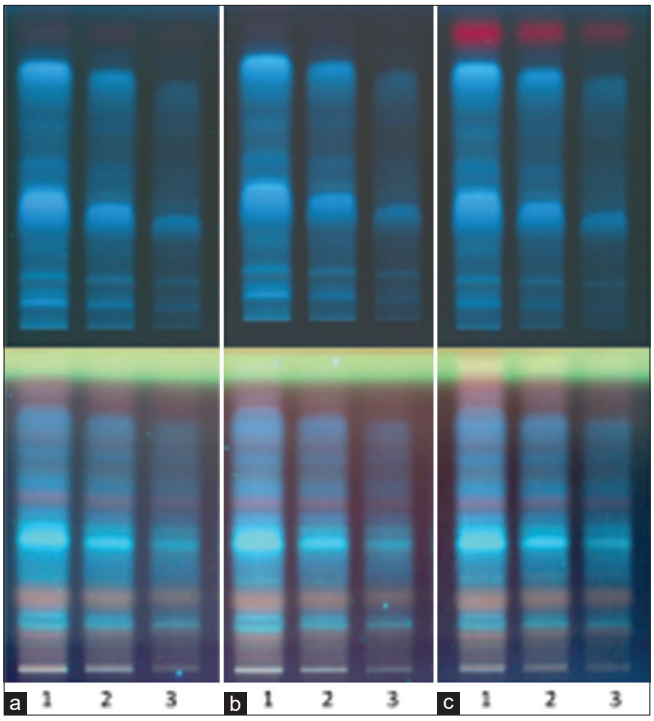

Figure 3: Extract optimization. (a) Samples extracted with 40% methanol before (above) and after (below) derivatization. (b) Samples extracted with 60% methanol before (above) and after (below) derivatization. (c) Samples extracted with 80% methanol before (above) and after (below) derivatization

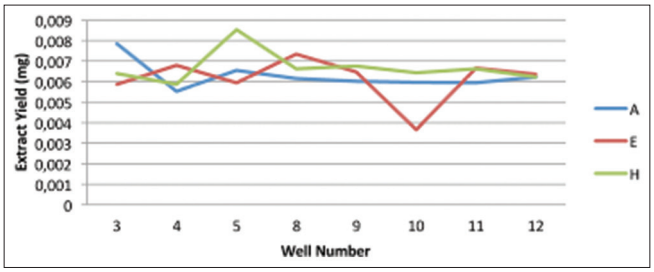

Figure 4: Extraction optimization, extract with 40% methanol

for separation technique. Miscibility of the three solvent mixtures in different proportions was tested before being used and mixtures that were not miscible were excluded from the optimization.[18]

Data analysis

Analysis of each plant (extracted with 12 different solvent combinations) was done using HPTLC on three separate plates on which samples of dilution series and reference samples. A volume of 2 uL from each sample and development was done in an automatic development chamber. For each plate 35 mL mobile phase, consisting of 25 mL for saturation and 10 mL for development, were freshly prepared and filled. After analysis, the plate was taken under UV 254 nm and UV 366 nm light whereby the picture quality was enhanced by high dynamic range mode.[19]

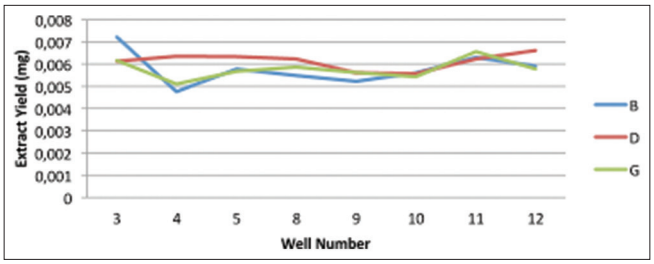

Figure 5: Extraction optimization, extract with 60% methanol

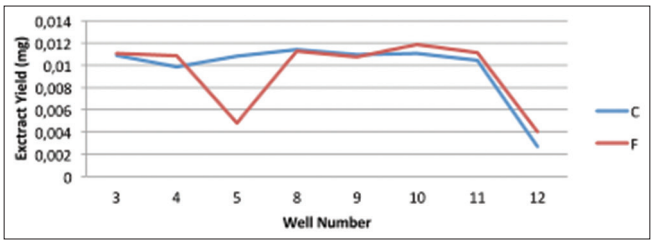

Figure 6: Extraction optimization, extract with 80% methanol

RESULTS

Detection of adulterated herbal medicines by FTIR spectroscopy

Class membership of the test samples can be predicted according to their distance from the class boundary (critical region). An adulterated test sample that lies above the class boundary can be considered successfully detected by the method. Our data indicate that contaminations below 5% are hardly detectable by this method. Only 56.2%of all test samples were correctly predicted as being adulterated using the 90% confidence limit. About 85.9% and 98.3% were correctly identified at 10% and 20% contamination levels [Figure 1]. Comprehensive detection (i.e., 100%) was achieved at 40% and 50% contamination. Our data further indicated that the detection rate (i.e., the probability for detection) is dependent on the similarity between sample and adulterant spectra [Figure 2].

Extraction solvent optimization for HPTLC fingerprinting

After the preparation of the dilution series of three solvent mixtures, extracts were filled in HPLC vials with inlets to carry out an HPTLC analysis [Figure 3]. Retention index shifts appear clearly in samples of concentrations of 25 and 50% shown in the figure the shifts are probably due to overloading of the stationary support [Figures 4-6].

CONCLUSION

Our study focused on addressing the critical issue of quality control in *Valerianae radix*, a natural preparation extensively used for treating insomnia and sleep disturbances. The pharmaceutical industry faces significant challenges due to the extensive variety of valerian species and the constant influx of new scientific findings. We suggest a certain range for extraction solvents, which give quantitative and qualitative better extract yield. However, the exact determination of a universal solvent is still a challenging task, which could not be solved by this method. Unlikely, we failed to find a way to normalize such a great amount of data obtained from different plates and affected by many external factors. Future development of convenient statistical methods would be a valuable support to overcome the difficulties that were defined in this study.

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