Published in final edited form as:

Anal Lett. 2016 March 1; 49(5): 711–722. doi:10.1080/00032719.2015.1045588.

DIFFERENTIATION OF *AURANTII FRUCTUS IMMATURUS* AND *FRUCTUS PONICIRI TRIFOLIATAE IMMATURUS* BY FLOW-INJECTION WITH ULTRAVIOLET SPECTROSCOPIC DETECTION AND PROTON NUCLEAR MAGNETIC RESONANCE USING PARTIAL LEAST-SQUARES DISCRIMINANT ANALYSIS

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Abstract

Two simple fingerprinting methods, flow-injection coupled to ultraviolet spectroscopy and proton nuclear magnetic resonance, were used for discriminating between *Aurantii fructus immaturus* and *Fructus poniciri trifoliatae immaturus*. Both methods were combined with partial least-squares discriminant analysis. In the flow-injection method, four data representations were evaluated: total ultraviolet absorbance chromatograms, averaged ultraviolet spectra, absorbance at 193, 205, 225, and 283 nm, and absorbance at 225 and 283 nm. Prediction rates of 100% were achieved for all data representations by partial least-squares discriminant analysis using leave-one-sample-out cross-validation. The prediction rate for the proton nuclear magnetic resonance data by partial least-squares discriminant analysis with leave-one-sample-out cross-validation was also 100%. A new validation set of data was collected by flow-injection with ultraviolet spectroscopic detection two weeks later and predicted by partial least-squares discriminant analysis models constructed by the initial data representations with no parameter changes. The classification rates were 95% with the total ultraviolet absorbance chromatograms datasets and 100% with the other three datasets. Flow-injection with ultraviolet detection and proton nuclear magnetic resonance are simple, high throughput, and low-cost methods for discrimination studies.

Keywords

flow-injection with ultraviolet spectroscopic detection; proton nuclear magnetic resonance; nutraceutical; partial least-squares discriminant analysis; *Aurantii fructus immaturus*; *Fructus poniciri trifoliatae immaturus*

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INTRODUCTION

Aurantii fructus immaturus is the dried, entire unripe fruit from *Citrus aurantium* L. (also known as the Seville orange or bitter orange) or *Citrus sinensis* Osbeck (sweet orange) (Fan et al., 2012). *Aurantii fructus immaturus* is widely used as a functional food or nutraceutical: in Asia it is a traditional herbal medicine to treat digestive ailments, and in the U.S. many dietary supplements for weight loss contain *Aurantii fructus immaturus* extracts (Fugh-Berman and Myers, 2004). *Aurantii fructus immaturus* contains numerous bioactive compounds, such as alkaloids (i.e., synephrine, octopamine, and tyramine), flavonoid glycosides (i.e., hesperidin, neohesperidin, naringin, and auranetin), and essential oils (i.e., limonene) (Fang et al., 2009). Besides treatment of digestive problems (Fugh-Berman and Myers, 2004, Fang et al., 2009), *Aurantii fructus immaturus* has other pharmacological properties including anticarcinogenic (Satoh et al., 1996), hemodynamic (Fugh-Berman and Myers, 2004), and P-glycoprotein inhibitory effects (Yoshida et al., 2005).

Fructus poniciri trifoliatae immaturus is the dried ripe fruit of the *Poncirus trifoliata* Raf. (also called trifoliolate orange or Chinese Bitter Orange), which is a close relative of citrus and was brought to North America as an ornamental plant (Soneji and Rao, 2011). Unlike *Aurantii fructus immaturus*, *Fructus poniciri trifoliatae immaturus* is inedible in the US, but in China, *Fructus poniciri trifoliatae immaturus* is used as a medicine to relieve congestion and obstruction by phlegm in the chest (Chuang et al., 2007b). *Fructus poniciri trifoliatae immaturus* has appeared as a counterfeit *Aurantii fructus immaturus* product in China (Xie, 1991).

Differentiation between *Aurantii fructus immaturus* and *Fructus poniciri trifoliatae immaturus* is important for authentication and quality assurance that maintains the safety and traceability of *Aurantii fructus immaturus* and/or *Fructus poniciri trifoliatae immaturus* related medications and dietary supplements. However, identification and authentication of botanical materials is a challenging task because of their complex chemistry. The most common method is to use their morphological characteristics (Rao, et al., 2011) which requires professional expertise but is also subject to human error. Also, if the botanical material is available as a powder, then morphological identification is impossible. Chromatographic fingerprint methods based on high-performance liquid chromatography (HPLC) techniques with ultraviolet spectroscopic detectors (Ding et al., 2007, Chen et al., 2012, Xu et al., 2010, Chuang, et al., 2007b, a) have been reported, which require long separation times in excess of sixty minutes and the methods are instrumentation-dependent with varying profiles for the same samples. HPLC coupled with mass spectrometry was reported for the determination of flavonoids, alkaloid compounds (Ding et al., 2007), and coumarins (Chen et al., 2012) in citrus materials, but are time-consuming, expensive, and require expertise to operate and maintain. Proton nuclear magnetic resonance (¹H-NMR) spectroscopy and flow-injection with ultraviolet spectroscopic detection are good alternatives. Both NMR and ultraviolet spectroscopic detectors are very stable thereby providing greater accuracy, reproducibility, and reliability (Betteridge, 1978, Krishnan, et al., 2005, Lenz et al., 2002). Other advantages of flow-injection coupled to ultraviolet spectroscopy are exceptional throughput (two minutes per sample analysis) and low cost.

In this study, flow-injection coupled to ultraviolet spectroscopy and $^1\text{H-NMR}$ were used for the discrimination of *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* for the first time. Models were built using classification models from datasets initially acquired to classify data collected two weeks later with no parametric changes to the procedure or classification models, which demonstrate the methods' robustness with respect to time. The prediction rates were 97.6% for models built from the flow-injection coupled to ultraviolet spectroscopy data and 95.0% for models built from well-plate reader data. For the $^1\text{H-NMR}$ method, a partial least-squares discriminant analysis model was constructed and validated using leave-one-sample-out cross-validation (Harrington et al., 2009).

EXPERIMENTAL

Reagents and Materials

Nine *Aurantii fructus immaturus* samples and thirteen *Fructus ponciri trifoliatae immaturus* samples were furnished by Professor Yuan-Shiun Chang from the School of Pharmacy at the China Medical University. All the samples were taxonomically authenticated in China. Acetonitrile and methanol were Optima grade from Fisher Scientific, Pittsburgh, PA, USA. Formic acid was MS grade from Sigma/Aldrich, St. Louis, MO, USA. Water was produced by a Thermo Barnstead Nanopure Life Science UV/UF purification system. Dimethyl sulfoxide- d_6 was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Disuccinimidylsuberate was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polyvinylidene fluoride syringe filters (17-mm i.d., 0.45 μm pore size) were purchased from VWR Scientific, Seattle, WA, USA.

Instrumentation

The HPLC system consists of an Accela 1250 binary pump, a Pal-Htc-Accela autosampler, an Accela 1250 photodiode array detector, and an Agilent column compartment (G1316A). The mobile phase was comprised of 0.1% formic acid in H_2O and 0.1% formic acid in acetonitrile with isocratic elution at 50:50 (v:v) at a flow rate of 0.5 mL/min for 1.0 min. An IEC Clinical Centrifuge (Damon/IEC Division, Needham Heights, MA, USA) was used for sample extraction. $^1\text{H-NMR}$ spectra were recorded at 289 K on a Bruker 600-MHz Avance III NMR spectrometer (600.13 MHz proton frequency).

All data were processed using Matlab R2014a (Math Works Inc., Natick, MA). All calculations were performed on an Intel Core i7 2.93 GHz personal computer with 12 GB Ram running a Microsoft Windows 8 Enterprise x64 operation system (Microsoft Corp., Redmond, WA).

Sample Preparations

One-half gram of each dried ground sample was mixed with 10.0 mL of 1:1 (v/v) methanol:water in a 15-mL centrifuge tube and sonicated for sixty minutes at room temperature. The samples were centrifuged at 5,000 g for fifteen minutes and passed through a polyvinylidene fluoride syringe filter. For the flow-injection with ultraviolet spectroscopic detection, the supernatant was diluted one-hundred fold with 1:1 (v/v) methanol:water and 5

μL were introduced for analysis. All analyses were completed within twenty-four hours of the extraction to minimize potential degradation of the chemical compounds.

For NMR analysis, 4 mL of supernatant of each sample extract was dried completely, dissolved in 1 mL of dimethyl sulfoxide-d₆ (containing 0.3 mM of disuccinimidyl suberate), and then transferred into a tube for analysis.

Data Processing

For flow-injection with ultraviolet spectroscopic detection, the averaged ultraviolet absorbance from 190 to 400 nm and 0 to 2 minutes and the total absorbance chromatogram of ultraviolet spectra from 0 to 2 minutes were exported to Excel (Microsoft, Inc., Bellevue, WA, US) and read directly into Matlab. The Accela data acquisition software was incapable of exporting the two-way data arrays for each measurement. Each total ultraviolet absorbance chromatogram comprised 2401 data points and each averaged ultraviolet spectrum included 211 data points. A total of 110 flow-injection with ultraviolet detection measurements (9 *Aurantii fructus immaturus* samples and 13 *Fructus ponciri trifoliatae immaturus* samples analyzed five times) were collected. All data were pretreated by normalizing to unit vector length (Zhang and Harrington, 2013).

For NMR, each spectrum consisted of 32 scans requiring 2.5 min acquisition time with the following parameters: 0.18 Hz/point, pulse width of 30° (12.7 μs) and relaxation delay of 5.0 s. ¹H-NMR spectra were processed using Mestrenova (version 5.2.5, Mestrelab Research, Santiago de Compostella, Spain). The resulting spectra were manually phase corrected, baseline corrected, and calibrated to disuccinimidyl suberate at 0.00 ppm. Spectral intensities were binned from δ 0 to 10 ppm with a 0.04 ppm width for each integrated region and then normalized so that the sum of the intensities was unity. The region of δ 4.7–5.0 ppm was excluded from the analysis because of the residual signal of water. The dimethyl sulfoxide-d₆ solvent peak was also removed from δ 2.4 to 2.6 ppm. Each ¹H-NMR data matrix consisted of 239 data points, and a total of 22 ¹H-NMR spectra (9 *Aurantii fructus immaturus* samples and 13 *Fructus ponciri trifoliatae immaturus* samples) were obtained.

Partial Least-Squares Discrimination

Partial least-squares discrimination is useful when the number of observations is small with respect to the number of variables for classification (Blanco-Gomis, et al., 1998). A binary answer (assigning a value of 1 to *Aurantii fructus immaturus* and 2 to *Fructus ponciri trifoliatae immaturus*) was established and multivariate regression between the criterions and the latent variables was carried out. The number of latent variables that yielded the lowest average prediction error for the training dataset was determined using an internal boot-strapped Latin partition (Zhang and Harrington, 2013).

RESULTS AND DISCUSSION

Differentiation of Plant Species by Flow-Injection

Typical averaged ultraviolet spectra for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* are shown in Fig. 1. *Aurantii fructus immaturus* extracts have

relatively higher absorbance values at 225 nm and 283 nm compared to *Fructus ponciri trifoliatae immaturus*. Fig. 2 is the principal component score plot obtained from the averaged ultraviolet spectra data of *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus*. The *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* scores were well separated. Fig. 3 shows the variable loadings for first principal component from the ultraviolet spectral dataset. The loadings indicate that the ultraviolet absorbances in the ranges centered at 193, 205, 255, and 283 nm contributed to the separation between *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* scores.

Typical total ultraviolet absorbance chromatograms for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* are given in Fig. 4. In this experiment, a guard column was used which offered only mild separation or virtual chromatography. The *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* chromatographic profiles were significantly different. The principal component analysis score plot in Fig. 5 indicates that the separation was adequate for the discrimination of *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus*.

Partial least-squares discriminant analysis models were constructed from averaged ultraviolet spectra and total ultraviolet absorbance chromatographic datasets. Each sample was measured five times using a random block design. A cross-validation method that leaves out one sample (five measurements) out was used to evaluate the model. This procedure was repeated so that each sample was used once for prediction and the results were pooled. The spectra and the chromatographic profiles yielded 100% prediction rates. The ultraviolet absorbance at 193, 205, 255, and 283 nm were selected to construct a reduced dataset comprising four variables. Similarly, partial least-squares discriminant analysis model and leave one sample out yielded a 100% prediction rate. According to the previous studies, the optimal wavelengths for the analysis of the major active biological constituents in citrus herbs such as synephrine, naringin and hesperidin were around 255 and 283 nm (Wu et al., 2013, Ding et al., 2007). Considering the ultraviolet absorbance at 193 and 205 nm may be influenced by many substances, a further reduced dataset comprised of two variables was constructed by selecting the ultraviolet absorbance at 255 and 283 nm, and a 100% prediction rate was achieved by the partial least-squares discriminant analysis model.

Discrimination of Plant Species by ¹H-NMR

The ¹H-NMR spectra for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* are provided in Fig. 6. Some obvious differences may be observed. For example, the *Fructus ponciri trifoliatae immaturus* spectra had a unique chemical shift peak at 2.3 ppm and six peaks between 3 and 4 ppm with greater intensities instead of four major peaks as in the *Aurantii fructus immaturus* spectra. These features contribute more to the separation of the two classes than the principal component analysis scores (Fig. 7). Fig. 8 is the loading plot for the first component of the NMR data. Several peaks, at 2.3, 3-4, 5.2, 5.6, 6, 6.1, 6.9, 7, and 7.4 ppm, contributed to the separation between *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* samples. Similarly, the partial least-squares

discriminant analysis model was constructed and evaluated from the $^1\text{H-NMR}$ dataset and the prediction rate also was 100% using leave-one- sample-out cross validation.

Classification of Flow-Injection for Blind Samples

Nine *Aurantii fructus immaturus* extracts and thirteen *Fructus ponciri trifoliatae immaturus* extracts were freshly prepared two weeks after the initial flow-injection with ultraviolet spectroscopic detection and analyzed. Each new preparation of each new sample was analyzed five times. The new flow-injection with ultraviolet spectroscopic detection data were treated as blind unknowns and classified by partial least-squares discriminant analysis like the initial dataset. The ultraviolet absorbance at four wavelengths (193, 205, 225, and 283 nm) or two wavelengths (225 and 283 nm) were selected to build new datasets.

The partial least-squares discriminant analysis models were constructed using the total ultraviolet absorbance chromatograms, averaged ultraviolet spectra, or reduced ultraviolet absorbance datasets collected earlier (training sets) and used to predict the validation data collected two weeks later. The numbers of latent variables used by partial least-squares were 9, 3, 3, and 2 when using total ultraviolet absorbance chromatograms, averaged ultraviolet spectra, and two reduced ultraviolet absorbance datasets. The prediction rate by partial least-squares discriminant analysis using total ultraviolet absorbance chromatograms was 95%; the prediction rate using averaged ultraviolet spectra was 100%; and the prediction rates using reduced ultraviolet absorbance data at four or two wavelengths were 100%. Therefore, the method using flow-injection with ultraviolet spectroscopic detection and partial least-squares discriminant analysis for classifying *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* was robust when averaged ultraviolet spectra or reduced ultraviolet absorbance at 193, 205, 225, and 283 nm or reduced ultraviolet absorbance at 225 and 283 nm data representations were used.

As illustrated in Fig. 9, larger variations are observed for *Fructus ponciri trifoliatae immaturus* using total ultraviolet absorbance chromatogram data (i.e., group D in Fig. 9B). More outlier scores (i.e., samples outside of the 95% confidence intervals ellipse) were found using the total ultraviolet absorbance chromatographic data, especially for *Fructus ponciri trifoliatae immaturus*. Because the scores of *Aurantii fructus immaturus* using either averaged ultraviolet spectra or total ultraviolet absorbance chromatogram datasets were centered and the *Aurantii fructus immaturus* validation scores corresponded well to the scores of the training data, the inconsistencies of the *Fructus ponciri trifoliatae immaturus* samples cannot be caused by instrumental drift that occurred between the new and old data collections, but may be from the variations in the *Fructus ponciri trifoliatae immaturus* samples. The results indicate the storage conditions may have been improper for *Fructus ponciri trifoliatae immaturus*. Although large variations were also observed in the *Fructus ponciri trifoliatae immaturus* validation set using the 4-wavelength (Fig. 9C) dataset, *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* scores were well separated, and a 100% prediction rate was achieved. Relatively small variations were observed among the samples for the two wavelength dataset (Fig. 9D). These results may be because the absorbance values at 225 and 283 nm were not as variable as those at 193 and 205 nm.

CONCLUSIONS

Aurantii fructus immaturus and *Fructus ponciri trifoliatae immaturus* were differentiated by flow-injection with ultraviolet spectroscopic detection and NMR. Flow-injection with ultraviolet spectroscopic detection was faster and employs less expensive instrumentation. Both methods provided reliable discrimination among samples when coupled to a version of partial least-squares discriminant analysis that automatically determines the number of latent variables. Furthermore, the ultraviolet spectral profiles of the fruits were sufficiently different by using absorbance measured at 225 and 283 nm and a prediction rate of 100% was obtained with a model built from the initial set for the validation set that was collected two weeks later. These results demonstrate the robustness of flow-injection with ultraviolet spectroscopic detection.

ACKNOWLEDGMENT

This research was supported by the Agricultural Research Service of the U.S. Department of Agriculture and an Interagency Agreement with the Office of Dietary Supplements of the National Institutes of Health of the U.S. Health and Human Services.

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Fig. 1. Ultraviolet spectra for (A) *Aurantii fructus immaturus* ($n = 45$) and (B) *Fructus ponciri trifoliatae immaturus* ($n = 65$).

Fig. 2. Principal component analysis score plot of ultraviolet spectra for *Aurantii fructus immaturus* and *Fructus poniciri trifoliatae immaturus*. The 95% confidence intervals are represented by ellipses.

Fig. 3. Loading for the first component of ultraviolet spectra and averaged ultraviolet spectra of *Aurantii fructus immaturus* and *Fructus poniciri trifoliatae immaturus*.

Fig. 4. Total absorbance chromatograms of (A) *Aurantii fructus immaturus* ($n = 9$) and (B) *Fructus ponciri trifoliatae immaturus* ($n = 13$).

Fig. 5. Principal component analysis score plot of total absorbance ultraviolet spectra for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus*. The 95% confidence intervals are represented by ellipses.

Fig. 6. NMR spectra for (A) *Aurantii fructus immaturus* and (B) *Fructus ponciri trifoliatae immaturus*.

Fig. 7. Principal component analysis score plot of NMR spectra for (A) *Aurantii fructus immaturus* and (B) *Fructus ponciri trifoliatae immaturus*. The 95% confidence intervals are represented by ellipses.

Fig. 8. Loading for first component of the NMR spectra plotted with the average *Fructus ponciri trifoliatae immaturus* spectrum and with the average of *Aurantii fructus immaturus* reflected around the origin for visualization.

Fig. 9.

Principal component analysis score plots of (A) ultraviolet spectra for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus*, (B) total absorbance ultraviolet spectra for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus*, (C) reduced ultraviolet absorbance at 193, 205, 225, and 283 nm for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus*, and (D) bivariate plot of reduced ultraviolet absorbance at 225 and 283 nm for *Aurantii fructus immaturus* and *Fructus ponciri trifoliatae immaturus* (D). The 95% confidence intervals are represented by ellipses. In the PCA score plots, 'A' is the training set of *Aurantii fructus immaturus*; 'B' is the training set

of *Fructus ponciri trifoliatae immaturus*; 'C' is the validation set of *Aurantii fructus immaturus*; and 'D' is the validation set of *Fructus ponciri trifoliatae immaturus*.

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