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Distinguishing *Smilax glabra* and *Smilax china* rhizomes by flow-injection mass spectrometry combined with principal component analysis

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Flow-injection mass spectrometry (FIMS) coupled with a chemometric method is proposed in this study to profile and distinguish between rhizomes of *Smilax glabra* (*S. glabra*) and *Smilax china* (*S. china*). The proposed method employed an electrospray-time-of-flight MS. The MS fingerprints were analyzed using principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) with the aid of SIMCA software. Findings showed that the two kinds of samples perfectly fell into their own classes. Further predictive study showed desirable predictability and the tested samples were successfully and reliably identified. The study demonstrated that the proposed method could serve as a powerful tool for distinguishing between *S. glabra* and *S. china*.

Keywords: Smilax glabra, *Smilax china*, rhizome, flow injection, mass spectrometry, PCA, OPLS-DA

Rhizomes of both *Smilax glabra* Roxb. (*S. glabra*) and *Smilax china* L. (*S. china*) are included in *Chinese Pharmacopeia* (1). Deriving from the same genus, rhizomes of both *S. glabra* and *S. china* share some similarities. They are both rich in resveratrol (2, 3) and look alike. In fact, they are two different species used as two medicines in clinical practice. There have been some studies so far on how to distinguish between them (4–7); nevertheless, these studies usually involved high or ultra-high performance liquid chromatography (HPLC or UHPLC) coupled with ultraviolet (UV) detection, or microscopic approaches (4, 5). Focusing on quantitation of several specific constituents or physical features, the reported methods were useful when it came to identifying and comparing specific components or visible identification, but they were also time-consuming and occasionally subjective. HPLC-based methods often take 30 min or even longer for such a distinguishing analysis. It is hence important to apply a rapid and robust approach to improve the existing ones, which, in this research, is flow injection mass spectrometry (FIMS) coupled with statistical methods, principal component analysis (PCA) and orthogonal partial least

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squares discriminant analysis (OPLS-DA). FIMS is quite an effective approach to maximize analytical throughput yet allow analyte discrimination in complex samples such as plant material. The major goal of this study was to distinguish between *S. china* and *S. glabra* in a rapid and simple way, so no analytical column was involved and the features of the flow injection method enabled this goal to be safely achieved.

The major edge of processing MS fingerprints by PCA is that it can greatly simplify the whole distinguishing procedure. Furthermore, the results can be analyzed visually when massive raw data are involved (8). This unsupervised method provides visually friendly patterns that are statistics-based, objective and reproducible (9). OPLS is a recently improved modification of the PLS method (10–12). As far as we know, there are no reports on distinguishing these two crude drugs by this approach.

Smilax glabra Roxb. and *Smilax china* L. are distributed over several provinces in China. *S. glabra* can modulate immunity, protect against liver injury, lower blood glucose and suppress cancer (13). Chemical constituents of *S. glabra*, as we know, are rather complex, the major ones considered pharmacologically effective are flavones, stilbenes, and phenylpropanoids, which are generally targeted constituents in quantification or qualification research (14, 15). Recent studies have reported that *S. china* L. can dispel inflammation, expel winddamp and induce urination, making it a different medicine from *S. glabra*. Chemically, there are big differences between these two plants: the major constituents of *S. china* are steroidal saponins (16–18). The two plants are similar in appearance, which leads to confusion. Given the mentioned situation, this work proposes a FIMS method, a relatively new analytical technique (19), to distinguish rapidly and reliably between the two groups of samples.

EXPERIMENTAL

Plant material and chemicals

S. glabra (SG) and *S. china* (SC) samples tested in the study were purchased in several provinces nationwide. The collected samples were authenticated by Xue-wen Lai, Jiangxi University of Traditional Chinese Medicine (JXUTCM, Nanchang, China). Voucher specimens are preserved in the Key Laboratory of Modern Preparation of Traditional Chinese Medicine of the Ministry of Education, JXUTCM.

Methanol of HPLC grade was purchased from Merck (Germany) and formic acid of HPLC grade was purchased from Xilong Chemical (China). Water was obtained using a Milli-Q Advantage A10 (Millipore, USA). Mobile phase consisted of 0.1 % formic acid in a mixture of water and methanol (50:50, *V/V*).

Sample preparation

Samples were ground into fine powders (0.841-mm sieve) using an analytical mill (Global Works, China). Ten mg of each sample powder was mixed with 5 mL of a mixture of methanol and water (70:30, *V/V*) in a 15-mL centrifuge tube, and then sonicated for 60 min at room temperature. Thereafter, it was centrifuged for 10 min at 5000 rpm (Centrifuge 5430 R, Eppendorf, Germany). Finally, the supernatant was diluted 10 times with methanol and filtered through a 0.22-μm filter prior to analysis. All samples were analyzed within 24 h after preparation to avoid any possible errors caused by degradation over time.

Flow injection-qTOF MS

The analytical procedure was performed on a platform consisting of a LTQ Orbitrap Velos Pro (Thermo Fisher Scientific Corporation, USA) for mass spectrometry and an Ultimate 3000 Rapid Separation LC (RSLC) system (Thermo Fisher Scientific Corporation) for UPLC. Flow injection was carried out through a guard column (Adsorbosphere All-Guard Cartridge, C18, 5 μm, 4.6 × 7.5 mm, Alltech Associates, USA) to minimize potential contamination of the MS system. Mobile phase consisted of 0.1 % formic acid in a water-methanol mixture (50:50, *V*/*V*) with isocratic elution at a flow rate of 0.3 mL min^{-1} for 2 min. Experiments were conducted using an electrospray ion (ESI) source in the negative ion mode. Mass spectra were recorded in high resolution and MS scan mode from *m*/*z* 120 to 1500 to obtain FIMS fingerprints. The following conditions were applied to the mass spectrometer: source temperature was set to 300 °C, capillary temperature was set to 320 °C, ion spray voltage was 3.5 kV with sheath gas of 275.8 kPa and auxiliary gas of 34.5 kPa. Each sample was measured with FIMS five times; analyses of 71 different samples thus provided 355 MS fingerprints in one continuous sequence.

Data processing

The MS data of each sample consisted of a matrix (ion counts *versus* mass-to-charge ratio over a range of 120–1500). The massive MS data obtained were handled with PCA (10, 20–22), placing the classification results on a sound statistical ground. Data of all samples were exported to Excel (Version 2013, 64-bit, Microsoft Corporation, USA) for pre-processing, and were then analyzed by statistical software SIMCA (Version 14.1.0.2047, 64-bit, MKS Umetrics, Umea, Sweden) for PCA and OPLS-DA. OPLS-DA separates the systematic variation in X into two parts; one part is correlated (predictive) to Y and one part is uncorrelated (orthogonal) to Y, which gives improved model interpretability. OPLS-DA was designed to address the differences between the two classes. This feature thus allowed it to serve as an ideal tool to achieve our goal. S-plot is an easy way to visualize an OPLS discriminant analysis model of two classes. It has been mainly used to filter out putative biomarkers from omics data (21, 22). It provides visualization of the OPLS/OPLS-DA predictive component loading to facilitate model interpretation.

The pre-processing by Excel involved combining spectra, sorting data, removing unnecessary or redundant information (such as headers, sample information and instrument information) and aligning masses (each spectrum is different in length given that all ions did not appear in each spectrum). Once pre-processing was done, the matrix was ready to be imported into SIMCA for further chemometric analysis. The scale type applied was Par (one of the data scaling options provided by SIMCA prior to the analyzing process of PCA) and four principal components were carefully selected; R2X (parameter of PCA model) of 0.68 was obtained.

RESULTS AND DISCUSSION

FIMS fingerprints of S. glabra *and* S. china

The outcome of FIMS fingerprinting is shown in Fig. 1. All acquisitions managed to be finished within 2 min for both kinds of crude drugs. Differences between the two spe-

cies were fairly clear visually. The most notable ions for *S. china* were at *m*/*z* 191 and 371, ion 191 dominated the *S. china* spectrum, which was a major difference compared to *S. glabra*. Ions for *S. glabra* were at *m*/*z* 449, 485, 433, with ion 449 dominating the whole spec-

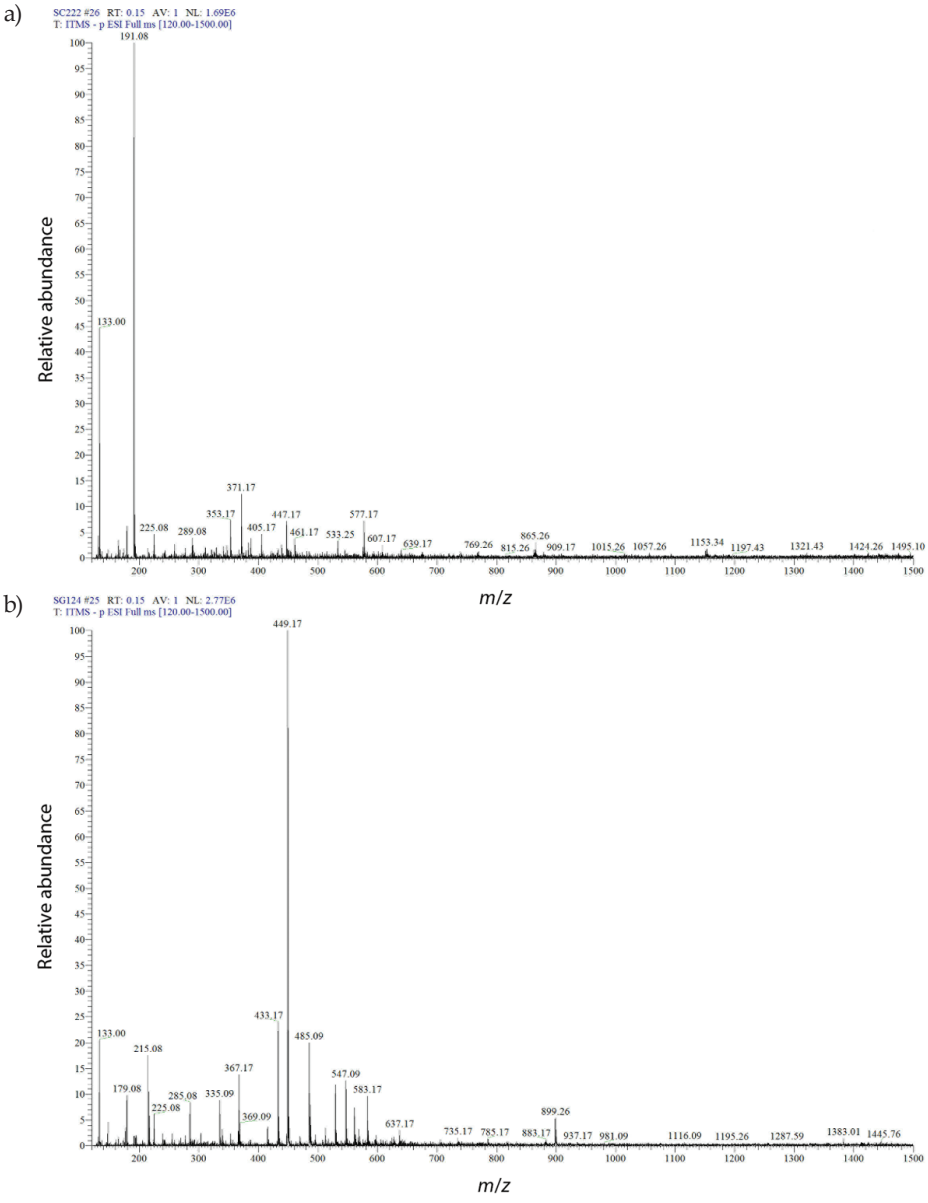

Fig. 1. Typical FIMS spectra of: a) *Smilax china* and b) *Smilax glabra*.

trum. On the basis of the foregoing, ions 191 and 449 were presumed to play significant roles in biological activities. Next, statistical methods PCA and OPLS-DA were employed to verify the validity of this hypothesis.

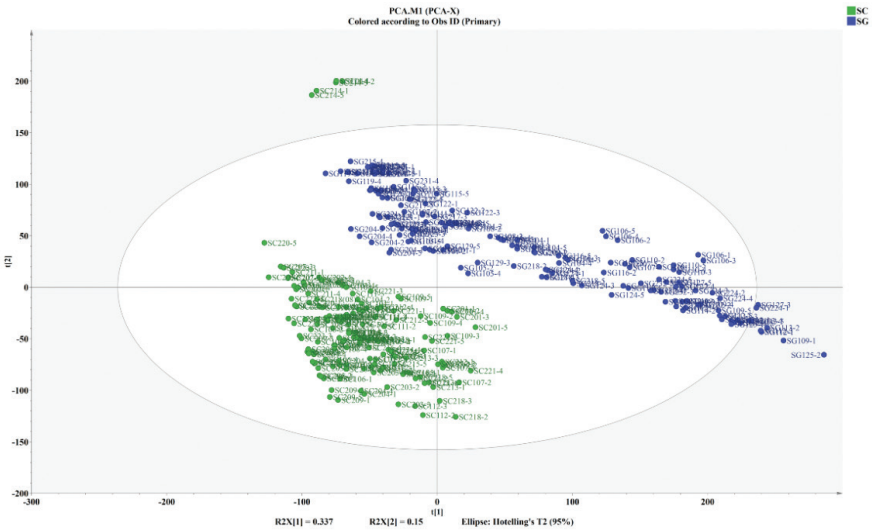

Fig. 2. PCA results for *Smilax china* (SC) and *Smilax glabra* (SG) samples.

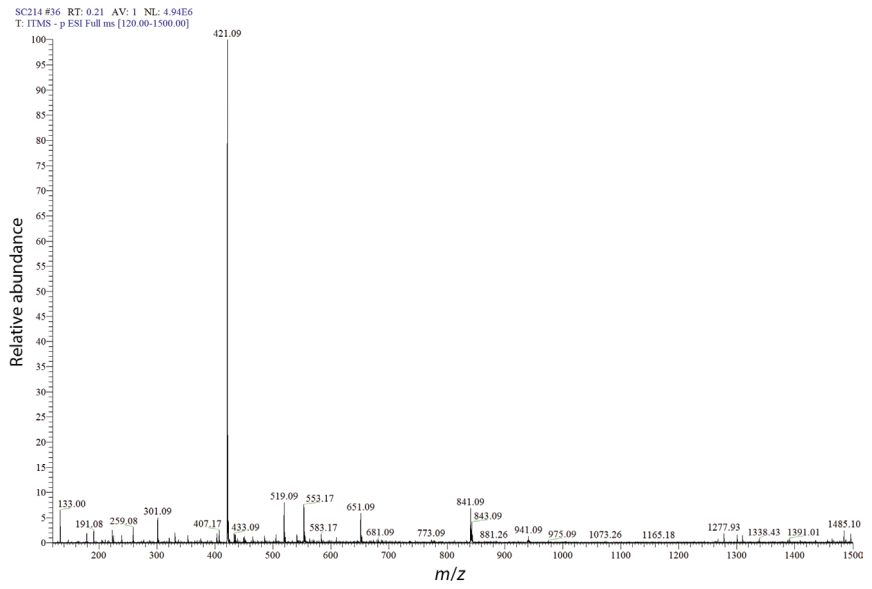

Fig. 3. FIMS spectrum of SC214.

PCA and OPLS-DA of FIMS fingerprints

Fig. 2 shows the PCA results for *S. glabra* and *S. china* samples. In this figure, green spots stand for *S. china* and the blue ones stand for *S. glabra*. This figure indicated that these two crude drugs were perfectly divided into two clusters with an outlier, SC214 (upper

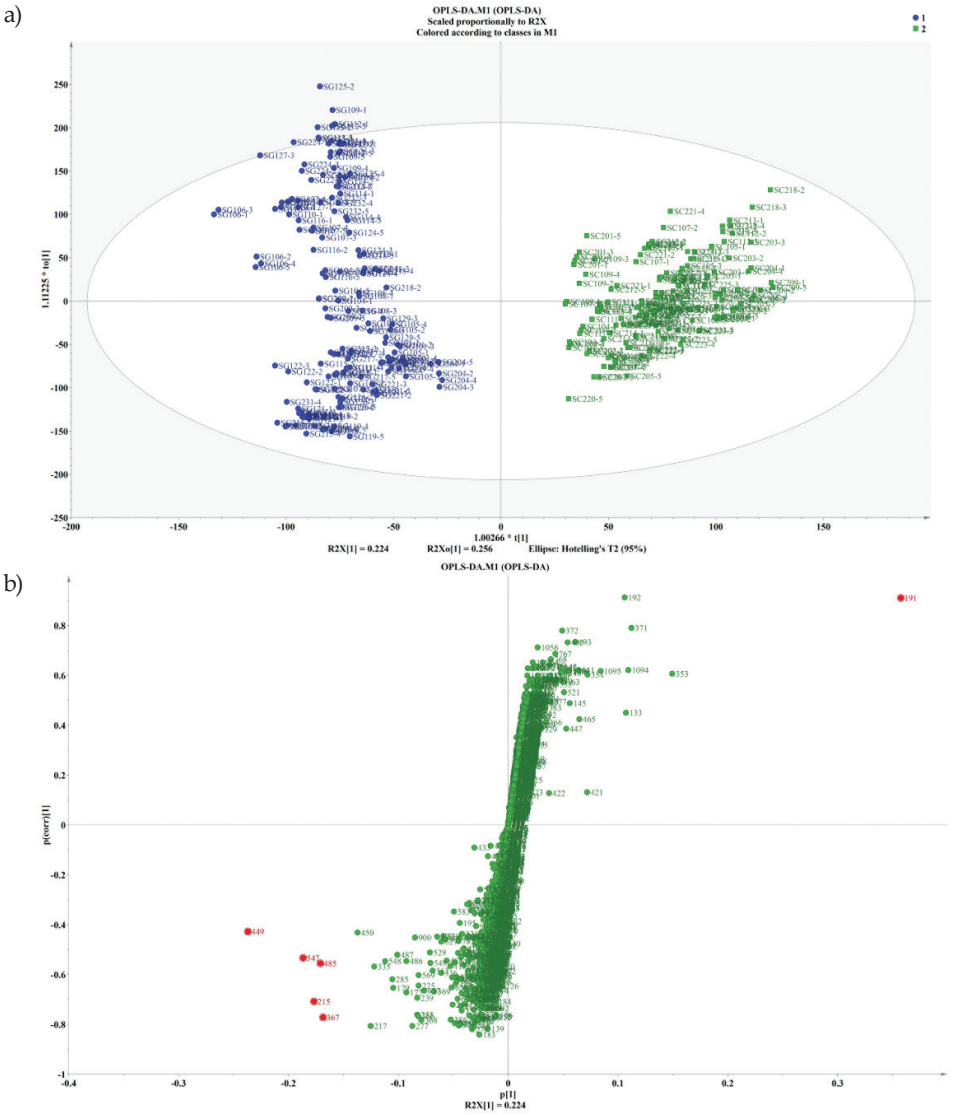

Fig. 4. OPLS-DA results for *Smilax china* (SC) and *Smilax glabra* (SG) samples: a) score plot of OPLS-DA, b) S-plot of OPLS-DA.

left). A closer inspection of the spectrum of sample SC214 was hence taken and the outcome showed that ion 421 dominated the spectrum but no other ions were the same as those of *S. china* or *S. glabra* (Fig. 3). This suggested that SC214 was most likely a fake *S. china* sample. A physical investigation later verified this hypothesis.

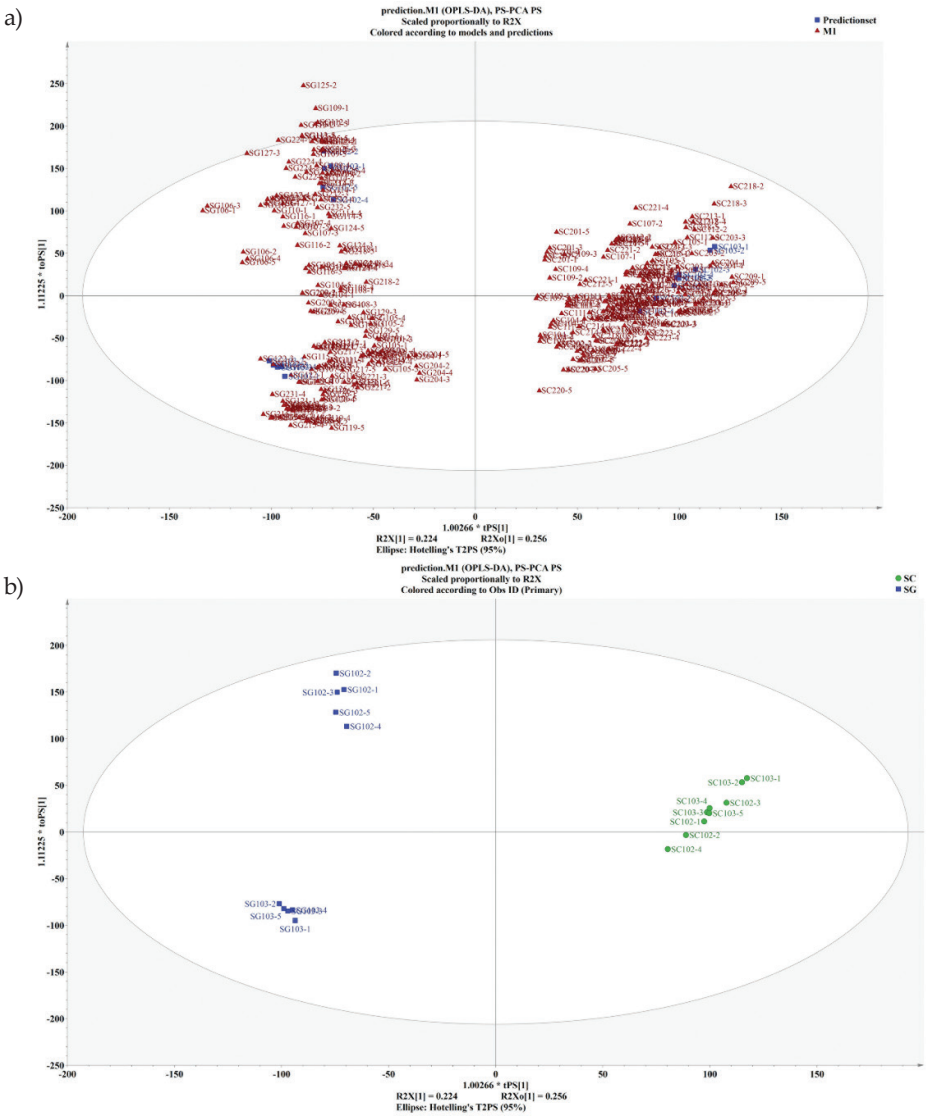

Fig. 5. Prediction results for known samples: a) score plot of prediction for all samples, b) score plot of prediction for tested samples. SC – *Smilax china* samples, SG – *Smilax glabra* samples.

To further identify the variables responsible for the discrimination, OPLS-DA was introduced. After performing OPLS-DA, a high predictive ability parameter Q2(Y) of 0.92 was obtained. Removing the outlier sample SC214, the score plot in Fig. 4a indicated that the two crude drugs were perfectly divided into two clusters with no mixed samples.

Fig. 4b is an S-plot to show how much each of the variables, that is, ions, contributes to classification by its distance to the origin. The most significant variables are therefore always situated far out on the wings of the "S" shape combining the high model influence with high reliability and are of relevance in the search for class differences. In this particular S-plot, it was obvious that some dozens of variables were relevant; ions at *m/z* 449 and 191 (marked red) sitting in the two wings stood out undoubtedly, which verified our previous hypothesis. It is worth noting that if more characteristic ions were required, ions at *m/z* 547, 215, 485 and 367 were all good options. In-depth research on these distinguishing ions is already underway.

Prediction

Prediction of a specific set is a special function of SIMCA to investigate if the developed model is good for predicting new samples using the current prediction set. In this work, four more known samples, two for each class, were introduced in the prediction for a test run. Results in Fig. 5a show that the new imported samples (blue) blended into the two existing classes (magenta). In Fig. 5b, the classes from previous OPLS-DA results were removed for closer inspection. As expected, the prediction outcome was quite good: all *S. glabra* samples were in the left, being right in previous *S. glabra* classes, and so were the two *S. china* samples. This suggested that the developed model was good and capable of predicting new unknown *S. glabra* or *S. china* samples.

CONCLUSIONS

This study addressed a FIMS method combined with multivariate analysis to be applied for distinguishing between *S. glabra* and *S. china* in a simple, fast and reliable manner. The proposed method suggests a model system in which FIMS fingerprinting is used to analyze a considerable number of samples fairly quickly. Hence, it is particularly helpful when crude drugs are in no condition for physical identification, such as powdered raw drugs and products containing a single medicine, or when large amounts of samples are involved. Characteristic ions found in this work, which are responsible for the classification of *S. glabra* and *S. china*, are worth further exploring in a next-step study.

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