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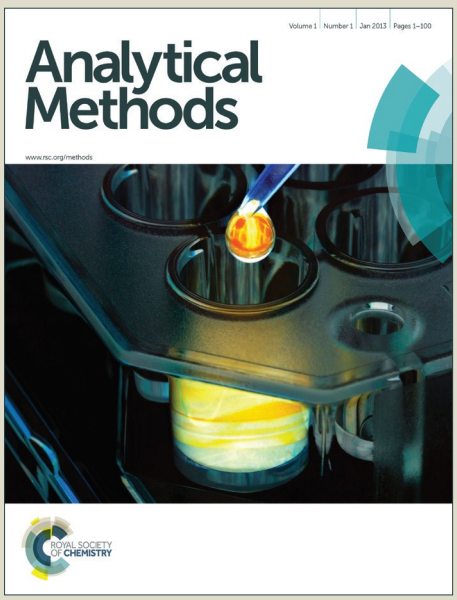

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ARTICLE

Rapid assessing the quality of *Qingkailing* products using wooden-tip electrospray ionization mass spectrometry combined with multivariate statistical analysis†

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This study demonstrates the application of wooden-tip electrospray ionization mass spectrometry (wooden-tip ESI-MS) combined with multivariate statistical analysis for achieving rapid quality assessment of *Qingkailing* products, a famous combinatorial herbal formula registered by *Chinese Pharmacopoeia*. By utilizing sharp wooden tips to load samples and induce ESI for mass spectrometric analysis, various ingredients with different chemical structures present in *Qingkailing* products were detected simultaneously without any sample pretreatment with analysis speed of approximately 1 min per sample. There were 26 characteristic peaks in the negative ion wooden-tip ESI–MS fingerprint of *Qingkailing*, and they were successfully identified by collision-induced dissociation experiments. The developed method was applied to evaluate the quality and trace the origins of 16 batches of *Qingkailing* oral liquid and 9 batches of *Qingkailing* granule samples. By using multivariate statistical analysis on the obtained MS fingerprints, different *Qingkailing* samples were successfully differentiated, and their quality stability and consistency were also easily assessed. In addition, the qualified and damaged samples were distinguished unambiguously. Our results demonstrated that the combined application of wooden-tip ESI-MS and multivariate statistical analysis could be a rapid strategy for pharmaceutical analysis, with promising prospects for tracing the origins and assessing the quality of herbal medicines and related products.

Introduction

Qingkailing is a famous combinatorial herbal formula consisted of eight medicinal materials (i.e., *Concha Margaritifera, Fructus Gardenise, Cornu Bubali, Radix Isatidis, Baicalinum, Flos Lonicerae,Cholic acid and hyodeoxycholic acid*) and reported to have excellent curative effects on circulation system disease, phlogistic disease, virosis, and some inexplicalbe fever.[1-5] Previous studies have revealed that *Qingkailing* formula contains active ingredients including bile acids, amino acids, nucleotides, organic acids, flavonoids, and iridoid glycosides, etc.[1-5] However, the contents of these active ingredients present in different *Qingkailing* products might vary significantly due to many cases such as variations in raw materials from different cultivation areas and climatic conditions as well as differences in production procedures by different manufacturers, which leads to fluctuations in quality and therapeutic efficacy. Hence, it's imperative to seek an efficient and comprehensive method for quality assessment and control of *Qingkailing* products.

In the past two decades, a few methodologies have been developed for investigating *Qingkailing* products as well as assessing and controlling their quality. Screening and identification of the active ingredients in *Qingkailing* injection was reported by using high performance liquid chromatography (HPLC) coupled with time-of-flight mass spectrometry (TOF-MS) and HPLC coupled with ion trap mass spectrometry (IT-MS).[1] Moreover, an off-line comprehensive two-dimensional (2D) HPLC-MS method was proposed by Ma *et al.*[2] for investigating the constituents in *Qingkailing* injection. To achieve quality control of *Qingkailing* injection, quantitative determination of cholic acid, deoxycholic acid, and chenodeoxycholic acid by HPLC-MS/MS was developed by Liu *et al.*[3] *Qingkailing* exerts its therapeutic effects through multi-components of its eight medicinal materials on multi-targets. Therefore, its overall quality could not be represented by only three marker compounds. For systematically and comprehensively controlling the quality of *Qingkailing* injection, a method of fingerprinting analysis combined with seven-compounds determination by HPLC coupled with evaporative light scattering detection (ELSD) was developed by Cao *et al*,[4] and a 2D HPLC fingerprinting method was developed by Yan *et al*.[5] However, these chromatography-based methods are tedious because the time-consuming steps of sample pretreatment and chromatographic separation must be included, and thus, they are difficult to meet the requirements of speediness and high-throughput in quality control of *Qingkailing* products. With the increased consumption of *Qingkailing* products, there is an urgent need to develop rapid and reliable methodologies for their quality control.

Benefited from the high sensitivity and specificity, MS becomes one of the premier methodologies for rapid analysis.

The rapid development of ambient ionization techniques[6-8] allows MS to carry out direct analysis for complex samples under ambient and open-air condition with minimal or no sample pretreatment. Since the introduction of desorption electrospray ionization (DESI)[9-11] by Cooks and his co-workers, a series of ambient ionization techniques such as direct analysis in real time (DART),[12-14] desorption atmospheric pressure chemical ionization (DAPCI),[15-17] electrospray laser desorption/ionization (ELDI),[18-20] extractive electrospray ionization (EESI),[21-23] laser ablation electrospray ionization (LAESI),[24-27] dielectric discharge barrier ionization (DBDI),[28] low-temperature plasma (LTP),[29-31] and desorption corona beam ionization (DCBI),[32, 33] etc., are playing increasingly important role in analytical practices. Among these ambient ionization techniques, a number of methods are based on electrospray ionization (ESI) mechanism and utilized various solid substrates to load samples and induce ESI for direct mass spectrometric analysis. These ambient ionization methods are summarized as solid-substrate ESI.[34] Representative solid-substrate ESI methods, which use a sharp solid as substrate to load sample and induce ESI，include direct electrospray probe (DEP),[35-39] probe electrospray ionization (PESI),[40-45] paper spray,[46-51] wooden-tip ESI,[52-56] pipette-tip ESI,[57, 58] aluminum foil ESI,[59] and touch spray,[60] etc. Among them, wooden-tip ESI is one relatively newly developed method that applies wooden tips as solid substrates for loading samples and ionization of analytes for direct mass spectrometric analysis. Compared with other solid-substrate ESI methods, wooden-tip ESI possesses the following advantages: 1) wooden tips are hard, slim, and easy for sampling in many cases; 2) the hydrophilic and porous surfaces of wooden tipss allows more effective adhesion of different forms of samples on the tip surface; 3) the narrow-stick shapes of wooden tips avoid rapid diffusion and vaporization of solvents, which enables longer signal durations; and 4) the disposable use of wooden tips avoids cross-contamination and memory effects of samples. Till now, wooden-tip ESI has been applied for the analysis of a wide variety of samples, including illicit drugs in raw urine[53], raw plant materials,[54] and pharmaceuticals,[55] etc.

In this study, wooden-tip ESI-MS combined with multivariate statistical analysis method was developed and employed to rapidly assess the quality stability and consistency of *Qingkailing* products as well as the difference between the qualified and damaged samples.

Experimental

Materials and reagents

Wooden toothpicks for the preparation of wooden tips were made of birch wood and purchased from a PARKnSHOP supermarket in Hong Kong. HPLC grade of methanol was supplied by Burdick & Jackson (Muskegon, MI, USA).

Twelve reference substances, i.e., 3-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, baicalein, baicalin, caffeic acid, cholic acid, deoxycholic acid, gluconic acid，malic acid, quinic acid, wogonin, and wogonoside were purchased from the National Research Center for Certified Reference Materials of China (Beijing, China).

Sixteen batches of *Qingkailing* oral liquid samples and nine batches of *Qingkailing* granule samples were purchased from different drugstores in Guangzhou, and all of them were made by companies that passed the authentication of Good Manufacturing Practice.

A damage experiment was performed by heating a sample at 70 ℃ for 30 days. An oral liquid sample was stored in an airtight bottle with a rubber plug. Before the damage experiment, the rubber plug of each sample was punctured to form a small hole for releasing the pressure. The samples of group A, i.e., 12 batches of Baiyunshan oral liquid samples, were applied for the damage experiment.

Wooden-tip ESI-MS analysis

Wooden-tip ESI-MS experiments were performed in a way similar to previous literatures.[52-56] Before sample loading, wooden tips were prepared by cutting the wooden toothpicks into a length of about 2 cm and sharping their tip-ends to the diameters of 0.15~0.20 mm. To load oral liquid samples, wooden tips were dipped into sample solution for about 10 s. While for loading granule samples, wooden tips were first prewetted with MeOH/H_2O (1/1), and then scraped with the sample until a layer of sample was adhered onto the tip surface. After sample loading, one loaded wooden tip was held by a copper clip and mounted onto a three-dimensional (3D) moving stage, and the tip-end was placed pointing to the MS inlet and adjusted to a position of 10 mm away from the MS inlet. An optimized high voltage was applied to the wooden tip through the copper clip. Then, some spray solvent was pipetted onto the tip surface for wooden-tip ESI–MS analysis. The total analysis time for a sample was approximately 1 min including sample loading.

Mass spectrometry

Mass spectrometric analysis was performed on a Trap XCT IT mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The ionization source was set up using the corresponding ESI configuration. Mass spectra were recorded in the range of *m/z* 50–1000. In order to identify the compounds, collision-induced dissociation (CID) experiments were performed to the interested precursor ions with an isolation window of 2.0 *m/z* units. The experimental control and data acquisition were conducted by the MSD Trap Control 5.2 software (Agilent Technologies, Santa Clara, CA, USA).

Multivariate statistical analysis

Each *Qingkailing* sample was analyzed three times by the developed wooden-tip ESI-MS method, and the average mass spectrum generated from the three paralleled measurements was regarded as the sample's fingerprint. The ASCII information of *Qingkailing* samples' fingerprints was converted into a Microsoft Excel (Microsoft Corp., Redmond, WA, USA) file, and the file was then transferred into SIMCA-P (11.5 Umetrics, Umea, Sweden) for multivariate statistical analysis. Principal component analysis (PCA), a well-known multivariate analysis approach, was applied for the study. The principle of PCA involves a mathematical procedure for the transformation of multiply correlated variables into a smaller number of uncorrelated variables called principal components (PCs). The relative abundances of each mass spectral peak were used as variables, and the samples were used as observations. The datasets were mean-centered scaled prior to modeling.

Results and discussion

Optimization of the experimental parameters and wooden-tip ESI-MS fingerprint of *Qingkailing*

Firstly, the experimental parameters of wooden-tip ESI-MS were optimized to obtain reliable and desirable mass spectra of *Qingkailing* products. The initial experiment was performed to investigate the positive and negative ion detection modes. As reported by previous studies,[1-5] the major active ingredients present in *Qingkailing* formula are bile acids, amino acids, nucleotides, organic acids, flavonoids, and iridoid glycosides, etc. These compounds are easily deprotonated and show their $[M-H]^-$ signals in the negative ion detection mode (Fig. 1a). While in the positive ion spectrum (Fig. 1b), abundance signals from glucose (*m/z* 181 $[M+H]^+$, *m/z* 203 $[M+Na]^+$, and *m/z* 219 $[M+K]^+$) and sucrose (*m/z* 365 $[M+Na]^+$, *m/z* 381 $[M+K]^+$, and *m/z* 707 $[2M+Na]^+$) were observed, and the signals from active ingredients were strongly suppressed. Thus, mass spectra from negative ion wooden-tip ESI-MS are more suitable for fingerprint analysis of *Qingkailing* products than those from positive ion wooden-tip ESI-MS.

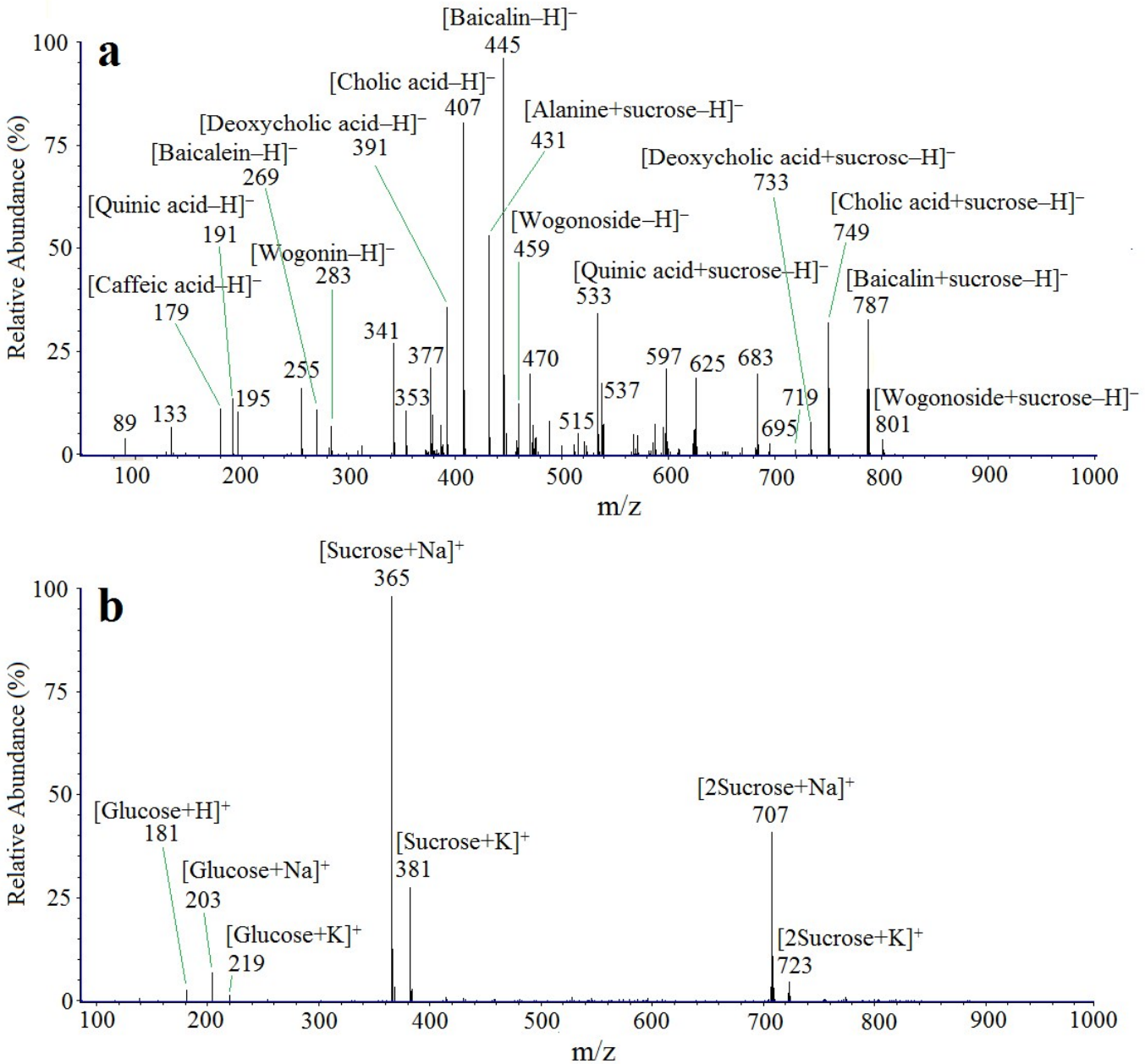

Fig. 1 Representative wooden-tip ESI mass spectra of one *Qingkailing* oral liquid sample with (a) negative and (b) positive ion detection mode.

Other parameters, including the high voltage, the distance between the tip-end and the MS inlet, and the type and amount of spray solvent, were all optimized by employing univariate designs. Results showed that a high voltage of –3.5 kV, a distance of 10 mm between the tip-end and the MS inlet, and a spray solvent of 5 μL methanol gave desirable sensitivity for the analysis.

As shown in Fig. 1a, the characteristic peaks in the negative ion wooden-tip ESI–MS fingerprint of *Qingkailing* include the ions at *m/z* 89, 133, 179, 191, 195, 269, 283, 341, 353, 377, 391, 407, 431, 445, 459, 515, 533, 537, 625, 683, 695, 719, 733, 749, 787, and 801. The identification of these ions was successfully achieved via CID experiments, by comparing their fragmental ions with those of reference substances as well as previous reported literature,[1] and the results were summarized in Table 1. For example, the ion at *m/z* 445 was identified as the signal of deprotonated baicalin, which yielded a fragment peak at *m/z* 269 via CID, suggesting the loss of a dehydrated glucuronic acid. The ion at *m/z* 407 was deduced as negatively charged cholic acid, which produced fragmental ions at *m/z* 389, 371, and 343, involving the loss of H_2O, $2H_2O$, and [$2H_2O$+CO], respectively. Ion at *m/z* 391 yielding ionic fragments at *m/z* 373, 355, 345, and 327 by the loss of H_2O, $2H_2O$, [H_2O+CO], and [$2H_2O$+CO], respectively, was identified as deprotonated deoxycholic acid.

Table 1 Characteristic MS peaks observed in negative ion wooden-tip ESI mass spectra and their fragment ions by CID experiments as well as the forms and origins of signals.

Peak (*m/z*)	CID fragment ions (*m/z*)	Form of signal	Origin of signal
89	45	$[M–H]^-$	alanine
133	115	$[M–H]^-$	malic acid*
179	161	$[M–H]^-$	caffeic acid*
191	173, 127, 111	$[M–H]^-$	quinic acid*
195	179, 135	$[M–H]^-$	gluconic acid*
269	251, 179, 89	$[M–H]^-$	baicalein*
283	268	$[M–H]^-$	wogonin*
341	179	$[M–H]^-$	sucrose
353	191, 179, 173	$[M–H]^-$	3-*O*-caffeoylquinic acid*; 4-*O*-caffeoylquinic acid; 5-*O*-caffeoylquinic acid
377	341	$[M+Cl]^-$	sucrose
391	373, 355, 345, 327	$[M–H]^-$	deoxycholic acid*; hypodeoxycholic acid; chenodexycholic acid
407	389, 371, 343	$[M–H]^-$	cholic acid*; hyocholic acid
431	341	$[M+sucrose–H]^-$	alanine
445	269	$[M–H]^-$	baicalin*
459	283, 268	$[M–H]^-$	wogonoside*
515	353, 173	$[M–H]^-$	3,4-di-*O*-caffeoylquinic acid; 4,5-di-*O*-caffeoylquinic acid; 3,5-di-*O*-caffeoylquinic acid*
533	191	$[M+sucrose–H]^-$	quinic acid
537	195	$[M+sucrose–H]^-$	gluconic acid
625	445	$[M+glucose–H]^-$	baicalin
683	341	$[2M–H]^-$	sucrose
695	353	$[M+sucrose–H]^-$	3-*O*-caffeoylquinic acid; 4-*O*-caffeoylquinic acid; 5-*O*-caffeoylquinic acid
719	377	$[2M+Cl]^-$	sucrose
733	683, 391, 341	$[M+sucrose–H]^-$	deoxycholic acid; hypodeoxycholic acid; chenodexycholic acid
749	407, 341	$[M+sucrose–H]^-$	cholic acid; hyocholic acid
787	445	$[M+sucrose–H]^-$	baicalin
801	459	$[M+sucrose–H]^-$	wogonoside

*signals identified by compared with the authentic compounds.

Many of active ingredients showed both deprotonated molecules and sucrose/glucose adductive signals in the mass spectral fingerprints because sucrose and glucose existed in both *Qingkailing* oral liquids and granules as additives with high contents. For instance, the $[M–H]^-$ and $[M+sucrose–H]^-$ signals of alanine were observed in the spectrum with *m/z* 89 and 431, respectively. The signals of $[M–H]^-$ and $[M+sucrose–H]^-$ for quinic acid were at *m/z* 191 and 533, respectively. The $[M–H]^-$ and $[M+sucrose–H]^-$ signals of gluconic acid were at *m/z* 195 and 537, respectively. Deoxycholic acid showed $[M–H]^-$ and $[M+sucrose–H]^-$ signals at *m/z* 391 and 733, respectively. Cholic acid showed the signals of $[M–H]^-$ and $[M+sucrose–H]^-$ at *m/z* 407 and 749, respectively. Baicalin showed abundant signals at *m/z* 445, 625, and 787, which represented $[M–H]^-$, $[M+glucose–H]^-$, and $[M+sucrose–H]^-$, respectively. In addition, abundant signals from sucrose, e.g. *m/z* 341 $[M–H]^-$, *m/z* 377 $[M+Cl]^-$, *m/z* 683 $[2M–H]^-$, and *m/z* 719 $[2M+Cl]^-$, were observed even in negative ion detection mode. All of these ions were also confirmed by their CID experiments.

Method validation

In general, the repeatability and reproducibility of a MS-based analytical method is validated by the parameter of relative abundance of mass spectral peaks (RAMSP).[51, 56, 61] For calculation of the RAMSP values, a reference mass spectral peak should be chosen firstly. In this study, a peak at *m/z* value of 445 (which is derived from baicalin) was selected as the reference mass spectral peak for calculating the RAMSP value for each mass spectral peak because it shows maximum abundance in most investigated samples' fingerprints. The RAMSP value of each peak was obtained by comparing its abundance of mass spectral peak (AMSP) with that of baicalin (*m/z*=445), using the formula: $RAMSP=AMSP_{m/z}/AMSP_{m/z=445}$. Peaks with RAMSP value of no less than 0.1 (i.e., relative abundances of no less than 10%) were selected to validate the method repeatability and reproducibility by calculating their relative standard deviations (RSDs).

To validate the method repeatability, one *Qingkailing* oral liquid sample was analyzed for six times continuously within a day, and the RSDs of the selective peaks' RAMSP values obtained from the six different measurements were less than 6.1% (n=6). For the validation of the method reproducibility, the experiment was performed at 4 different days (i.e., day 1, day 2, day 3, and day 6) by using one *Qingkailing* oral liquid sample with six paralleled measurements on each day. The RSDs of RAMSP values of mass spectral peaks with relative abundances >10% were less than 11.6% (n=24). These results are acceptable for fingerprinting analysis using an ambient MS method without any sample pretreatment.

Assessing quality and tracing origins of *Qingkailing* products

The developed wooden-tip ESI-MS method was subsequently applied to analyze 12 batches of Baiyunshan (samples A) and 4 batches of Shanxitaihang (samples B) *Qingkailing* oral liquid and 9 batches of Baiyunshan (samples C) *Qingkailing* granule samples. The obtained MS fingerprints (Fig. S1, ESI†) were applied for PCA analysis to assess their quality consistency and trace their origins. In this study, the relative abundance of each peak was used as variable to observe the variation of the investigated samples. After dimensionality reduction, three factors were extracted. The PC1 eigenvalue was 14.10 with a contribution of 56.6%, accounting for most of the variance. The PC2 eigenvalue was 4.12 with a contribution

of 16.5%, and the PC3 eigenvalue was 1.54 with a contribution of 6.16%. The first three PCs, accounting for 79.2% of the total variance, were selected for modeling.

The assessment of quality stability and consistency plays an important role in pharmaceutical quality control, and this process can be easily achieved via PCA score plot. The 2D PCA score plot of the 25 investigated *Qingkailing* samples are shown in Fig. 2. The data points of A and B groups showed tighter clusters in PCA score plot than those of C group did, which demonstrated the better quality stability and consistency of the *Qingkailing* oral liquid samples compared to the granule samples.

Differentiation of the origin are also critical for pharmaceutical quality control. As shown in Fig. 2, the data points of PCA score plot were clearly separated into three groups. The clustering trend unambiguously illustrated the quality differences among these groups. Thus, tracing the origins of different samples were readily achieved.

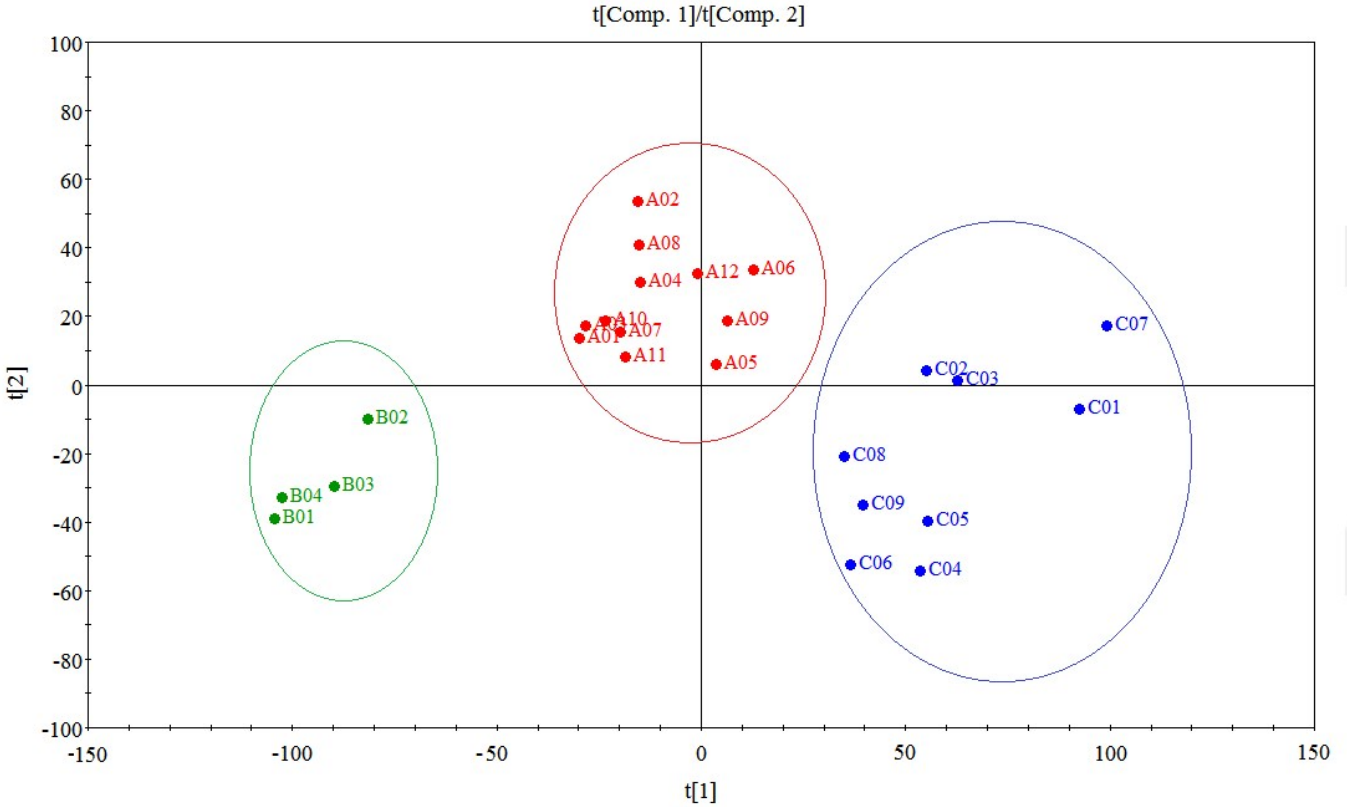

Fig. 2 2D PCA score plot of investigated sixteen batches of *Qingkailing* oral liquid samples and nine batches of *Qingkailing* granule samples.

Distinguishing the qualified and damaged samples

Distinguishing the damaged pharmaceutical samples from qualified samples plays also an important role in the pharmaceutical quality control. To demonstrate such discrimination power of wooden-tip ESI-MS method, samples of group A (12 batches of Baiyunshan oral liquid samples) were damaged by heating at 70 ℃ for 30 days. Both the qualified and damaged samples of group A were analyzed by our developed wooden-tip ESI-MS, and the obtained MS fingerprints (Fig. S2, ESI†) were used for PCA analysis. Three factors were extracted for modeling after dimensionality reduction. The PC1 eigenvalue was 15.80 with a contribution of 65.9%, the PC2 eigenvalue was 3.28 with a contribution of 13.7%, and the PC3 eigenvalue was 1.54 with a contribution of 6.41%.

In the obtained 2D PCA score plot (Fig. 3a), the data points of qualified and damaged samples were clearly separated into two groups, demonstrating the unambiguously discrimination of damaged samples from qualified samples. The data points of qualified samples, which showed a tight cluster in PCA score plot, exhibited the desirable quality stability and consistency of qualified Baiyunshan *Qingkailing* oral liquid. The data points of damaged samples are located far from those of qualified samples, indicating significant changes in the quality of the damaged samples. It is easily observed that the data points from damaged samples were much more discrete than those from qualified samples, indicating that the quality of damaged samples is unstable and inconsistent.

The 2D PCA loadings plot was utilized to recognize the mass spectral markers accounting for the classification of qualified and damaged groups. The data points with large loading values are considered to be the markers that can be attributed to the classification of the samples as reflected in the score plots. As shown in Fig. 3b, the preferential distribution of major markers are the ions at *m/z* 533 (0.954), 445 (0.698), 407 (0.673), 537 (0.362), and 391 (0.351). The ions at *m/z* 445, 407, and 391, signals of baicalin, cholic acid, and deoxycholic acid, respectively, showed great positive correlation to qualified samples, which demonstrated the contents of these active ingredients in qualified samples were significant higher than those in damaged samples as well as their decomposition in

damaged samples. The ions at *m/z* 533 and 537, signals of quinic acid and gluconic acid, respectively, showed great positive correlation with damaged samples, which demonstrated the production of thses compounds in damaged *Qingkailing* samples.

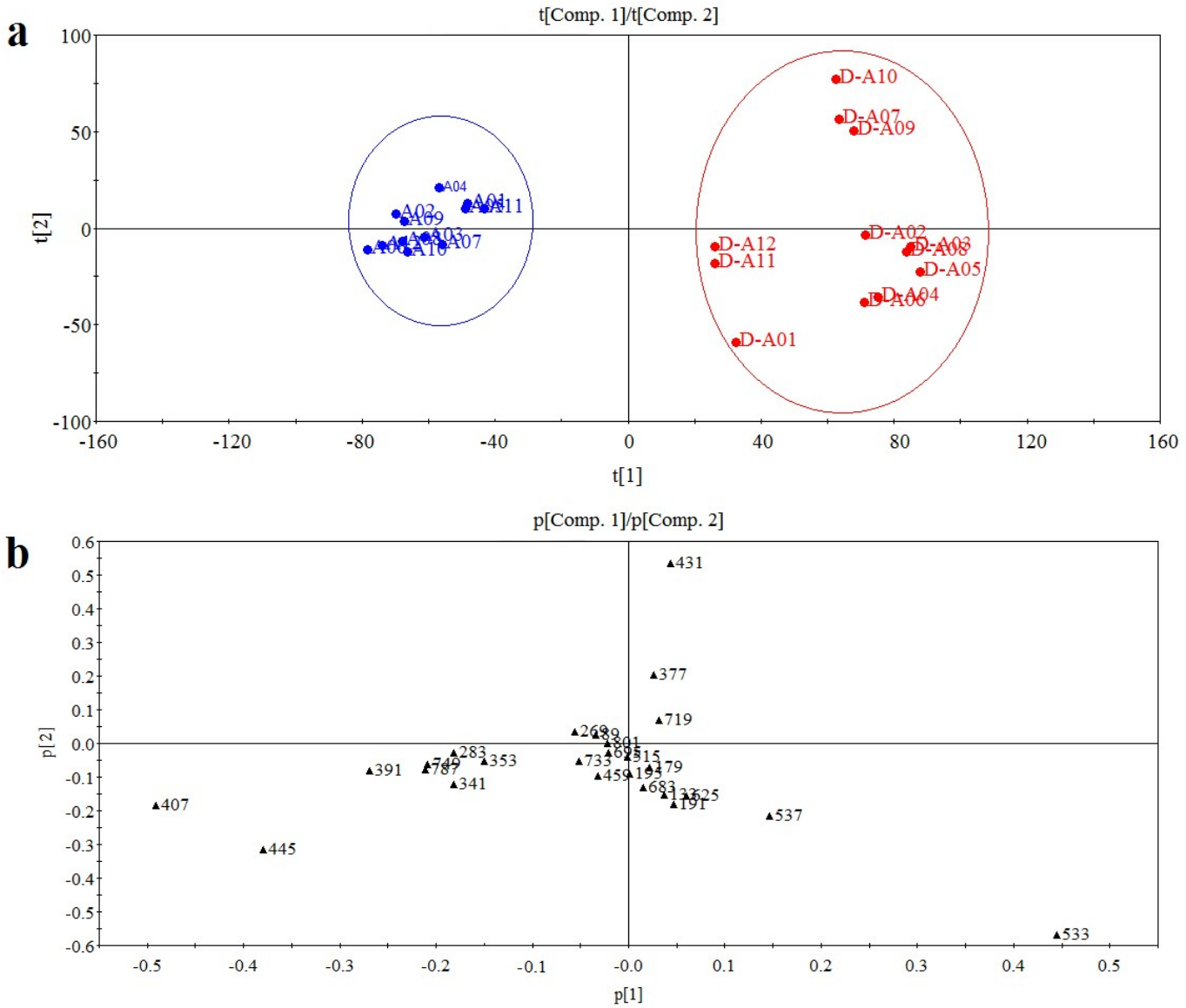

Fig. 3 (a) 2D PCA score plot of twelve investigated qualified and damaged *Qingkailing* oral liquid samples; (b) 2D PCA loading plot of chemical marker from qualified and damaged *Qingkailing* oral liquid samples.

Conclusions

This paper contributes to the development of a novel strategy for rapid accessing the quality of *Qingkailing* products by using a combination of wooden-tip ESI–MS and multivariate statistical analysis. Wooden-tip ESI-MS allows direct analysis of various *Qingkailing* samples under ambient and open-air conditions without the requirement of time-consuming sample pretreatment. As a result, rapid analysis is readily achieved and various active ingredients are simultaneously detected. The combined approach of wooden-tip ESI-MS and multivariate data analysis provides the possibility for rapidly assessing the quality stability and consistency of *Qingkailing* products, and discriminating the qualified and damaged samples.

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Notes and references

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