Processing Technology Investigation of Loquat (Eriobotrya japonica) Leaf by Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry Combined with Chemometrics

Labin Wu, Xue Jiang, Linfang Huang*, Shilin Chen

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Abstract

Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) and multivariate statistical analysis were used to investigate the processing technology of Loquat (Eriobotrya japonica) leaf (pipaye, PPY). The differences in samples processed using different methods were revealed by unsupervised principal component analysis (PCA). In the scores plot of PCA, honey-processed PPY (PPPY), crude PPY (CPPY), and heated PPY (HPPY) were clearly discriminated. Furthermore, samples processed at different temperatures could also be distinguished; indeed, our PCA results demonstrated the importance of temperature during processing. Two unique marker ions were found to discriminate between PPPY and CPPY by orthogonal partial least squares discriminant analysis (OPLS-DA), which could be used as potential chemical markers. The method was further confirmed by a verification test with commercial PPY. The orthogonal array experiment revealed an optimized processing condition with 50% honey at 140 °C for 20 min after 4 h of moistening time, a process that provides significant information for standardized production.

Citation: Wu L, Jiang X, Huang L, Chen S (2013) Processing Technology Investigation of Loquat (Eriobotrya japonica) Leaf by Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry Combined with Chemometrics. PLoS ONE 8(5): e64178. doi:10.1371/journal.pone.0064178

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: lfhuang@implad.ac.cn

Introduction

The leaf of Eriobotrya japonica (Thunb.) Lindl (loquat), commonly referred to as pipaye (PPY), is a well-known and commonly used herb in traditional Chinese medicine (TCM). Generally, PPY is used for the treatment of lung-related diseases, including cough, asthma, and chronic bronchitis, as well as for headache, lower back pain, and dysmenorrhea [1–3]. Various triterpenes, sesquiterpenes, flavonoids, tannins, and megastigmane glycosides have been identified in PPY, and some of them have been found to possess antitumor, antiviral, hypoglycemic, and anti-inflammatory properties [4–8].

In TCM, PPY should be processed before clinical use. According to ancient literature, multiple methods have been used to process PPY. These methods include removing the hair on the leaves, heating [9], and heating in the presence of honey or ginger [10]. Among these methods, removing the hair on the leaves is regarded as a necessary step before using PPY. Today, the honey-heating method is most commonly used as it has been found to be effective in curing cough and pulmonary diseases [11–13]. Honey-processed pipaye (PPPY) is used in decoctions and has also been developed as a patent drug in the medicinal market in China. However, chemical analysis and determination of optimal processing mechanisms for PPPY have not yet been investigated, despite the fact that PPPY has been used for hundreds of years.

Indeed, although PPPY has been recorded in all versions of the Pharmacopoeia of the People’s Republic of China [14], the specific steps and regulatory operations of PPPY have not been established. Therefore, in the current study, we sought to investigate the processing technology of PPY based on chemical analysis and chemometrics. The optimal honey-processing technology of PPPY is also discussed.

Ultra-performance liquid chromatography (UPLC) coupled with photodiode array detector (PDA) and quadrupole time-of-flight mass spectrometry (QTOF/MS) is a newly developed technique in quantitative and qualitative analysis as well as in metabolite analysis and identification of complex compounds in TCM [15]. To efficiently analyze and compare the information-rich spectroscopic data generated by UPLC-QTOF/MS analysis from different samples, MarkerLynx professional software is often used. MarkerLynx is a peak detection algorithm that analyzes rich spectroscopic data generated by UPLC-QTOF/MS analysis from 2 or
more group samples [16]. In the present study, CPPY, PPPY, and HPPY were analyzed by UPLC-PDA-QTOF/MS coupled with Markerlynx to explore the chemical differences and processing technologies of these different preparations for the first time.

In the chemical analysis of CPPY, HPPY, and PPPY, oleanolic acid (OA) and ursolic acid (UA) are regarded as indicative compounds in evaluation and quality control and are listed as chemical indicators in the Pharmacopeia of China (2010 version) [14]. OA and UA reportedly possess biological activity, including anti-inflammatory [17,18], antiprotozoal [19], and antimicrobial properties [20], as well as cytotoxicity to cancer cells [21]. OA possesses hepatoprotective [22] and anti-ulcer bioactivities [23], while UA also exhibits antitumor activity through enhancing the production of both nitric oxide and tumor necrosis factor-α via nuclear factor-kappaB activation in resting macrophages [24]. Therefore, in this study, we evaluated the significance of PPY processed using different methods and investigated the optimal processing technology of PPY based on measurement of OA and UA.

Materials and Methods

Reagents and plant materials

Reference substances for OA and UA (batch no. OA: 11090562, UA: 12020602, Must Co., Ltd., Sichuan, China); acetonitrile and methanol (CR, chromatographic reagents; Fisher Scientific Co., Ltd., MA, USA); ethanol (AR, analytical reagent) and ammonium acetate (AR; Xilong Co., Ltd., Shanxi, China); and honey (edible sophora flower honey, Baihua Honey Co., Ltd., Beijing, China) were purchased from the indicated companies. Leaves from *E. japonica* (Thunb.) Lindl. were collected from Changshou Feilong (Chongqing, China) on November 20, 2011. Random test samples of *E. japonica* leaves, including crude and honey-processed samples, were from Tong Ren Tang Co., Ltd. (Beijing, China).

Preparation of samples

First, honey (honey:PPY ratio, 1:1 w/w) was dissolved in water (50%, v/v). The honey solution was brushed onto the surface of PPY, which was then sealed in a container for 2–4 h. Next, the sample was heated in an oven at 80, 100, 120, 140, or 160°C for 20 min. Samples were then cut into slices (2 mm ×30 mm) to yield PPPY slices for analysis. This method has been submitted for an invention patent in China, with a patent application number of 201210384659.2. HPPY samples were heated in an oven at 80, 100, 120, 140, or 160°C for 20 min, respectively.

One gram of each sample was weighed accurately into a conical flask with a stopper. Then, 50 mL ethanol was added to the sample. The solution was extracted ultrasonically (250 W, 50 kHz) for 30 min. The sample solutions were subsequently filtered through a 0.22-μm membrane and then injected into the HPLC and UPLC-QTOF/MS system for analysis [14].

High-performance liquid chromatography (HPLC) and UPLC-MS methods

The HPLC system model 1525 (Waters, Milford, MA, USA), including binary gradient pump, vacuum degas machine, automatic sample injector, constant temperature column oven, dual wavelength ultraviolet detector model 2487, Breeze chromatographic working station model; chromatographic column model (C18 column, 250 mm ×4.6 mm, 5 μm, Waters). For UPLC analysis, the following systems/parameters were used: Waters Acquity system (Waters) equipped with binary solvent delivery pump, auto-sampler, and PDA detector and connected to a Waters Empower 2 data station; Waters Acquity UPLC BEH C18 column (2.1 mm ×100 mm, 1.7 μm, Waters); ultrasonication (250 W, 50 kHz, Kunshan Ultrasonic Instrument Co., Zhejiang, China); and an electronic analytical balance model AB135-2 (Mettler-Toledo., Greifensee, Zurich, Switzerland).

OA and UA were analyzed by HPLC. An acetonitrile-methanol-0.5% ammonium acetate solution (67:12:21) was set as the mobile phase. The wavelength was set to 210 nm. The references substances, OA and UA, were prepared with ethanol [14]. UPLC separations were carried out in a binary mobile phase at a flow rate of 0.25 mL/min. The optimized separation conditions were as follows: solvent (A), acetonitrile-methanol (5:1); and solvent (B), 0.5% ammonium acetate. The gradient

| Factors | Levels | Amount of honey (A, %) | Temperature (B, °C) | Heating time (C, min) | Moistening time (D, h) |
|---------|--------|------------------------|---------------------|-----------------------|------------------------|
| 1       | 50     | 100                    | 10                  | 2                     |
| 2       | 100    | 120                    | 20                  | 3                     |
| 3       | 200    | 140                    | 30                  | 4                     |

Table 1. Methodological validation.

| Standard compound | Interday precision (RSD%) N = 6 | Intraday precision (RSD%) N = 6 | Repeatability (RSD%) N = 6 | Mean (%) | RSD (%) | LOD (μg/mL) | LOQ (μg/mL) |
|-------------------|---------------------------------|-------------------------------|----------------------------|----------|---------|-------------|-------------|
| Oleanolic acid    | 0.0363                          | 0.9436                        | 1.1630                     | 97.20    | 2.12    | 0.9         | 2.8         |
| Ursolic acid      | 0.2332                          | 1.9917                        | 0.8563                     | 98.53    | 1.10    | 1.0         | 3.0         |

Table 2. Factors and levels of the orthogonal array design.

doi:10.1371/journal.pone.0064178.t001

doi:10.1371/journal.pone.0064178.t002
elutions were as follows: 0–10 min, 70%–80% A; 10–12 min, return to initial conditions. The sample volume injected was 5 mL.

The UPLC/MS analysis was performed on a QTOF Synapt G2 HDMS system (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operated in the negative ion mode. N2 was used as the desolvation gas. The desolvation temperature was set at 450°C at a flow rate of 800 L/h, and the source temperature was set at 120°C. The capillary and cone voltages were set to 2500 and 40 V, respectively. The data were collected between 50–1200 Da with a 0.1-s scan time and a 0.01-s interscan delay over a 12 min analysis time. Argon (Ar) was used as the collision gas at a pressure of 7.066 × 10^-2 Pa. All the MS data were collected using the LockSpray system to ensure the mass accuracy and reproducibility. The [M–H]^− ion of leucine-enkephalin at m/z 554.2615 was used as the lock mass in negative ESI mode.

Methodological evaluation

The calibration curve, inter- and intraday precision, repeatability and recovery rates were measured as above (Table 1). The calibration curve and precision were tested with OA and UA; the repeatability and recovery rates were tested with PPY. The calibration curves of OA and UA were Y = 416970 x −4012 (r = 0.9997) and Y = 798659x −662112 (r = 0.9991); and the linear ranges of OA and UA were 0.061–1.22 μg and 0.26–5.2 μg, respectively. Limits of detection (LODs) were established at a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQs) were established at an S/N of 10. The LODs of OA and UA were calculated to be 0.9 and 1.0 μg/mL, and the LOQs of OA and UA were calculated to be 2.8 and 3.0 μg/mL, respectively.

Data analysis

UPLC-QTOF/MS data for CPPY, HPPY, and PPPY samples were analyzed to identify potential discriminant variables. Peak finding, alignment, and filtering of ESI-raw data were carried out with MarkerLynx applications manager version 4.1 (Waters, Manchester, UK). The parameters used were as follows: retention time (tR) ranging from 0 to 12 min, mass ranging from 50 to 1200 Da, retention time tolerance of 0.02 min, and a mass tolerance of 0.02 Da. The noise elimination level was set at 6.00, and the minimum intensity was set to 15% of base peak intensity. For data analysis, a list of the intensities of the detected peaks was generated using retention time and mass data (m/z). An arbitrary ID was assigned to each of these tR–m/z pairs with the order of the UPLC elution. The ion intensities for each detected peak were normalized against the sum of the peak intensities within that sample using MarkerLynx software. Ion identification was based on the tR and m/z. The resulting 3-dimensional data comprising the peak number (tR–m/z pair), sample name, and ion intensity were analyzed by PCA and orthogonal partial least squares discriminant analysis (OPLS-DA) in MarkerLynx software [25].

Orthogonal array design

The orthogonal array design was performed on Orthogonality Experiment Assistant II software (Sharetop Software Studio, 2002, Beijing, China). The influential factors were set as the amount of honey, the heating temperature, the heating period, and the sealing period during the pre-experiment because these factors could affect the attribution of TCMs significantly in others herbs [26]. The levels of these factors are presented in Table 2 according to our pre-experiments and previous literature. The orthogonal array design was performed as L9 (3^4) (Table 3) with evaluation scores based on the determination of OA and UA, and the results were then analyzed with variance analysis.

Table 3. The L9 (3^4) experiment design of the orthogonal array design.

| Factors | Temperature | Heating time | Honey amount | Moistening time | Evaluation score |
|---------|-------------|--------------|--------------|-----------------|------------------|
| No. 1   | 1           | 1            | 1            | 1               | 93.7517          |
| No. 2   | 1           | 2            | 2            | 2               | 94.6764          |
| No. 3   | 1           | 3            | 3            | 3               | 84.1200          |
| No. 4   | 2           | 1            | 2            | 3               | 84.0749          |
| No. 5   | 2           | 2            | 3            | 1               | 82.1996          |
| No. 6   | 2           | 3            | 1            | 2               | 97.3241          |
| No. 7   | 3           | 1            | 3            | 2               | 82.8741          |
| No. 8   | 3           | 2            | 1            | 3               | 100.0000         |
| No. 9   | 3           | 3            | 2            | 1               | 90.3395          |
| Mean 1  | 90.849      | 86.900       | 97.025       | 88.764          |
| Mean 2  | 87.866      | 92.292       | 89.697       | 91.625          |
| Mean 3  | 91.071      | 90.595       | 83.065       | 89.398          |
| R       | 3.205       | 5.392        | 13.960       | 2.861           |

Table 4. Determination of OA and UA in CPPY, PPPY, and HPPY (N = 3).

| Temperature (°C) | PPPY (%) | HPPY (%) |
|-----------------|----------|----------|
| OA   | UA       | OA       | UA       |
| 80   | 0.0879   | 0.4912   | 0.1313   | 0.7756   |
| 100  | 0.1138   | 0.5776   | 0.1799   | 0.9190   |
| 120  | 0.1193   | 0.5711   | 0.1250   | 0.7651   |
| 140  | 0.1147   | 0.5944   | 1.1892   | 0.9244   |
| 160  | 0.1102   | 0.5503   | 0.1463   | 0.8081   |
| 0 (CPPY)| ---      | ---      | 0.1723   | 0.7387   |
Verification of the method
The method has been verified by randomly testing CPPY and POPY available in the medicinal market. The test samples were subjected to the methods described above. The data were then analyzed by PCA and OPLS-DA.

Results
Determination of OA and UA
The determination of OA and UA in CPPY, HPPY, and PPPY are presented in Table 4. Rankings of the contents in the samples were CPPY ≈ HPPY > PPPY. HPPY and CPPY contained

Table 5. Tentatively identified compounds from leaves of E. japonica.

| Peak no. | t_R (min) | Assigned identity                                                                 | Molecular formula | Mean measured mass (Da) | Theoretical exact mass (Da) | ppm | Fragments m/z | References |
|----------|-----------|-----------------------------------------------------------------------------------|-------------------|-------------------------|-----------------------------|-----|---------------|------------|
| 1        | 1.52      | euscaphic acid                                                                    | C_{30}H_{48}O_{5} | 487.3411                | 487.3423                    | −2.5| 469.3325      | [M-H-H_{2}O] , [M-H-H_{2}O-3H_{2}O] | [29,34] |
| 2        | 1.96      | 2α, 19α-dihydroxyurs-3-oxo-urs-12-en-28-oic acid                                 | C_{30}H_{46}O_{5} | 485.3231                | 485.3267                    | −7.4| 467.3167      | [M-H-H_{2}O] , [M-H-H_{2}O-3H_{2}O] | [30] |
| 3        | 2.31      | 3-O-p-coumaroyloctomeronic acid                                                   | C_{30}H_{46}O_{5} | 633.3813                | 633.3850                    | −5.8| 487.3415      | [M-H-H_{2}O] , [M-H-H_{2}O-3H_{2}O] | [30] |
| 4        | 2.87      | maslinic acid                                                                     | C_{30}H_{46}O_{4} | 471.3431                | 471.3474                    | −9.1| 427.3688      | [M-H-CO_{2}] , [M-H-CO_{2}-H_{2}O] | [29,34] |
| 5        | 3.16      | 2α-hydroxyursolic acid                                                            | C_{30}H_{46}O_{4} | 471.3431                | 471.3474                    | −9.1| 427.3583      | [M-H-CO_{2}] , [M-H-CO_{2}-H_{2}O] | [29,34] |
| 6        | 3.61      | linolenic acid                                                                    | C_{18}H_{30}O_{2} | 277.2168                | 277.2168                    | 0.7 | 255.2334      | [M-H-H_{2}O] | [31] |
| 7        | 5.08      | hyptadienic acid                                                                  | C_{11}H_{18}O_{2} | 469.3310                | 469.3318                    | −3.6| 425.3776      | [M-H-CO_{2}] | [30] |
| 8        | 5.20      | linoleic acid                                                                     | C_{10}H_{18}O_{2} | 279.2324                | 279.2324                    | 0.7 | 255.2333      | [M-H-H_{2}O] | [31] |
| 9        | 5.37      | 3β-O-coumaroyl-2α-hydroxyurs-12-en-28-oic acid                                   | C_{31}H_{50}O_{6} | 617.3852                | 617.3842                    | 1.6 | 471.3441      | [M-H-C_{6}H_{9}O_{5}] | [32] |
| 10       | 6.64      | oleanolic acid                                                                    | C_{30}H_{48}O_{3} | 455.3505                | 455.3525                    | −4.4| 411.3620      | [M-H-CO_{2}] | [29,34] |
| 11       | 7.27      | ursolic acid                                                                      | C_{30}H_{46}O_{3} | 455.3505                | 455.3525                    | −4.4| 411.3615      | [M-H-CO_{2}] | [29,34] |
| 12       | 7.45      | palmitic acid                                                                     | C_{10}H_{20}O_{2} | 255.2333                | 255.2324                    | 3.5 | 217.0050      | [M-H-CO_{2}] | [33] |
| 13       | 10.03     |                                                                                   | C_{10}H_{20}O_{2} | 283.2637                | 283.2637                    | 0   | 255.2333      | [M-H-CO_{2}] | [31] |
| 14       | 10.64     |                                                                                   | C_{10}H_{20}O_{2} | 283.2637                | 283.2637                    | 0   | 255.2333      | [M-H-CO_{2}] | [31] |
| 15       | 11.19     |                                                                                   | C_{10}H_{20}O_{2} | 283.2637                | 283.2637                    | 0   | 255.2333      | [M-H-CO_{2}] | [31] |
higher OA and UA contents than PPPY, while OA and UA in CPPY were similar to those in HPPY, indicating the chemical stability of OA and UA under heating conditions [27]. Two reasons may explain why HPPY contained more OA and UA than PPPY: 1) PPPY may contain less herb materials than HPPY because half of PPPY is made up of honey, and 2) Maillard reactions may occur during the heating process due to the existence of organic acids and polysaccharides [28]. According to ancient literature, HPPY and PPPY have the same significance in clinical practice but have different pharmacological effects. Interestingly, PPPY is more commonly used than HPPY in clinical practice nowadays. Additionally, HPPY has received less attention than PPPY. Considering the comprehensiveness and complexity of TCM, further investigation is required to determine the pharmacological values of HPPY and PPPY.

Tentative peak assignment by UPLC-QTOF/MS

Table 5 lists the tentatively identified compounds in CPPY, HPPY, and PPPY. A total of 15 compounds were identified by UPLC-QTOF/MS based on database interrogation, standard compounds, and references, as shown in Figure 1 [29–34]. Peaks 10 and 11 were identified as OA and UA, respectively, based on retention times, MS, and MS/MS fragment ions [28,33]. Peaks 1, 4, and 5 were identified as euscaphic acid, maslinic acid, and 2α-hydroxyursolic acid, respectively, based on retention times, and MS data for these peaks were consistent with references [29,34]. Peaks 2, 3, 7, 9, and 12 were identified as 2α,19α-dihydroxyurs-3-oxo-urs-12-en-28-oic acid, 3-O-p-coumaroyltormentic acid, hydratadienic acid, 3β-O-coumaroyl-2α-hydroxy-urs-12-en-28-oic acid, and palmitic acid, respectively, based on molecular mass and MS/MS fragment ions [30,32,33]. Linolenic, linoleic, stearic, and isomeric stearic acids were identified according to molecular masses and fragment ions and have been identified in the seeds or fruits of *E. japonica* [31]. All other compounds have been previously reported to be present in the leaves of *E. japonica*.

PCA of CPPY, HPPY, and PPPY

PCA uses an N-dimensional vector approach to separate samples on the basis of the cumulative correlation of all metabolite data and then identifies the vector (eigenvector) that yields the greatest separation among samples without requiring prior knowledge of the data sets [35]. Mean-centered and par-scaled (scaled to square root of SD) mathematical methods were performed to pretreat the data sets resulting from the above data. Samples processed using the same conditions were replicated with 3 individuals (N = 3). A total of 1058 variables were used to create the model. The 2-component PCA model cumulatively accounted for 50.51% of variation (PC1, 32.95%; PC2, 17.56%).

Figure 2 shows that CPPY, HPPY, and PPPY samples were divided into 3 main clusters observed in the PCA scores plot. Such division indicated that use of different processing methods could significantly alter the composition of compounds and that CPPY, HPPY, and PPPY were distinct from each other. This distinct separation could be representative of their multiple pharmacological effects.

The PCA score plot illustrates that samples processed at different temperatures could be clearly discriminated. In HPPY, samples treated at 80, 100, and 120°C differed from those treated at 140 and 160°C. Obviously, the samples changed dramatically after heating at 140°C, and chemical differences between 140 and 160°C were minimal.

**Table 6. Analysis of variance of the orthogonal array design.**

| Factors       | SS     | Degrees of freedom | F*   |
|---------------|--------|--------------------|------|
| Temperature   | 19.221 | 2                  | 1.419|
| Heating time  | 45.600 | 2                  | 3.366|
| Honey amount  | 292.594| 2                  | 21.598*|
| Moistening time| 13.547 | 2                  | 1.000|
| Error         | 13.55  | 2                  |      |

*The critical F value was 21.598 (*p<0.05).*  

doi:10.1371/journal.pone.0064178.t006
160°C treatments were not evident. PYPY samples treated at 80, 100, and 120°C were clustered into 1 group and separated from those samples treated at 140 and 160°C. Unlike HPPY samples, PYPY samples processed at 140 and 160°C were located far from each other. This finding indicated that dramatic chemical changes occurred when the processing temperature was raised to 140°C. In addition, honey treatment may lead to such results. Therefore, this experiment clearly demonstrated the importance of temperature and auxiliary materials, i.e., honey, during processing.

Orthogonal array design and standardized production of PYPY

The results of the orthogonal array design are shown in Table 2, 3 and 6. Table 3 shows that the optimized production procedure for PYPY was performed in experiment no. 8 (A3B2C1D3) depending on the quality scores (based on the determination of OA and UA). Therefore, the optimized production of PYPY was performed with 50% honey at 140°C for 20 min after 4 h of moistening time. Table 6 shows the analysis of variance, demonstrating that the most significantly influential factor was the amount of honey, which had the highest critical F value.

The orthogonal array design confirmed the results of the PCA above. The PCA showed that temperatures of 140 and 160°C could significantly alter the process, unlike temperatures of 80, 100, and 120°C. From the visual observation of PYPY, PYPY samples processed at 160°C showed a dark color, indicating an excessive heating process. Considering the results of PCA and the orthogonal array design, we regarded 140°C as the optimal temperature in the processing PYPY. The orthogonal array design experiment demonstrated a certain credibility in the standardization of processed TCMs. Through this experiment, we recommend the A3B2C1D3 processing steps for the production of PYPY.

OPLS-DA and marker identification

To identify markers for the discrimination between crude and processed samples, extended statistical analysis was performed to generate the S-plot of OPLS-DA. In the S-plot, each point represented a tR–m/z ion pair. The X axis represented the contribution of the ion. The distance of the tR–m/z ion pair pointed to the origin on the X axis and represented the contribution of this ion to the differences between the 2 groups. The Y axis represented the confidence of the ion. The distance of the tR–m/z ion pair pointed to the origin on the Y axis and represented the confidence level of this ion. Thus, the tR–m/z ion pointing to the 2 ends of the “S” represented the characteristic markers with the highest confidence in each group.

In Figure 3, pairs of these samples were compared in an S-plot. The circled points were regarded as the highest confidence markers, which could be used as potential points in distinguishing between markers. The results of OPLS-DA showed that UPLC-QTOF/MS could be applied to distinguish between raw and processed PYPY by the S-plot (Figure 3). A total of 6, 6, and 6 credible and significant markers are found to be available in distinguishing between CPPY/HPPY, CPPY/PYPY, and HPPY/PYPY samples, respectively (Table 7). Two identities of potential markers b and c (Table 7) were tentatively assigned (Table 5) [29,30,34]. The components correlated to these 2 ions were tentatively assigned as 2α-hydroxyursolic acid and 3-O-p-coumaroyltormentic acid. Therefore, significant differences existed between crude and processed PYPY according to the S-plot of OPLS-DA, and these credible markers could be considered in distinguishing between and identifying these different samples.

Verification test

In the verification test, CPPY and PYPY could be separated by the PCA score plot (Figure 4). This demonstrated that UPLC-QTOF-MS could be used as the method for identification between commercial CPPY and PYPY. Additionally, OPLS-DA was performed to generate an S-plot (Figure 3D). Two marker ions, marker a (0.95_221.0600/0.94_221.0670) and marker b (3.32_471.3449/3.17_471.3453), were successfully verified. Marker a could be detected in experimental and test PYPY samples, but could not be detected in CPPY (Figure 5A, 5B). The ion intensity of marker b in CPPY was higher than that in PYPY in experimental samples, and this was verified in test samples.
Table 7. Marker tᵢᵢ–m/z ion pairs in the S-plot.

|                | Experimental Loquat leaves | Commercial Loquat leaves |
|----------------|-----------------------------|--------------------------|
|                | CPPY/PPPY | CPPY/HPPY | HPPY/PPPY | CPPY/PPPY |
| CPPY           | 3.32–471.3449 (b) | 1.48–293.2056 | 2.33–633.3749 (c) | 3.17–471.3453 (b) |
|CPPY           | 0.96–415.0948 | CPPY       | 0.95–341.0990 | HPPY       | 1.57–826.5356 |
|                | 0.93–517.2170 | 0.93–517.2170 | 1.56–572.3066 | 2.87–471.3481 |
|                | 0.93–503.1484 | 2.33–633.3749 (c) | 0.93–503.1484 | 0.94–341.1066 |
|PPPY           | 0.95–221.0600 (a) | HPPY       | 2.42–663.3751 | PYYPYY     | 1.56–667.3883 |
|                | 0.93–539.1216 | 0.99–331.2377 | 0.95–221.0600 (a) | PYYPYY     | 0.98–191.0561 |

A Novel Strategy for the Study of TCM Processing

doi:10.1371/journal.pone.0064178.t007
Figure 4. PCA (scores plot) of commercial CPPY and PPPY.
doi:10.1371/journal.pone.0064178.g004

Figure 5. Ion intensities of markers a and b. (●, CPPY; ■, PPPY. A, marker a in experimental samples; B, marker a in test samples; C, marker b in experimental samples; D, marker b in test samples).
doi:10.1371/journal.pone.0064178.g005
Conclusion

This investigation explored the processing technology of Loquat leaves by UPLC–QTOF/MS and chemometrics. PCA successfully illustrated the differences in samples processed using different processing methods. We were able to distinguish between CPPY, HPPY, and PPPY, and the differences between samples processed at different temperatures were also presented, which indicated the dramatic differences caused by processing methods. OPLS-DA identified 2 unique marker ions that could discriminate between CPPY and PPPY, for the first time. This finding was verified by experiments using test samples. The optimized processing condition used 50% honey at 140°C for 20 min after 4 h of moistening time, in an orthogonal array design. This investigation provides insights into the development of processing technology in TCM.

Author Contributions

Conceived and designed the experiments: LH SC. Performed the experiments: LW XJ. Analyzed the data: LW. Wrote the paper: XJ.

References

1. Lin SQ (2007) World loquat production and research with special reference to China. Acta Horticult 750: 37–44.
2. Lin JY, Tang CY (2008) Strawberry, loquat, mulberry, and bitter melon juices exhibit prophylactic effects on LPS-induced inflammation using murine peritoneal macrophages. Food Chem 107: 1597–1596.
3. Ito H, Kobayashi E, Takamatsu Y, Li SH, Hatano T, et al. (2000) Polyphenols from Eriobotrya japonica and their cytotoxicity against human oral tumor cell lines. Chemical and Pharmaceutical Bulletin 48: 687–695.
4. Shimizu M, Fukushima H, Tsuji H, Tanaka M, Hayashi T, et al. (1986) Antiinflammatory constituents of topically applied crude drugs. I. Constituents and anti-inflammatory effect of Eriobotrya japonica Lindl. Chemical and Pharmaceutical Bulletin 34: 2614–2617.
5. De Tommasi N, De Simone F, Cirino G, Cicela C, Piazza C (1991) Hypoglycemic effects of sesquiterpene glycosides and polyhydroxylated triterpenoids of Eriobotrya japonica. Planta Medica 57: 414–416.
6. Chen J, Li WL, Wu JL, Ren BR, Zhang HQ (2008) Hypoglycemic effects of a sesquiterpene glycoside isolated from leaves of loquat (Eriobotrya japonica (Thunb.) Lindl.) Phytomedicine 15: 98–102.
7. Norihito B, Toshiohi A, Harunuki T, Ken Y, Yusuke T, et al. (2005) Anti-inflammatory and Antitumor-Promoting Effects of the Triterpene Acids from the Leaves of Eriobotrya japonica. Biological and Pharmaceutical Bulletin. 28: 1995–1999.
8. Kim SH, Shin TY (2009) Anti-inflammatory effect of leaves of Eriobotrya japonica correlating with attenuation of p30 MAPK, ERK, and NF-kappaB activation in mast cells. Toxicology In vitro 23: 1215–1219.
9. Su J (1981) Xin xiu Ben Cao. Hefei: An hui Science & Technology Publishing House. 445 p.
10. Chen JM (1987) Ben Guo Meng Quan. Hefei: An hui Science & Technology Publishing House. 202 p.
11. Li SC (1958) Lei Gong Pao Zhi Yao Xing Jie. Shanghai: Shanghai Science and Technology Publishing House. 445 p.
12. Wang A (1997) Ben Cao Bei Yao. Shenyang: Liaoning Science and Technology Publishing House. 172 p.
13. Wu YL (1958) Ben Cao Cong Xin. Shanghai: Shanghai Science and Technology Publishing House. 201 p.
14. State Pharmacoepia Committee (2010) Pharmacoepia of People’s Republic of China, Beijing: People’s Medical Publishing House. 190 p.
15. Churchwell MI, Twaddle NC, Meeker LR, Doregga DR (2005) Improving LC-MS sensitivity through increases in chromatographic performance: Comparisons of UPLC-ES/MS/MS to HPLC-ES/MS/MS. J Chromatogr B 825: 134–143.
16. Zhou W, Su SL, Duan JA, Guo JM, Qian DW, et al. (2010) Characterization of the Active Constituents in Shizhao San Using Bioactivity Evaluation Followed by UPLC-QTOF and Markerlynx Analysis. Molecules 15: 6217–6230.
17. Alvez E, Rotelli AF, Pelzer LE, Saadbf JR, Giordano O (2000) Phytochemical study and anti-inflammatory properties of Lampaya hieronymi Schum ex Moldenke. Farmaco 55: 505–505.
18. Chiang LC, Chiang W, Chang MY, Ng LT, Lin CC (2003) Antileukemic activity of selected natural products in Taiwan. Am J Chin Med 31: 37–46.
19. Cunha WR, Camila M, Ferreira DeS, Crotti AE, Lopes NP, et al. (2003) In vitro trypanocidal activity of triterpenes from Miconia species. Planta Med 69: 470–472.
20. Diaz AM, Abad MJ, Fernandez L, Recuero C, Villaescusa L, et al. (2000) In vitro anti-inflammatory activity of indoloids and triterpenoid compounds isolated from Phyllirea latifolia L. Biol Pharm Bull 23: 1307–1313.
21. Farina C, Pinza M, Pifferi G (1998) Synthesis and anti-ulcer activity of new derivatives of glycyrhetinic, oleanolic and ursoic acids. Farmaco 53: 22–32.
22. Jeong HG (1999) Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. Toxicol Lett 105: 215–222.
23. Woldemariam GM, Singh MP, Maiese WM (2003) Constituents of antibacterial extract of Caesalpinia paraguariensis Burk Z. Naturforschung A 58c: 70–73.
24. You HJ, Choi CY, Kim JY, Park SJ, Hahn KS, et al. (2001) Ursolic acid enhances nitric oxide and tumor necrosis factor-alpha production via nuclear factor-kappa B activation in the resting macrophages. FEBS Lett 509: 136–140.
25. Li ML, Song JC, Qiao CF, Zhou Y, Xu HX (2010) UPLC-PDA-TOFMS based chemical profiling approach to rapidly evaluate chemical consistency between traditional and dispensing granule decoctions of traditional medicine combinatorial formulae. J Pharm Biomed Anal 52: 468–478.
26. Lin GM, LAI YX, YU XL, JIA TZ (2010) Optimal Technique of Bran-processed Fructus Aurantii Immaturus. Chinese Journal of Experimental Traditional Medical Formulare 16: 21–25.
27. Qi YF, Li K (2011) Quality evaluation of Eriobotryae Folium collected during different harvest and study on its stability. Chinese Traditional and Herbal Medicine 6: 1217–1220.
28. Zheng WH, Xu X (2005) Research Progress on Maillard Reaction. Progress In Chemistry 17: 122–129.
29. Je JH, Zhou L, Lin G, Liu D, W LW (2005) Studies on constituents of triterpene acids from Eriobotrya japonica and their anti-inflammatory and antitussive effects. Chin Pharm J 38: 752–757.
30. Taniguchi S, Imayoshi Y, Kobayashi E, Takamatsu Y, Ito H, et al. (2002) Production of bioactive triterpenoids by Eriobotrya japonica calli. Phytochemistry 59: 315–323.
31. Chen PP, Zhou JH, Chang H, ZENG FK (2012) Production of bioactive triterpenoids by Eriobotrya japonica calli. Phytochemistry 79: 315–323.
32. Shao M, Wang Y, Huang XJ, Fan CL, Zhang QW, et al. (2012) Four new triterpenoids from Eriobotrya japonica: Eriobotrya japonica. Phytochemistry 79: 315–323.