New monitoring strategy for the quality control in the processing practice of *Scutellariae Radix*

Lili Li[a,#], Chunsong Cheng[b,#], Qi Huang[a,c], Daiyin Peng[a*], Cun Zhang[c*]

[a] *College of Pharmacy, Anhui University of Traditional Chinese Medicine, Hefei, 230000, China*

[b] *Faculty of Chinese Medicine, Macau University of Science and Technology, Macau 999078, China*

[c] *Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences Beijing 100700, China*

[#] These authors contributed equally to this work.

* Corresponding author. E-mail address：pengdaiyin@163.com (D. Peng) & zhc95@163.com (C. Zhang)

ABSTRACT: *Scutellariae Radix* (*Huangqin*) is an important herb medicine used for anti-infective, anti-oxidation, immunotherapy and other clinical treatments in China. Every year, about 10, 000 tons of dry herbs are processed and put into clinical use in Chinese medicine. Inhomogeneity of quality of the processed products has been clinically concerned for many years. In this research, the basic chromatographic information for quality control was first provided for processed *Huangqin* products. Wogonin and baicalein, as well as their glycosides were proposed as obvious indicators to control the quality of processed products and to monitor the processing practice. Among them, the content of wogonin (95 %~105 % of crude *Huangqin*) is best suited as a chemical indicator for carbonated *Huangqin*. In addition, the ratio of peak areas of baicalein (about from 2 to 1) has been regarded as a quick identification signal to guide production of carbonized *Huangqin* in our processing practice. Accordingly, we have provided a multivariate monitoring strategy for the quality control in processing practice of *Hquangqin.*

*Keywords***:** Huangqin; Paozhi; processing; Chinese herbal medicine; wogonin; baicalein.

1. Introduction

According to traditional Chinese medicine (TCM) theory, the raw herbal drugs need to be processed into "decoction pieces" and then proceed for their medical applications [1]. In general, different processed products of herbal materials are used in different clinical practices. Currently, Chinese herbal processing (Paozhi) is often produced with supplementary materials and heated by fire which represents a unique Chinese pharmaceutical technique to facilitate the clinical use in the guidance of Traditional Chinese Medicine (TCM) theory [2]. It is generally believed that the degree of processing and the level of technology directly alter the qualitative and quantitative chemical composition of herbal materials and can thus affect the efficacy of Chinese herbs [1, 3]. So, the processing industry must strictly observe the processing standards in every province in China. However, with the development of mechanization and the decrease of experienced processing workers, Chinese herbal processing has faced more and more challenges on the quality control of processed products, which is also aggravated by the practices of the different and even inconsistent contemporary processing standards.

Huangqin, is a popular Chinese herbal drug produced from the taproot of the medicinal plant *Scutellaria baicalensis* Georgi. Clinical practices indicated it has good therapeutic effects on upper respiratory tract infection, acute gastroenteritis, and is also widely used for the treatment of various diseases, such as viral hepatitis, bronchitis, as well as tumors prevention [4, 5]. Baicalin, baicalein, wogonin and oroxylin A are its main active components [6]. In fact, *Huangqin* needs processing with different methods for clinical use (Fig.1). Now, products of different specifications can be purchased in the market, such as raw *Huangqin*, alcohol *Huangqin* or carbonized *Huangqin* (Fig.2).

Traditional Chinese medicine [7] consider that the crude raw *Huangqin* is suitable for the treatment of gastrointestinal and respiratory tract infections, processing with alcohol can enhance the anti-infection effect of *Huangqin* on the respiratory system, and carbonized *Huangqin* is suitable for infection and bleeding symptoms in gastrointestinal tract and respiratory tract. This basic treatment theory was established before the Tang Dynasty, 1,000 years ago, and has been inherited and developed by Chinese medicine from generation to

generation. So, a serious challenge is how to ensure the stability of different processed products, and how to guarantee the quality, which is also our concern in this paper. In particular, carbonization of medicinal materials is very easy to overheat, resulting in poor quality of herbal medicines. Taking *Huangqin* as an example, the purpose of carbonization is to delay the oxidation and enzymatic hydrolysis of internal chemical components in the maximum extent. [8] So it is considered that completely surrounded surface carbonization is the expected processing result for *Huangqin*'s processing. However, the size of the sliced herbs (Fig.2 A) is usually not uniform that lead to a non-uniform heating on the surface of the medicinal materials and thus directly led to a local serious carbonization and greatly reduced the content of chemical components and quality of medicinal medicines. Therefore, an accurate quality control method is inseparable from the control of index components. [9] But, the testing cost of existing control methods greatly exceeded the expectations of routine quality control in Chinese pharmaceutical companies. [10, 11]

Metabolomics is considered as a powerful technology to describe the similarities and differences between herb samples [12]. Metabolomics analysis has been established for investigating metabolic differences food classes and plant species [13] over recent years. So here, a new strategy we proposed is selection of index components with full reference to metabolomics and accurate quantification based on HPLC technology with good separation effect. Moreover, the OPLS-DA model with excellent performances is also used here to quickly distinguish different processed products and unqualified ones. This study might provide a feasible strategy for quality control of *Scutellariae Radix* and facilitate better understanding of their different traditional uses.

2. Materials and methods

2.1 Samples collection

Three different specifications of *Huangqin* herbs, total 48 batches of samples, including raw *Huangqin*, alcohol *Huangqin* and carbonized *Huangqin* were collected from the traditional four largest producing areas of *Neimeng*, *Heibei*, *Shanxi* and *Gansu* provinces in China (Table 1). All products were identified by Professor Yong-Qing Xiao (Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences) according to their

morphological characteristics. The samples were stored in College of Pharmacy, Anhui University of Traditional Chinese Medicine, Hefei, China.

2.2 Equipment and reagents

The chromatographic evaluation was performed on an Agilent HPLC-PDA system (Agilent-1260, USA). The data were acquired and processed by using a LC-Solution workstation. An Agilent HC-C18 (2) column (250 mm×4.6 mm, 5 μm) was used to separate the ingredients in the products. A rotary evaporator (EYELA, Japan) and an ultrasonic cleaner (Kunshan, China) were also employed. All standard products including scutellarin, baicalin, wogonoside, baicalein, wogonin, chrysin and oroxylin–A, were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All standard solutions were prepared with HPLC grade methanol from Merck (Darmstadt, Germany), and stored at 4 ∘C. Working standard solutions were prepared by diluting with an appropriate volume of methanol to the desired concentrations. Methanol and ethanol were used as the organic modifiers, which were also HPLC grade and purchased from Merck. Other reagents were all of analytical grade. Water used throughout the experiments was generated by a Milli-Q academic water purification system (Milford, MA, USA).

2.3 Chromatographic conditions

The mobile phase was consisted of methanol and 0.1% solution of formic acid with gradient elution. The mobile phase was also filtered through a 0.22 μm membrane filter and degassed by ultrasound before using. The sample injection volume was 10 μL. The solvent flow rate was 1.0 mL/min. The column temperature and detection wavelength were 35°C and 280 nm, respectively. Chromatographic peaks were identified by comparing the retention time with standards. The mobile phase gradient was shown in Table 2.

2.4 Preparation of standard solutions

Seven compounds (Scutellarin, baicalin, wogonoside, baicalein, wogonin, chrysin and oroxylin-A) were precisely weighed and placed into a volumetric flask, and then added methanol (10 mL) into this volumetric flask. The concentrations of the 7 reference compounds were 0.0464 mg·mL^{-1}, 1.03 mg·mL^{-1}, 0.214 mg·mL^{-1}, 0.221 mg·mL^{-1}, 0.1095 mg·mL^{-1}, 0.01176 mg·mL^{-1}, 0.0985 mg·mL^{-1} respectively.

2.5 Production of standard curves

The standard curves were obtained by plotting the peak areas and the gradient concentrations with different standard solutions. The regression equations were calculated in the form of $Y = aX + b$, where X and Y are the concentration of the standard solution ($\mu g \cdot mL^{-1}$) and the corresponding peak area, respectively. The results revealed that the peak area of each standard was linearly correlated to the injected concentration within a specific range, as shown in Table 3. Besides, all the calibration curves exhibited good linearity (r>0.9995). In addition, the mixed standard solutions were further diluted to series of standard solutions; the solutions below the minimum concentration of the standard curve were performed to evaluate the limits of detection (LODs) and the limits of quantification (LOQs) of the compounds. LOD and LOQ values represented 3 times and 10 times the signal-to-noise ratio were acquired for methodological evaluation.

2.6 Preparation of sample solutions

Various extraction solvents, extraction times and methods were evaluated in an effort to optimize the extraction procedure. The results (Supplementary Table 1) revealed that reflux extraction was the best method for dissolution of target compounds. So, further experiments were carried out with reflux extraction. Solvents including 30 % methanol, 50 % methanol, 70 % methanol and anhydrous methanol were screened with reflux to evaluate the efficiency of the solvent extraction. The results (Supplementary Table 2) showed that 50 % methanol was the most suitable extraction solvent, since it allowed the extraction of all the major constituents with high yields. The influence of the extraction time was 1 hour. All solutions were filtered with 0.22 μm before injecting in HPLC.

2.7 General methodological studies

The precision of the method was tested by evaluating the precision test. The mixed standard solutions was analyzed 6 times under the optimized conditions within 1 day (n = 6). The RSD % values were calculated by the contents of the 6 standard compounds, and the results (Supplementary Table 3) showed that the RSD % of all compounds were less than 2.1 %; Six independent sample solutions were prepared and analyzed for repeatability test using *Hebei* crude sample, the results showed that the RSD % of the calculated contents of the

7 compounds was less than 3.0 % (Supplementary Table 4); the sample was also analyzed in triplicate every 3 h within 24 h, and the RSD % of the calculated contents for the 7 compounds was also less than 2.1 % (Supplementary Table 5); *Hebei* crude sample was selected to be extracted for recovery test. In this study, 6 invidious were quantified first and the same volume of the mixed standard solution was added in the sample solutions for intervention, and the intervened samples were then extracted, and prepared as described above. The average recoveries were estimated in the form of Recovery (%) = (amount found – original amount) / amount spiked × 100%, and RSD = (S.D / mean) × 100%. As showed in the Fig.3, the results showed here that the developed method was really reliable and accurate. Overall recoveries were between 95% and 105%, and the RSD % of the calculated contents of 7 compounds were less than 3.0 %.

2.8 The determination of different products and data analysis

All products were prepared as described methods, and total 10 μL solution of each sample was injected into the HPLC system and the contention of the flavonoids was then calculated. All data were collected within Excel 2010; SPSS 13.0 was used for T-test and one way ANOVA; SIMCA 14.1 was used for analysis of PCA and OPLS-DA; and Excel 2010 was also used for some basic description statistical analysis in this research.

3. Results and discussion

Metabolomics, as a new system developed branch of biology, its studied contents are usually used to distinguish biological samples of different systems [14]. However, complex metabolomics' networks may take a lot of time and money in determination and analysis which are also the main barriers to analytical technology now. In fact, the reports on pharmacological activity of compounds have provided almost all active ingredients, such as the *Huangqin* we studied in this paper. So the main chemical components that *Huangqin* produces efficacies are the 7 kinds of flavonoids (Supplementary Fig.1 & Supplementary Fig.2) we detected. Although the detection of flavonoids is not difficult under the modern analytical techniques, and up to now, several analytical methods, including HPLC–UV [15,16], HPLC–ECD [17], UPLC–MS [18-19], and LC–MS/MS [20], have been developed for the determination of baicalin, wogonoside, baicalein, wogonin and oroxylin-A in

biological samples. However, as far as we are aware, no one has reported a simple and accurate method of 7 compounds simultaneously for the quality control of *Huangqi* herb. Since the HPLC has been almost configured to all Chinese herbal medicine enterprises, therefore a HPLC-based accurate quantitative technique is reliable, valuable and expected to provide. Furthermore, a kind of new monitoring strategy for the quality control of *Huangqin* processing is still expecting to be established. Therefore, we have performed the chemical fingerprint and identified the chromatographic peaks of the 7 compounds.

3.1 Chemical fingerprints and chromatographic peak identification

Considering that crude herb could retain flavonoids in the greatest extent, so we have first separated and analyzed 16 batches of crude medicinal herbs by HPLC-DAD under the above methods. As shown in Fig.4, total 20 peaks are separated significantly in the 80 minutes retention time. Benefiting from the previous separation results of phytochemical research in our team, total 14 peaks were identified by using the separated products and purchased standard compounds. This result provided basic chromatographic information for quality control in medicinal materials analysis. Therefore, we preliminarily compared the fingerprints of different processed products. As shown in Fig.5, we obtained some obvious changes after processing that the processing with carbonization made the chemical composition relatively simple than the crude herb, and a large number of flavonoids within 40 minutes of the reservation have been disappeared; but it was interesting that the peak areas of baicalein (Peak 15) and wogonin (Peak 17) had not decreased but a tendency to increase in proportion. So, the proportional characteristics of chemical composition in different processed products were our important investigation direction.

3.2 Content of active ingredients in different processed products

All samples including crude *Huangqin* and other 2 kinds processing products were determined and the content of 7 compounds was accurately calculated using the established standard curves. The results revealed that the proportion of different ingredients in the medicine is different, and the contents of baicalin and wogonoside were the main components in the crude *Huangqin* herb, their average contents were up to 12 % and 4 % respectively which indicated that all the crude *Huangqin* satisfied the standard of *Chinese Pharmacopoeia*

[21]. As shown in Fig.6-A, we discovered that *Huangqin*, processed and parched with alcohol was almost identical to the chemical composition of the crude ones, or more often, the contents of wogonin and baicalein were slightly more than the crude herb, due to the hydrolysis of glycosides as shown in Fig.7. Therefore, this information can be used as important monitoring indicators to control the quality of alcohol *Huangqin* in practical production.

In general, the carbonization of medicinal materials causes a sharp drop in effective ingredients. It seems that carbonized herbs are not a good idea for clinic use. But the traditional Chinese medicine theory supports that carbonized medicinal materials have hemostatic effects. In particular, the degree of carbonation of *Huangqin* needs to be paid attention in its clinical use. In the description of ancient herb standards, we also noticed that the carbonization of *Huangqin* herb needs "keeping its natural instincts". So we were looking forward to finding indicators that can dynamically demonstrate the degree of carbonization in herb processing. As shown in Fig.6-B, the results clearly presented the changes in the chemical compositions after carbonization. First of all, the glycosides including scutellarin, baicalin, wogonoside, are almost completely decomposed; Baicalei, chrysin, oroxylin A also are significantly reduced. It is very interesting that wogonin seem to maintain the same level of content with the crude medicinal materials. Then, whether high content of wogonin is the main medicinal substance of carbonized *Huangqin* to exert hemostasis effect? We were so honored to have searched a recent pharmacology literature [22], which has showed that wogonin prolonged activated partial thromboplastin time and prothrombin time and inhibition of the activities of thrombin and activated coagulation factor X (FXa), as well as inhibited production of thrombin and FXa. In addition, Wogonin also inhibited thrombin catalyzed fibrin polymerization and platelet aggregation and elicited anticoagulant effects in mice. All of those revealed that the wogonin possesses antithrombotic activities. Therefore, wogonin actually hold great potential to become a special pharmacodynamic marker to control the production of carbonized *Huangqin*.

3.3 The effect of processing on the quality of *Huangqin* of different origins

It is indeed that the cultivated geographical area has important influences on the quality

of medicinal materials [23-24]. So the raw medicinal materials from different origins seriously affect the stability of traditional Chinese medicines. However, excellent processing techniques have always been considered to improve this situation. So the test data we obtained have been further analyzed with PCA (Fig.8) and OPLS-DA (Fig.9). The results showed that PCA can only clearly distinguish the crude and carbonized herbs, but cannot distinguish the sources of medicinal materials. The results also showed that the OPLS-DA presented a better analytical model for origin identification that 97 % of samples are correctly distributed in four separate areas. However, artificially grouped OPLS-DA modeling requires a large amount of data for machine learning, so, at present, the amount of data we use for training is not enough to propose more reliable and practical solutions. Just like the Fig.10-A showing, different origins affect the chemical composition, but the intervention on the uniformity of medicinal materials is still unknown. In this study, the dispersion of the contents of each component was statistically analyzed. As shown in Fig.10-B & C, seven tested compounds are prompted that the dispersion of processed herb was significantly reduced. Thus our research showed that current processing techniques can make the quality of medicinal materials more uniform, and our systematic detection method of flavonoids provided clear chemical indicators for quality control in *Huangqin* processing practice.

4. Conclusion

A new monitoring strategy for *Huangqin* processing have been designed and proposed here. First of all, detection of changes in the content of glycosides and aglycones can be used to examine the degree of processing and the uniformity of the products in the processing practice. The important observing targets are wogonin and baicalein, as well as their glycosides. When their contents are slightly higher than the crude herb (>0.85 % & 0.38 %, the specific content parameters should refer to the content of crude medicinal materials of different batch), the processing of the alcohol *Huangqin* should be completed. In addition, since wogonim is directly related to herb efficacy, and relatively difficult to degrade and reduce in the processing practice, so the content of wogonin is best suited as a chemical indicator for carbonated *Huangqin*. The results suggested that the content of wogonim should satisfy 95 % ~ 105% of the content of crude herb. The ratio of the different peak area can also

be used as an indicator of quick judgment in *Huangqin* production. The ratio of peak areas of baicalein (Peak 15) and wogonin (Peak 17) will gradual decline from 2 to nearly 1, which has been regarded as a quick identification signal to guide production of carbonized *Huangqin* in our processing practice. In conclusion, we have developed a simple, cheap, stable and repeatable monitoring approach for *Huangqin* processing and production, this strategy and practice can be promoted and adapted to the quality control of at least 100 products of Chinese medicines in future. In fact, our practice has also provided an effective and real-time technical plan for the quality control of the processing production of Chinese medicines.

Contributions

L.L. LI and Q. HUANG conducted the experiments and data analysis, L.L. L and C.S. CHENG conducted the data analysis and wrote the paper, C. Zhang, and D.Y. Peng designed the experiments.

References

[1] Sheridan, H., Kopp, B., Krenn, L., et al. 2015. Traditional Chinese herbal medicine preparation: invoking the butterfly effect. Sci. **350**(6262), S64-S66.

[2] Xu, W., Wang, S., Lu, J., et al. 2018. Seeing the unseen of Chinese herbal medicine processing (paozhi): advances in new perspectives. Chin. Med. **13**(1), 4.

[3] Zhao, Z.Z., Liang, Z.T., Lu, G.H., et al. 2010. A unique issue in the standardization of chinese materia medica: processing. Planta. Med. **769**(17), 1975-1986.

[4] Zhang, D.Y., Wu, J., Ye, F., et al. 2003. Inhibition of cancer cell proliferation and prostaglandin E2 synthesis by scutellaria baicalensis. Cancer Res. **63**(14), 4037.

[5] Jeong, Y.C., Shin, S., Park, J.S., et al. 2011. Ethanol extract of *scutellaria baicalensis*

Georgi prevents oxidative damage and neuroinflammation and memorial impairments in artificial senescense mice. J. Biomed. Sci. **18**(1), 14.

[6] Li, H.B., Jiang, Y., Chen, F., 2004. Separation methods used for *Scutellaria baicalensis* active components. J. Chromatogr. B. **812**(1-2), 277-290.

[7] Choi, Y.A., Kang, O.H., Park, H.J., et al. 2005. Effect of processed *scutellaria baicalensis* on dextran sulfate sodium-induced colitis in mice. Int. J. Mol. Med. **16**(4), 667-672.

[8] Park, J.S., Park, H.Y., Kim, D.H., et al. U.S. 2016. Patent No. 9,327,007. Washington, DC: U.S. Patent and Trademark Office. 2-3.

[9] Torreggiani, D., Bertolo, G., 2001. Osmotic pre-treatments in fruit processing: chemical, physical and structural effects. J. Food Eng. **49**(2-3), 247-253.

[10] Zhao, J., Wang, M., Avula, B., et al. 2015. Change of chemical composition in Flos lonicerae: An investigation of herbal processing (PAO ZHI) using NMR spectroscopy. Planta. Med. **81**(11), 11.

[11] Zhao, J., Wang, M., Avula, B., et al. 2015. Effect of Processing on the traditional Chinese herbal medicine flos lonicerae: An NMR-based chemometric approach. Planta. Med. **81**(09), 754-764.

[12] Sumner, L.W., Pedro, M., Richard, A., 2003. Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry*. **62**(6), 817-836.

[13] Li, M.X., Liu, X.M., Zhang, X.F., et al. 2014. Prognostic role of neutrophil to lymphocyte ratio in colorectal cancer: A systematic review and meta-analysis. Int. J. Cancer. **134**(10), 2403-2413.

[14] Wang, M., Lamers, R.J., A., Korthout, H.A., et al. 2005. Metabolomics in the context of systems biology: bridging traditional Chinese medicine and molecular pharmacology. Phytother. Res. **19**(3), 173-182.

[15] Lu, T., Song, J., Huang, F., et al. 2007. Comparative pharmacokinetics of baicalin after oral administration of pure baicalin, *Radix scutellariae* extract and Huang-Lian-Jie-Du-Tang to rats. J. Ethnopharmacol. **110**(3), 412-418.

[16] Li, C., Zhang, L., Lin, G., et al. 2011. Identification and quantification of baicalein, wogonin, oroxylin A and their major glucuronide conjugated metabolites in rat plasma after

oral administration of Radix scutellariae product. J. Pharmaceut. Biomed. **54**(4), 750-758.

[17] Kotani, A., Kojima, S., Hakamata, H., et al. 2006. HPLC with electrochemical detection to examine the pharmacokinetics of baicalin and baicalein in rat plasma after oral administration of a Kampo medicine. Anal. Biochem. **350**(1), 99-104.

[18] Wang, Y., Yao, Y., An, R., et al. 2009. Simultaneous determination of puerarin, daidzein, baicalin, wogonoside and liquiritin of GegenQinlian decoction in rat plasma by ultra-performance liquid chromatography–mass spectrometry. J. Chromatogr. B. **877**(20-21), 1820-1826.

[19] Tong, L., Wan, M., Zhang, L., et al. 2012. Simultaneous determination of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin of Radix scutellariae extract in rat plasma by liquid chromatography tandem mass spectrometry. J. Pharmaceut. Biomed. **70**, 6-12.

[20] Ye, J.X., Wei, W., Quan, L., et al. 2010. An LC–MS/MS method for the simultaneous determination of chlorogenic acid, forsythiaside A and baicalin in rat plasma and its application to pharmacokinetic study of Shuang-huang-lian in rats. J. Pharmaceut. Biomed. **52**(4), 625-630.

[21] China Pharmacopoeia Committee. 2015. Chinese pharmacopoeia. China Medical Science Press: Beijing, China. 300-301.

[22] Ku, S.K., Bae, J.S., 2014. Antithrombotic activities of wogonin and wogonoside via inhibiting platelet aggregation. Fitoterapia. **98**, 27-35.

[23] Cheng, C., Liu, Z., Tan, T., et al. 2016. Investigation on stress factors causing dao-di production area change of astragalus membranaceus using current and historical geographic and climatic information. Modernization of Traditional Chinese Medicine and Materia Medica-World Science and Technology. **18**(1), 11-17.

[24] Cheng, C., Yuan, Q., Zhou, H., et al. 2016. Nondestructive estimation of growth year in ginseng cultivars using the means of mathematical modeling on the basis of allometry. Microsc. Res, Techniq. **79**(2), 98-105.