DEVELOPMENT OF THE QUANTIFIED RELATIONSHIP BETWEEN CHEMICAL COMPOSITIONS AND ANTIOXIDANT ACTIVITY OF RADIX ET RHIZOMA SALVIAE MILTIORRHIZAE, RADIX PUERARIAE LOBATAE AND THE HERBAL FORMULA OF DANSHEN-GEGEN TANG (DGT) USING LIQUID CHROMATOGRAPHY AND CHEMOMETRIC TECHNIQUES

CHAN HOI YAN

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Department of Applied Biology and Chemical Technology

Development of the quantified relationship between Chemical Compositions and Antioxidant Activity of Radix et Rhizoma Salviae Miltiorrhizae, Radix Puerariae Lobatae and the herbal formula of Danshen-Gegen Tang (DGT) using Liquid Chromatography and Chemometric Techniques

Chan Hoi-yan

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

April, 2010

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

CHAN Hoi-yan

ABSTRACT

Abstract of the thesis entitled

"**Development of the quantified relationship between Chemical Compositions and Antioxidant Activity of Radix et Rhizoma Salviae Miltiorrhizae, Radix Puerariae Lobatae and the herbal formula of Danshen-Gegen Tang (DGT) using Liquid Chromatography and Chemometric Techniques**"

submitted by Chan Hoi-yan
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Herbal medicine (HM) is widely used for preventing and healing diseases in these years. It is necessary to ascertain its consistency, safety and efficacy through getting related information and evidences from scientific investigation. Correlation of the chemical composition with traditionally intended medication is one of the important targets. It is difficult to achieve this owing to the complexity of HM and the holistic concept of the working principle involved in disease treatment. Currently, the authentication of HM is based on "compound-oriented approach" and "pattern-oriented approach". The former approach mainly monitors the contents of several selected chemical markers for the measurement and it is widely accepted by authorized agencies in the world. As for the latter approach, it considered all the detectable components appeared in the chromatographic fingerprint for assessment. Still, the efficacy of HM cannot be reflected from these two approaches. The major reason is that the bioactive constituents are not the key concern. Thus, it is interested to explore the bioactive components in HM as they are responsible for the pharmacologic activity. This could aid in improving the quality control measures, getting better understanding on the therapeutic effect of HM and finding drug lead candidates from the natural sources. Usually, only a few so called active ingredients are considered even if the bioactivity aspect is under investigation. How to

include more active ingredients and significant ones is still a great challenge to scientists. In this study, the quantified relationship between the chromatographic fingerprint and bioactivity of HM through the Quantitative Pattern-Activity Relationship (QPAR) approach was proposed and chemometric techniques were developed and applied to these two types of experimental data. The QPAR model thus established by us provides two pieces of crucial information about HM. They are (1) a model for predicting total bioactivity from the chromatographic fingerprint and (2) the features in the chromatographic profile responsible for the bioactivity. In this work, the Chinese herbal medicine Radix et Rhizoma Salviae Miltiorrhizae (DS), Radix Puerariae Lobatae (YG) as well as the herbal formula of Danshen–Gegen Tang (DGT) and a synthetic mixture system (MIX) were studied by both "compound-oriented approach" and "pattern-oriented approach". Their chemical fingerprints through HPLC-DAD-MS and RRLC-DAD instruments and antioxidant activities from FRAP assay were measured for data processing. In addition, the QPAR approach was applied to YG and MIX also to evaluate the performance of our QPAR methods developed and information mined.

The bioactivity of HM is affected by the inherent variations of HM, the manufacturing process and other factors. Definitely, one of them is sample extraction. The extraction parameters affect the class of components and their quantities to be extracted. This leads to variation in the bioactivity level of the extract obtained when different sample preparation procedures are used. In this work, the optimal extraction condition of Radix et Rhizoma Salviae Miltiorrhizae (DS) in response to the antioxidant activity was explored and determined by response surface methodology (RSM). Through which, we investigated the relationship of bio-response and extraction parameters. In this way, the optimal antioxidant activity of DS was achieved by using 80% methanol with the volume of 90ml for 45minutes under ultrasound assisted extraction. Twenty DS samples collected from different provinces of P. R. China were investigated under the optimal extraction conditions. It was found that the antioxidant activity of DS is related to the amounts of phenolic acids instead of diterpenoids.

The antioxidant activity of another HM, Radix Puerariae Lobatae (YG), was also studied.

Both the bioactivity and chemical analysis were carried out on seventy eight YG samples. Through these two types of experimental data acquired, the quantified relationship between the chromatographic fingerprint and antioxidant activity of YG was established using the chemometric methods, PLS and UVE-PLS. Through which, on top of the chemical composition information, the antioxidant activity level of any YG sample including the unknown one can be reflected from its chromatographic fingerprint. The performance of determining the antioxidant activity level from the established relationship is good with the root mean square error for training set of 76.88 (6.37%) and the root mean square error of prediction for validation set of 79.59 (6.60%). The correlation coefficients of the predicted and experimental antioxidant capacities of the samples in the training and validation sets are 0.9318 and 0.8926 respectively.

Exploration of bioactive components in YG was further attempted through establishing the QPAR relationship by another chemometrics algorithm, Target Projection coupled with Selectivity Ratio (TP/SR). The potential bioactive candidates were revealed at three regions. Their respective retention times are (G1) 12.5min, (G2) 13.3min and (G3) 15.6min. This prediction was proven by our experimental fractionation of YG samples. Furthermore, the discovery of bioactive components was done on another system which we called it as synthetic mixture system (MIX). They were made up of twelve different classes of reference compounds commonly found in HM. The most active components in this system were picked up by this algorithm. They were gallic acid (GA) and quercetin (QT). These findings show that our quantified relationship of chemical fingerprint and bioactivity could aid in studying the pharmacological activity of HM in more detail, drug discovery from the natural source, and upgrading the quality assessment HM with both chemical composition and bioactivity considered at the same time.

In another study, the optimal ratio of two component herbs, DS and YG, in the DGT preparation was determined with the application of the chromatographic technique and in-vitro antioxidant assay. Rapid resolution liquid chromatography (RRLC) was utilized in this investigation because of its rapid analysis, improved resolution and sensitivity. It helped markedly in analyzing complex systems like DGT with many components

involved. Concerning both chemical composition and antioxidant activity, the best combination ratio of DS and YG was determined to be 6:4. The outcomes and methods proposed could provide a useful tool in finding out the optimal combination of the component herbs in other herbal formulae.

PUBLICATION AND PRESENTATION

Journal Articles

1. F.T. Chau, H.Y. Chan, C.Y. Cheung, C.J. Xu, Y.Z. Liang and O.M. Kvalheim. "**Recipe for Uncovering the Bioactive Components in Herbal Medicine**." Analytical Chemistry, 2009, 81: 7217-7225.

2. O.M. Kvalheim, H.Y. Chan, I.F.F. Benzie, Y.T. Szeto, A.H.C. Tzang, D.K.W. Mok, and F.T. Chau. "**Chromatographic Profiling and Multivariate targeted analysis for revealing and quantifying the contributions from individual components to the Bioactive Signature in Herbal Medicine**." Article submitted.

3. Z.D. Zeng, F.T. Chau, H.Y. Chan, C.Y. Cheung, T.Y. Lau, S.Y. Wei, D.K.W. Mok, C.O. Chan and Y.Z. Liang. "**Review: Recent advances in the compound-oriented and pattern-oriented approaches to the quality control of herbal medicines**" Chinese medicine 2008, 3**:**9

Conference/ Symposium Presentation

1. H.Y. Chan and F.T. Chau, **"Chemical analyses of saikosaponins A and D in** Radix ***Bupleurum*** **by HPLC"**. Poster presentation at the 13th Symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 22 April, 2006.
2. H.Y. Chan and F.T. Chau, **"Evaluation of the quality of *Ginkgo Biloba* commercial products by using the Chromatographic Pattern Approach"**. Poster presentation at the 26th International Symposium on Chromatography, Denmark, 21-25 August, 2006.
3. H.Y. Chan and F.T. Chau. **"Evaluation of antioxidant activity of *Ginkgo biloba* dietary supplement product under different sample preparation"**. Poster presentation at the 14th Symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 28 April, 2007.

4. H.Y. Chan and F.T. Chau. "**Studying the effect of extraction conditions using Uniform Design on antioxidative activity of *Ginkgo biloba* dietary supplement products"**. Poster presentation at 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 16 August, 2007.
5. C.J. Xu, T.Y. Lau, H.Y. Chan, C.Y. Cheung and F.T. Chau. "**Pre-processing of liquid chromatography/mass spectrometry data in herbal medicine system-a comparison study**". Poster presentation at 14th EuroAnalysis, Antwerp, Belgium, 9-14 September, 2007.
6. H.Y. Chan and F.T. Chau. "**Validation of analytical method using Pattern Approach and Marker Approach"**. Poster presentation at the 15th symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 26 April, 2008.
7. H.Y. Chan, C.O. Chan, C.Y. Cheung, T.Y. Lau and F.T. Chau. "**Comparing the antioxidant activity of the extract of Radix *Puerariae Lobatae* with the mixtures of its isoflavonoid components"**. Poster presentation at 2008 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 15 August, 2008.
8. H.Y. Chan, C.Y. Cheung, T.Y. Lau, C.J. Xu and F.T. Chau. "**Estimation of the antioxidant activity of Radix *Puerariae Lobatae* (Gegen) from its chemical-antioxidant activity fingerprint**". Poster presentation at 11th Scandinavian Symposium on Chemometrics, Norway, 8-11 June, 2009.
9. F.T. Chau, O.M. Kvalheim, H.Y. Chan and Y.Z. Liang. "**A very powerful tool for herbal medicine: QPAR technique**". Poster presentation at the 4th International Functional Food Symposium, Hong Kong, 29 October, 2009.
10. F.T. Chau, H.Y. Chan, T.Y. Lau and D.K.W. Mok. "**Recent progress in quality control of Chinese Medicine using both chemical and bioactivity information**". Poster presentation at the International Conference on Resource Development of Chinese Medicines from Northwestern China, Hong Kong, 10-11 December, 2009.
11. H.Y. Chan, T.Y. Lau, O.M. Kvalheim, D.K.W. Mok, C.O. Chan and F.T. Chau. "**Revealing the potential antioxidants in Radix Puerariae Lobatae (YG) using QPAR technique**." Poster presentation at the 17th symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 24 April, 2010.

Courses attended

1. **A one day training course on: TCM fingerprint techniques and chemometrics**. Lectured by Prof. F.T. Chau, Prof. O.M. Kvalheim, Prof. Y.Z. Liang and Prof. P.S. Xie. A course offered in the Hong Kong Polytechnic University, 22 February, 2006.
2. **Multivariate data analysis in chromatography.** Lectured by Prof. Rasmus Bro, in the 26th International Symposium on Chromatography, Denmark, 21 August, 2006.
3. **Workshop on recent development of TCM fingerprint analysis techniques and the New SFDA TCM stability Guidelines**. Lectured by Prof. F.T. Chau, Prof. P.S Xie, Prof. Y.Z. Liang, Dr. Daniel K.W. Mok and Dr. C.O. Chan. The workshop offered by the Department of Applied Biology and Chemical Technology of the Hong Kong Polytechnic University, 7 June, 2007.
4. **Workshop on recent progress in chemical fingerprint-bioactivity analysis techniques of Chinese Medicine.** Lectured by Prof. F.T. Chau, Prof. O.M. Kvalheim, Prof. Y.Z. Liang and Prof. P.S. Xie. The workshop offered by the Department of Applied Biology and Chemical Technology of the Hong Kong Polytechnic University, 18 October, 2008.
5. **Workshop on UGC-Areas of Excellence Project and Innovation and Technology Fund Project.** The workshop offered by the Institute of Chinese Medicine, the Chinese University of Hong Kong, 14 February, 2009
6. **Workshop on System Biology for TCM research**. The workshop offered by the School of Chinese Medicine, Hong Kong Baptist University, 23 April, 2009
7. **Workshop on recent advancement in linking bioactivities and chemical fingerprints of herbal medicine together for plant drug development.** Lectured by Prof. F.T. Chau and Prof. O.M. Kvalheim. The workshop offered by the Department of Applied Biology and Chemical Technology of the Hong Kong Polytechnic University, 27 October, 2009.
8. **International Conference on Resource Development of Chinese Medicines from Northwestern China**. The conference organized by The Hong Kong University of Science and Technology, 10-11 December, 2009

Committee for Symposium

1. Committee member of 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 16 August, 2007.
2. Committee member of 2008 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 15 August, 2008.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

β-PE	β-phycoerythrin
AAPH	2,2'-azo-bis(2-amidinopropane) dihydrochloride
$ABTS^{+\cdot}$	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)
AHP	American Herbal Pharmacopoeia
AMWFA	Alternative Moving Window Factor Analysis
ANN	Artificial Neural Networks
BA	Betulinic acid
BHMA	British Herbal Medicine Association
CASE	Computer Aided Similarity Evaluation
CE	Capillary Electrophoresis
CMA	Coumaric Acid
COW	Correlation Optimized Warping
CP	Chinese Pharmacopoeia
CRM	Chemometric Resolution Methods
DA	Danshensu
DAD	Diode Array Detector
DE	Daidzein
DGT	Danshen-Gegen Tang
DPPH	2, 2-diphenyl-1-picrylhydrazyl radical
DS	Radix et Rhizoma Salviae Miltiorrhizae
DSA	Ultrasonic extract of Radix et Rhizoma Salviae Miltiorrhizae
DSB	Boiling extract of Radix et Rhizoma Salviae Miltiorrhizae
DZ	Daidzin
ED	Emodin
ELSD	Evaporative Light Scattering Detector
EMEA	European Medicines Agency
EWOP	Evolving Window Orthogonal Projection
ESI	Electrospray Ionization
FA	Ultrasonic extract of Danshen-Gegen Tang
FA2	Mixtures of ultrasonic extracts of Radix et Rhizoma Salviae Miltiorrhizae and Radix Puerariae Lobatae
FB	Boiling extract of Danshen-Gegen Tang
FB2	Mixtures of boiling extracts of Radix et Rhizoma Salviae Miltiorrhizae and Radix Puerariae Lobatae
FDA	the Food and Drug Administration
FG	*Pueraria Thomsonii* Benth.
FRAP	Ferric Reducing Antioxidant Power assay
FSMWEFA	Fixed Size Moving Window Evolving Factor Analysis
GA	Gallic Acid
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
GE	Genistin

GS	Genistein
HB	Hydroxybenzoic Acid
HELP	Heuristic Evolving Latent Projections
Hep-2	Laryngeal Epidermoid Carcinoma
HKCMMS	Hong Kong Chinese Materia Medica Standards
HM	Herbal Medicine
HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography – Diode Array Detector
HPLC-DAD-ELSD	High-Performance Liquid Chromatography – Diode Array Detector – Evaporative Light Scattering Detector
HPLC-DAD-MS	High-Performance Liquid Chromatography – Diode Array Detector – Mass Spectrometer
HPLC-UV	High-Performance Liquid Chromatography – Ultraviolet
KF	Kaempferol
KHP	Korean Herbal Pharmacopoeia
k-NN	k-nearest neighbor
KP	Korean Pharmacopoeia
LC-MS	Liquid Chromatography-Mass Spectrometer
MIX	Synthetic Mixture
MS	Mass Spectrometer
MSCC	Multi-Component Spectral Correlative Chromatography
NIR	Near-Infrared
O-PLS	Orthogonal Partial Least Squares
ORAC	Oxygen Radical Absorbance Capacity
PCA	Principal Component Analysis
PHPLC	Preparative High-Performance Liquid Chromatography
PLS	Partial Least Squares
PU	Puerarin
QPAR	Quantitative Pattern-Activity Relationship
QT	Quercetin
R	Correlation Coefficient
RA	Rosmarinic Acid
RBC	Red Blood Cell
RMSE	Root Mean Square Error
RMSECV	Root Mean Square Error of Cross Validation
RMSEP	Root Mean Square Error of Prediction
ROS	Reactive Oxygen Species
RRLC	Rapid Resolution Liquid Chromatography
RRLC-DAD	Rapid Resolution Liquid Chromatography – Diode Array Detector
RSD	Relative Standard Deviation
RSM	Response Surface Methodology
RT	Rutin
SAB	Salvianolic Acid B
SFA	Subwindow Factor Analysis

SFDA	The State Food and Drug Administration
SI	Similarity Index
SPA	Successive Projections Algorithm
SR	Selectivity Ratio
SVD	Singular Value Decomposition
TCM	Traditional Chinese Medicine
TEAC	Trolox Equivalent Antioxidant Capacity
TOF	Time-Of- Flight
TP	Target Projection
TP/SR	Target Projection coupled with Selectivity Ratio
TSI	Tanshinone I
TSIIA	Tanshinone IIA
UA	Ursolic acid
UPLC	Ultra-Performance Liquid Chromatography
UPLC-DAD	Ultra-Performance Liquid Chromatography – Diode Array Detector
USP	United States Pharmacopoeia
UV	Ultraviolet
UVE	Uninformative Variable Elimination
UVE-PLS	Uninformative Variable Elimination by Partial Least Squares
VIP	Variable Importance Projection
WHO	World Health Organization
YG	Radix Puerariae Lobatae
YGA	Ultrasonic extract of Radix Puerariae Lobatae
YGB	Boiling extract of Radix Puerariae Lobatae

Symbols

$\mathbf{U}_p$	Scores matrix of SVD	Eq [2.1]
$\mathbf{V}_p$	Loading matrix of SVD	Eq [2.1]
$\mathbf{S}_p$	diagonal matrix	Eq [2.1]
E	Matrix of **X**-residuals	Eq [2.1], Eq [2.6] and Eq [2.10]
$\mathbf{a}_k$	Linear combination coefficient of **E**	Eq [2.2]
$\mathbf{b}_k$	linear combination coefficient of **F**	Eq [2.2]
$\mathbf{E}_x$	Orthogonal matrix of **X**	Eq [2.2]
$\mathbf{F}_y$	Orthogonal matrix of **Y**	Eq [2.2]
$\mathbf{s}_k$	Common component	Eq [2.2]
T	Scores matrix	Eq [2.6] and Eq [2.10]
P	Loading matrix	Eq [2.6] and Eq [2.10]
X	Matrix of predictors variables	Eq [2.6], Eq [2.7] and Eq [5.1]
Y	Matrix of response variables	Eq [2.7]
B	Matrix of regression coefficients of all **Y**'s	Eq [2.7] and Eq [2.13]
F	Matrix of **Y**-residuals	Eq [2.7] and Eq [2.11]
t	**X** scores	Eq [2.8]
w	**X** weights	Eq [2.8]
u	**Y** scores	Eq [2.9]
q	**Y** weights	Eq [2.9]
b	Regression coefficient vector	Eq [2.12] and Eq [5.1]
$\mathbf{X}^+$	Generalized Inverse of reproduced **X**	Eq [2.12]
$\mathbf{X_{TP}}$	Single component TP model of **X**	Eq [2.14] and Eq [6.1]
$\mathbf{t}_{TP}$	Target projected scores	Eq [2.14] and Eq [6.1]
$\mathbf{p}_{TP}$	Target projected loading	Eq [2.14], Eq [2.16] and Eq [6.1]
$\mathbf{E}_{TP}$	Residual of target projection	Eq [2.14], Eq [2.17] and Eq [6.1]
$\mathbf{w}_{TP}$	Normalized regression coefficients vector	Eq [2.15]
$\mathbf{X}_{ort}$	Orthogonal part of **X**-matrix	Eq [2.18]
$\mathbf{X}_{pred}$	Predictive component of **X**-matrix	Eq [2.18]
$\mathbf{T}_{ort}$	Orthogonal scores	Eq [2.18]
$\mathbf{P}_{ort}$	Orthogonal loading	Eq [2.18]
$\mathbf{t}_{pred}$	Predictive scores	Eq [2.18]
$\mathbf{p}_{pred}$	Predictive loading	Eq [2.18]
$\mathbf{b}_j$	Regression coefficient of j variable	Eq [2.19] and Eq [5.2]
$\mathbf{s}_j$	Stability of the variable	Eq [2.19] and Eq [5.2]
$\mathbf{V_{expl,i}}$	Explained variance of variable	Eq [2.20] and Eq [6.2]
$\mathbf{V_{res,i}}$	Residual variance of variable	Eq [2.20] and Eq [6.2]
X_i	Input parameter or factor at i^{th} level	Eq [4.1] and Eq [4.2]
Y_i	Response at i^{th} level	Eq [4.1] and Eq [4.2]
β	Regression coefficient of X factors	Eq [4.1] and Eq [4.2]
y	Response vector	Eq [5.1]
f	Residual vector	Eq [5.1]
y^i	Predicted value of i^{th} sample	Eq [5.3] and Eq [5.4]
y_i	Experimental value of i^{th} sample	Eq [5.3] and Eq [5.4]
y_m	Mean of experimental result	Eq [5.4]

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Chapter 1

Introduction

The usage of Herbal Medicine (HM) for preventing and healing disease has been a history of thousands of years. There is an increasing acceptance of HM in the treatment of disease in these years. More than 1.5 billion people over the world believe in the efficacy of the HM [1]. It may be related to the beneficial effect of HM as it is a natural source and its side effect is supposed to be less than that of the western drug. More that that, it could deal with the limitation of western drug in curing certain complex disease [2]. It is mainly because the western medicine targets on individual part of the human body while HM concerns the balance of the whole body. The therapeutic effect of HM is more effective on this. Furthermore, application of HM is not only focused on the remedy but also concerning the prevention of disease. For example, the combination of Danshen and Gegen could help in the secondary prevention of atherosclerosis [3]. Nowadays, drug discovery is facing the bottleneck. The rich sources of drug candidates can be obtained from the nature including herbal medicine. Because of these, HM is catching more attention by the public.

However, the scientific information on the effectiveness and the systematic mechanistic studies of HM are insufficient. One of the difficulties have to be encountered in studying HM is its complexity. Here, complexity is related to the chemical compositions and the practice of treatment. Firstly, there are thousands of compounds in HM. Most of them remained unknown resulting in that the possible bioactive components are unidentified for further mechanistic study. Besides, the activity of HM is aimed at system level via the interaction with multiple targets in human body [4]. This is the second complexity of HM. It is because the working principle of HM aims at maintaining the balance of the whole body [5]. Thus, it is totally different from that of the western medicine in diagnosis and disease treatment. Another challenge for thorough study of HM is the safety and quality concern. It is crucial to maintain the batch to batch or manufacturer to manufacturer consistency of the HM product. But it is unavoidable to have intrinsic variations originating from seasonal variation, geographical variation, growing condition, harvesting method and post-harvesting formulation process [6]. This would result in chemical non-equivalence including the contents of the constituents and the chemical profiles. Moreover, the manufacturing procedures have not been standardized for

processing HM and, hence, the therapeutic effect of the final products may be inconsistent. Eventually, appropriate quality control measures are required in ascertaining the effectiveness, safety and consistency.

Currently, quality control measures of HM included “compound-oriented approach” and “pattern-oriented approach”. The authentication of HM is mainly based on a few selected chemical reference compounds or chemical markers in the former approach. The quality evaluation is assessed by measuring the discrepancy of selected compounds in HM. But the bioactivity of the reference compounds or sometimes called markers may not represent the therapeutic effect of HM. That is, these chemical markers are not the most active or even inactive ingredients under scrutiny. Therefore, it is unable to reflect the quality of the HM from the pharmacological point of view. Moreover, these markers may also be constituents in other HMs. This leads to confusion during adulteration [7, 8]. In the “pattern-oriented approach”, the whole chromatographic fingerprint of the HM is used in quality assessment by taking the comprehensive strategy. Thus, the variations among the HMs are evaluated by utilizing all the detectable components displayed in the fingerprint. Still, the biological activity of HM is not appraised.

In fact, the ideal situation in modernization of Chinese medicine requires thorough quality proof of HM and correlation of its chemical composition with the traditionally intended medication [9]. The existing quality control approaches are insufficient in reflecting the therapeutic effectiveness of HM. Moreover, the theory of HM in prevention, diagnosis or treatment of disease is based on an integrity concept. The same theory should also be applied in the quality control in the ideal situation such that multiple components are considered during quality assessment of herbal medicine. The objective of this research work is trying to assess the quality of HM through the above two approaches as well as to correlate the chemical composition and biological activity of HM at the same time. As a result, the consistency, safety and efficacy are considered in our newly proposed quality control method.

Since HM is composed of multiple constituents, separation is necessary for the chemical

analysis. In recent years, characterization of chemical constituents of HM is carried out by the advanced modern technology. It is common to use high-performance liquid chromatography (HPLC) or gas chromatography (GC) to analyze non-volatile or volatile compounds. More and more studies on chemical analysis of HM were preformed by improved chromatographic techniques. Now, better resolution can be obtained by rapid resolution liquid chromatography (RRLC) or ultra-performance liquid chromatography (UPLC). It is because the separation column used is made up by the smaller particle size which could sustain the high pressure. Subsequently, it could enhance the separation of the very complicated HM system. As a whole, all these instruments are coupled with different kinds of detectors. The most commonly utilized detector is diode array detector (DAD), a multi-channel UV detector. However, it is limited to compounds with chromophores. Another one which has been widely utilized for identifying compounds is mass spectrometer (MS). Through which, the chemical structures of these compounds can be elucidated from the mass to charge ratio and the subsequent MS/MS analysis. It helps a lot in getting more information about the constituents of HM.

Apart from studying the chemical compositions, HM is subjected to different biomolecules or function of the cell, tissues and organs to examine the interaction among them. Various kinds of experimental processes have been targeted to determine the bioactivity and discovery of bioactive components within [10, 11]. One of the screening methods involves the investigation of the metabolite profiles in biofluids or interaction with the target cells or molecules such as enzyme. The development of biofingerprinting chromatogram is an alternative in exploring relationship of HM and the biomolecules [10-17]. Also, the finding of bioactive components is done by bioassay guided fractionation. In this way, the fractionation and bioassay were investigated step by step aiming at finding out the bioactive compounds [18, 19]. There is no doubt that these approaches allow better understanding on the pharmacological property of HM. Indeed, chemometric techniques can also help improving the interpretation of chemical and biological information together. Furthermore, the established relationship enables detection of bioactive components of HM and the estimation of the bioactivity capacity from the chromatographic profiles simultaneously.

In this research work, the chemical composition of two herbal medicines, Radix et Rhizoma Salviae Miltiorrhizae (DS) and Radix Puerariae Lobatae (YG) were recorded by HPLC-DAD-MS instrument. Apart from this, the combination of these two herbs which is known as Danshen-Gegen Tang (DGT) was examined through the newly developed RRLC-DAD instrument. Their antioxidant properties were focused in the meantime. The balance of antioxidant and oxidant is necessary to avoid having the oxidant stress which would result in different diseases such as cancer, Alzheimer disease, cerebral ischemic damage, cardiovascular disease or diabetes. The oxidant or known as reactive oxygen species (ROS) would attack the cellular system, DNA or protein. Because of this, there is increasing concern on the protective effect exerted by HM on the cellular system in these years. Ferric Reducing Antioxidant Power assay (FRAP) was adopted in this work to evaluate the antioxidant properties.

In addition, the relationship between the chemical composition and biological activity of HM is going to be established. This would require the help of chemometric technique to do so. In Chapter 2, the recent chemical analyses and in-vitro bioassay on antioxidant activity will be reviewed and discussed. Also, the chemometric techniques that are commonly applied in analyzing HM will be described briefly. Furthermore, the establishment of the relationship via multivariate modeling will be mentioned. Apart from these, the current quality control approaches are introduced as well.

It is well known that there are inherent variations among the HM due to different cultivation practice and conditions. Not only this, but also the manufacturing process is another factor influencing the efficacy of the final products. Usually, the extraction procedures are optimized to obtain the assigned amounts of a few selected chemical components. Actually, the therapeutic effect of HM is due to the contribution of various constituents. Hence, optimization of the procedures should be based on attaining the assigned or desired levels of biological activities. Here, the response surface methodology (RSM) was applied in finding the best combination of different extraction parameters with respect to biological activity. In Chapter 4, optimization of the

antioxidant capacity of the Radix et Rhizoma Salviae Miltiorrhizae (DS) sample was undergone. This Chinese medicine has been used for prevention and treatment of coronary arteriosclerosis, angina pectoris and hyperlipaemia, promotion of the circulation, improvement of blood stasis and protection of the heart against ischemia-reperfusion injury [20-25]. Also, it is good for treating hepatitis, chronic renal failure, dysmenorrheal, hepatocirrhosis and neuroasthenic insomnia [26-33], clearing away heat, relieving vexation, nourishing blood and tranquilizing the mind [22]. These pharmacological activities of DS were attributed to the phenolic acids and diterpenoids. They are the major classes of components that are well known antioxidants and are related to the antioxidant activity of DS. Twenty DS samples collected from different regions of P. R. China will be compared according to their chemical compositions and antioxidant activities in this study using the optimal extraction condition found.

Both chemical composition and the biological activities are crucial information to have better understanding about HM. As mentioned previously, the current quality control measures are mainly focused on identification and quantification of selected chemical reference compounds. An alternative for quality control is to measure the variations of both the target and non-target compounds that are displayed in the chromatographic fingerprints or detected by the instrument or method used. Both methods are inadequate in ascertaining the therapeutic effectiveness of HM. In Chapter 5, we will focus on the establishment of the relationship between the chromatographic fingerprint and related biological activity of Radix Puerariae Lobatae (YG). YG exhibits important pharmacological activities such as relieving fever and dysentery, promoting the production of body fluid, facilitating eruption and lessening stiffness and pain of the nape, and for the treatment of cardiovascular diseases like hypertension [34-38]. Isoflavonoid is the major class of constituents in YG which is phytoestrogen. It can prevent cancers, have antiproliferative effects, reduce the risk of osteoporosis, lower plasma cholesterol and decrease the risk of coronary heart disease [39-42]. They showed protection against oxidative DNA damage or attenuated oxidative stress because of their antioxidant activities [41, 43]. Here, the chemometric techniques including Partial Least Squares method (PLS) and Uninformative Variable Elimination by Partial Least Squares (UVE-

PLS) are utilized in correlating these two pieces of information. Based on the model thus established, the antioxidant activity of a YG sample can be estimated from its chromatographic fingerprint. In this way, the chromatographic fingerprint provides both chemical composition and the quality level of the HM for a specific bioactivity simultaneously.

Complexity of HM is one of the major difficulties in examining its treatment mechanism on disease. Most constituents in HM are unknown and it is very hard to find out which components are responsible for the therapeutic effect. Multiple studies were focused on the exploration of the bioactive components like bioassay guided fractionation or various screening procedures. This would help in further investigation of the interaction of the components with the cellular system. Also, this could deal with the drawback of the current "compound-oriented approach" as mentioned previously. Very often, the selected chemical reference compounds did not represent the therapeutic effect of the HM well. Thus, doubt is casted on the quality of HM. Not only this, the finding of the bioactive candidates from HM could assist in the drug discovery. In this study, discovery of the components that are related to the biological activity involved can be preformed through the established relationship. We tried to identify the bioactive candidates from YG utilizing Target Projection coupled with Selectivity Ratio (TP/SR) with results presented. In this study, we found that application of appropriate chemometric technique could greatly shorten the time in the discovery process with high accuracy. Furthermore, fractionation of YG was preformed in order to prove the finding from TP/SR. Apart from this, the revealing of the bioactive components in another complex system, synthetic mixtures (MIX), was also taken place using TP/SR. The results will be discussed in Chapter 6.

In the previous chapters, the quantitative relationship between the chemical composition and the bioactivity was discussed on the single herbal medicine. Traditionally, diagnosis through the use of HM was based on the usage of several herbs together. It is believed that the synergistic or additive effect among the components in different herbs could enhance the biological activity and related therapeutic effects. On the contrary, the

toxicity of the HM can be reduced due to the antagonistic effect of the constituents [1]. This is regarded to be the consequence of the specific combination of HMs in a formula. The working principles of the compound formulation in traditional Chinese medicine are mainly based on this. The herbal formula, Danshen-gegen Tang (DGT), composed of the two herbs, Danshen and Gegen. It can be applied in the treatment of cardiovascular disease. DGT could modulate the key early events in atherosclerosis which had the significant effects on foam cell formation in vitro [44, 45] and it was able to exhibit the secondary prevention of the atherosclerosis in the clinical trial [3]. Protection on the cardiovascular system by DGT is due to the antioxidant activity which may be affected by the relative amounts of DS and YG present in DGT. In Chapter 7, the effect of different combination ratio of these two herbs on antioxidant activity and chemical composition of DGT will be discussed based on our study. Not only this, but also the relationship between the contents of the identified reference components and the antioxidant activity will be inspected.

To summarize, the use of HM in diagnosis and curing disease is widely accepted these years. But more solid scientific proofs and evidences are required. It is also important in ensuring the quality of HM in modernization of HM. To have quality control of HM in more advanced level, linkage between the chemical composition and the biological activity is a must so as to ensure the consistency, safety and efficacy of HM. From the established relationship, the bioactive candidates of HM can be explored. This could assist in quality control and studying the practice of treatment using HM. The confident on using HM can be raised in order to have a more substantiate quality evaluation measures.

Chapter 2

Review on quality control of herbal medicine, antioxidant assays and chemometric techniques through different approaches

2.1 Introduction

Herbal Medicines (HM) are plant-derived materials and preparations with therapeutic or other human health benefits, which contain either raw or processed ingredients from one or more plants, inorganic materials or animal origin based on World Health Organization (WHO) definition [46]. In these years, many patients who suffered cardiovascular disease, cancer, AIDs and others would like to take HM for treatment. It is believed that the side effect of HM is much lower than that of the western drug. Not only this, but also their therapeutic effect are complementary to those of western drugs [47]. According to WHO, 65% -80% of the world's population is using herbal medicine for healthcare [48]. The usage of HM is expanding and gaining acceptance. Hence, scientific researches were conducted in different aspects to explore the pharmacological activity, discover the bioactive ingredients, study the toxicity, and access the efficacy of HM. These could aid in learning the fundamental traditional theory of HM. There is no doubt that these studies could resolve the mysteries of HM to certain extent. But the quality of HM should be secured beforehand.

It is well known that HM is composed of various classes of components while most of the chemical constituents with unknown identities. Moreover, the holistic concept of HM is applied in the treatment of the disease which is different from the use of western drugs. That is, HM aims at restoring and maintaining the dynamic balance of a person through the multiple components in HM. These are related to the therapeutic effect [4]. Additionally, the whole herbal medicine is considered as one active ingredient because the multi-components in HM are indivisible and interactive. The multi-target approach is applied in the treatment of disease [7]. Because of these characteristics of HM, we have encountered difficulties during the quality control of HM which have to ensure the safety, consistency and efficacy of the product.

General quality control approaches are capable of monitoring the safety and consistency of HM through identification and characterization utilizing selected chemical markers and chromatographic fingerprint respectively. Moreover, the application of chemometric technique such as Principal Component Analysis (PCA) [49], Artificial Neural Networks

(ANN), k-nearest neighbor (k-NN) [6] would enhance the assessment of HM by discriminating different species based on chromatographic fingerprints. The chemometric resolution methods (CRM) were used extensively to obtain the "pure" chromatographic peak profiles for qualitative and quantitative analysis. Unfortunately, the current quality control approaches are insufficient in evaluating the efficacy of the HM. Although a few active components may be selected as the chemical markers mostly, they are unable to represent the pharmacological activity of the HM. Consequently, evaluation of the HM bioactivity is based on outcomes of various bioassays. Then, assurance on the quality of HM can be obtained by considering the information of the chemical composition of HM and measured bioactivity simultaneously.

The pharmacological activity of HM is strongly related to its chemical composition. There is lack of quality control method nowadays in correlating the bioactivity and chemical composition of HM together. If the relationship between the chemical composition and bioactivity can be established, the HM chromatographic fingerprint used does not just reveal the identity of HM only but its bioactivity as well. Apart from that, the identification of bioactive components could help in choosing the appropriate chemical markers for quality control. Selection of suitable chemical markers is still the major pitfall of using chemical marker in quality evaluation. Multivariate modeling, a kind of chemometric technique, can be applied to link the bioactivity with chromatographic fingerprint. In this research study, quantified relationship between the chromatographic fingerprint and antioxidant activity of Radix Puerariae Lobatae was set up based on these two properties of HM (Chapter 5). At the same time, it is possible to discover the potential antioxidant components and the synthetic mixtures system (Chapter 6). This helped in developing a new quality control methodology at much higher level.

In this chapter, the current quality control approaches on HM using recent advanced chromatographic technology and detection system in characterizing the chemical components in HM are briefly described. Also, the in-vitro antioxidant assays are introduced which would be applied in screening the bioactivity of HM. Besides, the application of chemometric technique in quality control is discussed.

2.2 Current quality control approaches of herbal medicine

2.2.1 Background of different quality control approaches

Quality control of HM is a challenging task. The first reason is that HM is a complex system which has multiple constituents and most of them are unknown. Another reason is that the profound impact on the contents of the constituents and chemical composition of HM is imposed by variation of harvesting time, geographic location, soil condition, season, processing, storage and other factors [4, 50]. In turn, they would affect the safety or efficacy of the HM and influence its consistency on the pharmacological activity. Therefore, quality control of HM is utmost important and a challenging issue. The proposed approaches in this aspect are attempting to ensure consistency, safety and efficacy. Because of this, the two commonly used approaches are described here. They are "compound-oriented approach" and "pattern-oriented approach". "Compound-oriented approach" involves the use of selected chemical markers or unknown chemical components with partially known chemical properties in the authentication of HM. Another one is known as "pattern-oriented approach" that makes use of all the data acquired from analytical instrument such as chromatographic fingerprint [51]. These approaches can help to certify the safety and consistency of HM to certain degree.

2.2.1.1 Compound-oriented approach

We have limited knowledge of HM, so a few chemical components were generally chosen for quality evaluation. Consequently, the authentication of HM was based on identification and quantification of a few selected chemical markers or target compounds. It is widely accepted in Chinese Pharmacopoeia (CP), American Herbal Pharmacopoeia (AHP), United States Pharmacopoeia (USP), European Medicines Agency (EMEA), Korean Pharmacopoeia (KP), Korean Herbal Pharmacopoeia (KHP) and Hong Kong Chinese Materia Medica Standards (HKCMMS). According to the Chinese Pharmacopoeia (2005 edition), a total of 282 chemical markers are listed for quality assessment of HM [52]. Chemical markers can be categorized into analytical markers and active markers based on the EMEA herbal quality guidelines [53]. Active markers represent the constituents contributing to the therapeutic activity but not responsible for

the full therapeutic effect. It could indicate part of the efficacy of HM. As certain HMs are not studied thoroughly, the chemical components which may not have bioactivity but present at very high amount would be selected for analytical purpose. They are known as analytical markers. The purpose of using the main components is to evaluate the stability and consistency. Besides, this approach can be utilized to assess safety of HM also with quantification of toxic compound which is known as negative marker based on the definition of Srinivasan [50], for example, Ginkgolic acid in *Ginkgo biloba* or aristolochic acid in Radix Aristolochiae Fangchi, Caulis Aristolochiae Manshuriensis and Radix Aristolochiae [52].

Sometimes, authentication of HM is based on multiple target components instead of one component. Chemical constituents from different classes of compounds are determined simultaneously. Like Radix et Rhizoma Salviae Miltiorrhizae, salvianolic acid B and tanshinone IIA are the authorized chemical markers in CP 2005. They belong to two classes of compounds which are phenolic acid and diterpenoid compound respectively. Additionally, this way is found to be useful in quality evaluation of the decoction or commercial products. For example, multiple classes of marker constituents were simultaneously determined in Gegen Tang granule [54], Gengen-danshen capsules and Xin-Ke Shu tablets [55] and Danggui Buxue Tang [56]. The chemical markers utilized in these works were selected from their component herbs. Hence, this approach provides quantitative evaluation of the quality of HM and HM formulae.

Still, the above approach is not good enough in quality assurance. Currently, the majority of HM is authenticated by one chemical maker only. Sometimes, the same reference standards were selected for several herbal medicines for the purpose. For example, identification of Radix Puerariae Lobatae and Radix Puerariae Thomsonii was based on puerarin as recommended by CP 2005. They displayed different antioxidant activity [36]. Radix ginseng acts as tonic and stimulant while Radix notoginseng eliminates blood stasis and arrests bleeding [57]. But they were identified by the same chemical marker, ginosenosides. Furthermore, the selected reference standard may not be bioactive. Stilbene glucoside is the chemical marker for identifying zhiheshouwu. However,

zhiheshouwu is processed before used and the stilbene glycoside is transformed into free anthraquinone [4]. As mentioned above, they just served for analytical purpose and are classified as analytical markers. Moreover, quite often, the contents of the markers are extremely variable due to the variations in growing conditions and harvesting time [4, 6]. It is hard to determine an appropriate limit for the amount of the marker in HM. More crucially, many reference standards are not commercially available for certain herbal medicines [52]. Poria is one of the HM that is lack of chemical markers. Above all, it is very difficult to detect the spike reference compounds in dietary supplements or commercial products via the "compound-oriented approach". Detection of the presence of one or a few of target compounds would indicate the presence of the constituent herbs in the commercial products. Indeed, this procedure is lack of evidence to confirm the identity of the constituent herbs in the products [58]. To cope with these difficulties, "pattern-oriented approach" is an alterative quality control measure. Instead of investigating the quantities of a few selected chemical compounds, all the compounds that are detected by the analytical instrument or method are included for comparison. Also, if instrument like HPLC-DAD is used, the peak heights or areas in the chromatogram represent the contents of the related components.

2.2.1.2 Pattern-oriented approach

The advanced development of chromatographic technology such as gas chromatography coupled with mass spectrometry (GC-MS) or high-performance liquid chromatography coupled with diode array detector in series with mass spectrometry (HPLC-DAD-MS) aids in characterizing the chemical composition of HM. They recorded all the detectable components including target and non-target components in a chemical system. Thus, the identities of the non-target components can be determined from different type of data including their retention times, UV-spectra and/or even mass spectra. It is believed that the therapeutic effect of HM comes from the combined action of different components together. Hence, the authentication of HM which is based on all known, partially known and unknown components is named as "pattern-oriented approach". This approach has gained more and more attention in these years. It has been introduced and accepted by the World Health Organization (WHO), the State Food and Drug Administration (SFDA), the

Food and Drug Administration (FDA), the EMEA in addition to British Herbal Medicine Association (BHMA) as one of the strategies for assessing HM in year 2000 [20, 28, 59, 60]. Those samples with similar chromatographic fingerprints were considered to have similar properties.

Actually, this approach is a comprehensive strategy which could solve some problems encountered in the "compound-oriented approach". Through the "pattern-oriented approach", the variation of both the target and non-target compounds is evaluated. In fact, the consistency of the HM may also be affected by the change of non-target compounds [4]. In addition, it is important for us to consider all the detectable components as the therapeutic effect is attributed from the multiple components. Sometimes, the selected markers may not an active compound. Thus, the whole chromatographic profiles can represent the entity of HM in a better way.

Furthermore, the whole chromatographic fingerprint is very helpful in distinguishing different species. Many HMs shared the same chemical markers for identification as mentioned previously. There are a variety of species for the same HM. For example, in recent studies, two species of Radix Paeoniae Rubra, *Paeonia lactiflora* Pallas and *Paeonia veitchii* Lynch were cultivated in different regions. This resulted in the difference in their chemical compositions of unknown compounds in addition to the variation in quantity of the same and the sole chemical marker, paeoniflorin [5]. The same problem came across for another HM, Bupleuri Radix when they were identified by the chemical markers, saikosaponin A and D only. Definitely, the "compound-oriented approach" alone was not good enough to distinguish different species of Bupleuri Radix and the toxic species *B. longiradiatum* in this aspect. Yet, their chromatographic profiles are different from the official species, *B. chinense*, with most of the peaks being absent or unique [6]. These demonstrated that the variations of other unidentified compounds should be considered also.

In order to obtain as much information as possible for confirmation of the identities of HM, multiple chromatographic fingerprints instead of one chromatographic profile are

used. It is because a specific chromatographic profile may represent only a subset of all the components especially herbal formulae. Two different chromatographic fingerprints were obtained under two chromatographic conditions for Danshen Dropping Pill [20]. Besides, the combination of different chromatographic pattern could capture more information on the HM. In Li et al.'s study, the binary chromatographic fingerprints of total alkaloid from *Caulophyllum robustum* was obtained from GC-MS and HPLC-DAD instruments for quality assessment [61]. The consistency and fraud can be deduced from the information rich multiple chromatographic profiles. Recently, the chromatographic fingerprint and activity fingerprint were combined for the quality evaluation of HM. The chromatographic fingerprints of HM before and after the addition of free radical were compared and the bioactive compounds can be revealed through this on-line screening method. The construction of the activity integrated fingerprint is able to demonstrate the integrity and fuzziness of the bioactivity and chemical characteristic among the samples [10].

From these, the usage of "pattern-oriented approach" is able to reduce the pitfall of "compound-oriented approach". The variations of the unnoticed compounds are considered with the application of the whole chromatographic profiles. Thus, it gained the attention of scientists in authentication and identification of HM. Still, this approach requires the reference chemical markers for the accurate quantification. Furthermore, the efficacy information cannot be obtained from the chromatographic profile of HM only. These are the limitations of the "pattern-oriented approach".

2.2.2 Chemical analysis of components in herbal medicine

HM composes of thousands of chemical constituents from different classes of components including saponin, flavonoid, phenolic acid, anthraquinone and volatile oil. The development of analytical strategy is crucial in the quality control of HM so that the identity of the HM can be confirmed and distinguished from others. This required advanced chromatographic technique coupled with various kinds of detector for analyzing the constituents of HM. As mentioned previously, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the commonly used separation

method for analyzing the volatile and non-volatile compounds, respectively. Recently, the newly developed advanced chromatographic technique, ultra-performance liquid chromatography (UPLC) or rapid resolution liquid chromatography (RRLC), becomes more popular in the HM authentication. Besides, there is an increasing trend in application of capillary electrophoresis (CE) in identification of the constituents in HM [7]. Both UPLC or RRLC and CE are powerful separation tools which have high resolution, well separation efficiency and short analytical time. These merits are beneficial to the analysis of HM.

Chromatographic technique plays an important role in separating the components in a complicated system. At the same time, various kinds of detectors are required in recording the separated components. Generally, most of the components like phenolic acid, anthraquinone, and flavonoid are able to be detected by diode array detector (DAD). The identities of these components with chemophores can be confirmed from their UV spectra and retention time. For those compounds without the chemophores, such as saponin, triterpenoids or fatty acid, evaporative light scattering detector (ELSD) is a suitable option for analysis [7]. The detection is based on the light scattering on the surface of the component after vaporizing the mobile phase. Thus, it is known as universal detector. But it cannot provide any structure information as retention time is the only information for identifying the components. In order to detect these two kinds of compounds, DAD and ELSD are connected in series to provide complementary information. The HPLC coupled with DAD and ELSD was applied in the analysis of the Danggui Buxue Tang [7]. Recently, the application of mass spectrometer (MS) for the authentication of HM is increasing in popularity. The components in the HM can be matched with the standard compounds via their retention times and mass to charge ratios. Also, it is possible to elucidate the structure of non-target compounds with or without chemophores utilizing MS/MS study.

In our study, RRLC-DAD and HPLC-DAD-MS were utilized in analyzing Radix et Rhizoma Salviae Miltiorrhizae, Radix Puerariae Lobatae and their combination called Danshen-Gegen Tang (DGT). In the following section, the recently developed

chromatographic technology and currently used detection system in chemical analysis of HM were introduced.

2.2.2.1 Newly developed advanced liquid chromatographic techniques

Rapid resolution liquid chromatography (RRLC, product of Agilent Technologies with 1.8 μm particles) or ultra-performance liquid chromatography (UPLC, products of Waters Corporation with 1.7 μm particles) [62] are a relatively new technique in the liquid chromatography. They could reduce both analytical time and the solvent consumption when compared with the traditional HPLC system with the application of the sub-2μm particle size. Simultaneously, the solvent can be delivered under high pressure. According to the van Deemter equation that relates the linear velocity (flow rate) with the equivalent plate height (column efficiency), the efficiency of chromatographic process is enhanced when the particle size decreases [63]. At the same time, the efficiency does not lower with increasing flow rate [64]. Actually, the high back pressure would be generated when the small particle size in the conventional HPLC system is applied. In order to reduce this, the separation was preformed at elevated temperature to reduce the solvent viscosity.

The working principle of RRLC or UPLC is basically the same as HPLC. Therefore, the RRLC-DAD chromatogram is comparable with the HPLC-DAD chromatogram and the analytical method was switched from HPLC to UPLC in Jin et al. study [64]. Despite that, the resolution, peak capacity and sensitivity of the chromatographic fingerprint using RRLC or UPLC system are greatly improved [65]. Comparison between UPLC and HPLC analysis in pharmaceutical laboratory was undertaken by Novakova et al. [66]. Four samples, Triamcinolon cream, Hydrocortison cream, Indomethacin gel and estrogel gel, were tested using both UPLC and HPLC instruments. The results showed that the analytical method can be transferred from HPLC to UPLC analysis with the reduction of injection volume and the change of flow rate in addition to better performance of the chromatographic separation to the UPLC device.

With the advantages of RRLC or UPLC separation technology mentioned above, it is very suitable for analysis of complex systems especially HM. Currently, various studies

on quality control of HM [48, 67-69], analysis of Chinese herbal prescription such as Wen-Pi Tang [65] and metabolomics study of GegenQinlian decoction [70] were carried out through UPLC-DAD or UPLC-MS instrument. It is no doubt that the constituents in the complex herbal medicine system and traditional Chinese medicine formulation can be well separated and identified in short analytical time. Rapid routine analysis of HM is possible. Moreover, this would aid in exploring the non-target compounds in herbal medicine and gain better understanding of this complex system.

2.2.2.2 Detection systems

Recently, mass spectrometry has been used extensively in such as metabonomic study, discovery of potent active components or authentication of HM [71]. It has gained widespread acceptance as the analytical tool for qualitative and quantitative analysis of a complex system. As mentioned above, most of the HM constituents are in mystery. But it is necessary to have identification of both known and unknown compounds during the authentication. Mass spectrometer (MS) is able to do so when it coupled with different kinds of chromatographic techniques such as gas chromatography (GC), liquid chromatography (LC) and even capillary electrophoresis (CE). Thus, there is growing popularity of using MS in analyzing HM and the associated decoction these years [72]. Time-of-flight (TOF), ion trap or quadrupole are the commonly utilized mass spectrometer for phytochemical analysis of HM. Especially TOF, its high resolution could discriminate the compounds from exact mass instead of nominal mass.

With the availability of MS, more detailed study on HM can be carried out. MS is able to identify different classes of components in HM simultaneously. From the mass to charge ratio, the molecular weight of a component can be learned. Although many compounds shared the same molecular weight, the identity can be confirmed via MS/MS study on structure elucidation. The new steroidal glycosides were identified based on MS/MS analysis in the roots of *Cynanchum chekiangense* [72]. Another MS/MS analysis was preformed on another HM, ginger. Based on the characteristic fragmentation in both positive and negative mode, it is able to identify 26 diarylheptanoids [72]. This is one of the merits of MS over other detection systems. The sample did not absorb radiation from

the electromagnetic spectrum. Instead, it formed the ions in the ion source during MS analysis. Generally, identification of the HM components is based on UV spectrum and retention time using DAD detector. However, compounds in the same class usually have similar UV spectra. Then, the identification of these compounds is not easy to get. In addition, retention time is the sole characteristics of the detected components while utilizing the ELSD detector. It is inadequate to confirm the identity without reference standards available although it is useful in the detection of the compounds without chemophores. Previously, the analysis of Radix Astragali was carried out by the HPLC-DAD-ELSD hyphenated instrument. It is because it composed of two classes of compounds, isoflavonoids and saponin. Saponin could be detected by DAD detector at 200nm-210nm which is under the influence of strong background absorption. Introduction of MS could help to detect two classes of compounds simultaneously for the quality control purpose [73]. Besides, the sensitivity of MS is relatively higher than the other detection system such as DAD or ELSD. It is capable of detecting the compounds in HM at the low concentration levels which may be significant to the pharmacological activity. In summary, it is a powerful tool in analyzing the chemical composition of HM.

As mentioned before, RRLC or UPLC is a rapid separation technology with enhanced resolution. It is now coupled with MS in providing well resolved chromatographic profiles of HM in relatively short analytical time in addition to the mass measurement of all the detectable components. More and more studies on HM using this advanced analytical instrument are undergoing [74-76]. Through UPLC-MS analysis, 40 components in addition to the sulfonate compound in Radix Paeoniae could be identified in 12min. This method is able to detect the herb that is preprocessed by burning sulphur from identifying sulfonate compound which was inhibited to do so [75]. Also, nine diterpenoids of *Salvia* species could be identified through structure elucidation through MS/MS study besides the existing twelve referent standards. The components were analyzed in 10minutes. This definitely assisted the quality control of *Salvia* species [76].

Though MS has many advantages as mentioned above, yet, like other techniques, quantification of the identified chemical marker is troublesome since an internal standard

is required for quantification. It is very difficult to find a suitable one with similar structures to the target components and is unable to interact with other analytes in HM. Moreover, the detection of the analytes depended on their ionization capability which makes the selection of suitable internal standard more difficult. Furthermore, the MS database utilizing LC has not yet well developed as the database of GC-MS. The identification of unknown components could not be carried out solely by matching the mass to charge ratio obtained.

2.3 In-vitro antioxidant assays for the measurement of the antioxidant activities

Consistency and safety of the herbal medicine can be evaluated by existing quality control approaches. Yet, HM is a holistic concept. Thus, the HM efficacy evaluation is insufficient by just quantification of a few selected chemical markers. In addition, characterization of HM by the chromatographic fingerprint usually does not take care of the efficacy aspect. Information on the therapeutic effect and pharmacological activity of HM is provided through measurements from different in-vitro bioassays. They are available for screening the bioactivity of HM with the consideration of all compounds involved.

Antioxidant activities of HM or food stuff are in concern in these years. It was found that the antioxidants could prevent cardiovascular disease, cancer, Alzheimer's disease, diabetes, neurological disorders and ageing [77]. These diseases are the results of oxidative damage of large biomolecules such as DNA, protein or lipid owning to overproduction of reactive oxygen species (ROS) or lipid radicals. The deficiency of antioxidant defense leads to oxidant stress. It is important to have antioxidants to counteract and balance the production of ROS in order to reduce the chance of cellular damage. Antioxidant, the substance that present in low concentration compared to the oxidizable substrate, can significantly delay or inhibit oxidation of that substrate.

Antioxidant can be classified as enzymatic antioxidant and non-enzymatic antioxidant in

our body. Heme-oxygenase, Glutathione Peroxidase and Superoxide Dismutase are known as enzymatic antioxidant. Ascorbic acid, α-tocopherol or carotenoids are regarded as non-enzymatic antioxidant. Furthermore, antioxidants can be obtained from external sources such as food or HM. Phenolic acids and flavonoids are well known antioxidants that can be obtained from our diets.

In different assays, how good an antioxidant works against ROS is measured by its ability in protecting the oxidizable substrate. Screening of potential antioxidants in natural products can be carried out by in-vitro antioxidant assays including the chemical assay and cellular assay. In our study, the antioxidant activities of two herbal medicines, Radix et Rhizoma Salviae Miltiorrhizae and Radix Puerariae Lobatae, and a decoction based on these two were measured by the Ferric Reducing Antioxidant Power (FRAP) assay. It is a commonly used method for screening the antioxidant activities of herbal medicine. Besides, there are other kinds of in-vitro bioassays available. The working principles of these bioassays are briefly introduced below.

2.3.1 Ferric-reducing antioxidant power (FRAP) assay

FRAP assay measures the ferric reducing ability of antioxidant because the redox reaction would be involved in the reaction with ROS. Thus, the reducing capacity could reflect the antioxidant activity of the samples. In this assay, the ability of the sample in reducing ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) into ferrous tripyridyltriazine complex (Fe^{2+}-TPTZ) at low pH is measured at 593nm [78, 79]. Fe^{2+}-TPTZ absorbs most at 593nm and the production of this complex is detected. The recorded absorbance of the ferrous ion is multiplied by the conversion factor. This conversion factor was found from the absorbance of 1000 μM of standard ferrous ion and its concentration. Thus, the recorded absorbance is converted into FRAP value which serves as the indicator of the strength of the antioxidant activity and is related to the concentration of Fe (II) ion complex formed.

Another antioxidant assay that is similar to the FRAP assay is known as Phosphomolybdenum method [80]. But this method measured the ability of the samples

in reducing Mo(VI) phosphate complex into Mo(V) complex in green colour at 695nm instead. At the same time, the standard antioxidants such as Trolox or quercetin were evaluated. Hence, the antioxidant activity of the sample is expressed equivalent to the concentration of these standard antioxidants.

2.3.2 Metal chelating activity assay

Evaluation of the antioxidant activity is based on the lowering of the concentration of ferrous ion complex. It may be considered as the reverse from FRAP antioxidant assay which measures the formation of ferrous ion complex. In this assay, the antioxidant capacity is evaluated by the ability in chelating the metal ion, ferrous ion but not the reducing power. It is because metal ion would catalyze the production of ROS. In lipid peroxidation, ferrous ion would decompose the lipid into hydroxyl radical through Fenton reaction. This could reduce the production of ROS when metal ion can be chelated. The potential antioxidant instead of ferrozine would form complex with metal ion. Hence, the decrease in absorption at 562nm in which the ferrozine complex absorbs most is recorded. From the percentage of lowering in absorbance, the antioxidant activity of the samples is determined [81, 82].

2.3.3 Oxygen radical absorbance capacity (ORAC) assay

The most popular type in measurement of total antioxidant capacity is oxygen radical absorbance capacity (ORAC) assay. It measures the protective effect of antioxidants to the protein against the free radicals, hydroxyl and peroxyl radicals, produced during the assay. Hydroxyl radical involves in ischemia reperfusion injury in brain, heart and retina [83].

In this assay, the oxidation of porphyridium cruentum β-phycoerythrin (β-PE) is induced by 2,2'azobis(2-amidinopropane) dihydrochloride (AAPH). There is red photoreceptor pigment in β-PE which would give fluorescence. The conformation of the protein, β-PE, changed when it is under the oxidative damage of AAPH and it cannot give any fluorescence. Thus, the measurement is based on the detection of the chemical

damage to PE through the decrease in the fluorescence emission [84]. The protection against the loss of fluorescence reflected the antioxidant activity. In parallel, the protection is evaluated using Trolox, water soluble vitamin E, which is used as standard antioxidant to compare the antioxidant activity of the testing sample. Thus, the antioxidant capacity is expressed as Trolox equivalent antioxidant capacity (TEAC) [78, 79].

2.3.4 Hemolysis of red blood cells assay

This assay is similar to the ORAC assay but different oxidizable substrate is involved. It measured the protective effect on the red blood cell instead of the phycobiliprotein [85]. Red blood cell (RBC) is separated from the plasma of the rat blood which is obtained from abdominal aorta and added with 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). This leads to the oxidation of lipids and proteins in cell membrane and induce the hemolysis. Hemolysis is the process of the release of hemoglobin into surrounding fluid. The inhibitory effect against RBC hemolysis by scavenging the free radical is proportional to the concentration of the antioxidants in the incubation mixtures. The antioxidant capacity can be measured from the ability in scavenging the free radical, AAPH, at 540nm.

2.3.5 Trolox equivalent antioxidant capacity (TEAC) assay

In this assay, the free radical scavenging ability of the antioxidants is determined from the inhibitory effect on the blue green radical cation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)($ABTS^{+\cdot}$). The antioxidants are able to quench the long lived $ABTS^{+\cdot}$ through the donation of hydrogen to ABTS radical cation. It is produced from the peroxidase activity of metamyoglobin with hydrogen peroxide, and ABTS. Another way in production of ABTS radical cation involves the oxidation of ABTS with potassium persulfate [86]. The change of the absorbance at 734nm is measured because of the reaction between the antioxidant and the radical cation, $ABTS^{+\cdot}$. $ABTS^{+\cdot}$ absorbs strongly at this wavelength [78, 79, 87]. Afterwards, the antioxidant activity is expressed as TEAC which is the same for ORAC assay.

2.3.6 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) antioxidant assay

2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) antioxidant assay is another assay for determining the ability in scavenging free radical. The working principle of this method is similar to that of TEAC. But DPPH free radical is commercially available and does not require the production of this radical beforehand. The antioxidant capacity is detected by the decolorization at 515nm which DPPH radical absorbs most and exhibits blue violet colour and it is expressed in terms of the percentage of free radical scavenging [88]. Once the antioxidant donates hydrogen atom, the electron of DPPH becomes paired off and the absorption decreased.

Among the in-vitro antioxidant assays, FRAP assay measures the reducing power of the testing components. The property of chelating of metal ion is measured by metal chelating activity assay. ORAC assay, Hemolysis of Red Blood cells, TEAC assay and DPPH assay measure the ability of the samples in scavenging the free radical or inhibiting the activity of free radical. Moreover, Hemolysis of Red Blood cells assay is cellular in-vitro assay which is different from the rest of in-vitro assays. The remaining in-vitro assays mentioned here measure the antioxidant property based on chemical reaction. With the help of these bioassays, the antioxidant activity of HM can be assessed in details. Doubtless, this kind of information is related to the efficacy of HM although it is incomplete.

2.4 Chemometric techniques for different quality control approaches of herbal medicine

Chemometrics is a discipline of chemistry that applies mathematical and statistical techniques to retrieve more information from the chemical data [89]. It could aid in searching the hidden structure in data sets and extracting useful information within. In “compound-oriented approach”, quantification of multiple chemical markers is involved in determining whether the complex HM system meets the requirements or not. It is almost unavoidable to have overlapping peaks in the HM chromatographic fingerprints.

Resolution of these overlapping peaks is needed for finding the amount of the related components if adjustment of experimental condition cannot help to do so. Moreover, multiple chemical components are of concern during quality control based on their partial chemical information available such as retention times, UV spectra or mass spectra even though their chemical structures are not elucidated. Introduction of chemometric resolution method (CRM) assists in this aspect in getting the pure spectra and more accurate retention times of the resolved peaks from overlapping profiles for both identification and quantification. Thus, quality assessment data can be also greatly improved. CRMs have been used extensively in getting the pure chromatographic peak profile. They include heuristic evolving latent projections (HELP), subwindow factor analysis (SFA) or evolving window orthogonal projection (EWOP) and alternative moving window factor analysis (AMWFA), In addition, useful components can be found selectively with the help of local rank analysis method.

More intensive quality control can be carried out through the "pattern-oriented approach" coupled with chemometric technique [4]. For instance, the chromatographic fingerprints of different HM samples can be compared through the similarity evaluation. This approach can assist in measuring the consistency of the HM, distinguishing different species of HM through similarity or difference with the standardized herbal medicine and others [5, 6, 90]. The quantified similarity evaluation can be done by the pattern recognition program called Computer Aided Similarity Evaluation (CASE) [91]. Furthermore, the pattern recognition and classification assessment such as principal component analysis (PCA) could aid in evaluating the quality of herbal medicine. Combination of chemometric techniques with the chromatographic fingerprints had been proven to enhance the information obtained. In this section, the methodologies of AMWFA, SI and PCA are briefly described.

2.4.1 Alternative Moving Window Factor Analysis (AMWFA)

Alternative moving window factor analysis (AMWFA) is useful for detecting the characteristic chemical components and determining the common compounds among various samples [92]. Thus, it can be utilized to extract pure chromatographic and

spectral profiles of common components of the HM for identification and quantification. Also, it can help to find the similarity and difference between different HM samples. It is useful in both "component-oriented approach" and "pattern-oriented approach".

AMWFA is an extension and combination of multi-component spectral correlative chromatography (MSCC) and subwindow factor analysis (SFA). The common components appear in two different chromatographic profiles can be found from MSCC while the spectrum of the corresponding common components can be obtained through SFA [93, 94]. It involved two main steps in discovering the common components and resolving the corresponding pure spectra.

To begin with, **X** and **Y** are decomposed by singular value decomposition (SVD).

$$\mathbf{X} = \mathbf{U}_p\mathbf{S}_p\mathbf{V}_p^T + \mathbf{E}. \quad [2.1]$$

where $\mathbf{U}_p$ and $\mathbf{V}_p$ are score and loading matrices respectively. $\mathbf{S}_p$ and p denote as diagonal matrix and the principal component respectively. The same expression applies to Y. The orthogonal projection of **X** and **Y** were carried out alternatively in order to investigate the relationship between **X** and **Y** via MSCC and the inverse projection MSCC. The orthogonal bases of loading, $\mathbf{E_x}=\{e_1, e_2, e_3,\ldots, e_m\}$, of **X** and $\mathbf{F_y}=\{f_1, f_2, f_3,\ldots,f_n\}$, of **Y** can be obtained. Here, m and n represents the number of components in **X** and **Y** respectively. This could find out the common components, $\mathbf{s}_k$, among **X** and **Y**.

$$\mathbf{s}_k = \mathbf{E_x}\mathbf{a}_k = \mathbf{F_y}\mathbf{b}_k \quad [2.2]$$

where $\mathbf{a}_k$ and $\mathbf{b}_k$ are linear combination coefficient of bases $\mathbf{E_x}$ and $\mathbf{F_y}$ with k denoting the number of common components.

As it is inevitable to have noise during chromatographic analysis, $\mathbf{E_x}\mathbf{a}_k$ is not absolutely equal to $\mathbf{F_y}\mathbf{b}_k$. Thus, the agreement between these two parameters can be determined by the following expression.

$$f(\mathbf{a}_k, \mathbf{b}_k) = \left\| \mathbf{E_x}\,\mathbf{a}_k - \mathbf{F_y}\mathbf{b}_k \right\|^2 = 2\,(1- \mathbf{a}_k^T\,\mathbf{E_x}^T\mathbf{F_y}\mathbf{b}_k) = 2\,(1-d_k) \quad [2.3]$$

$$\text{and} \quad d_k = \mathbf{a}_k^T\,\mathbf{E_x}^T\mathbf{F_y}\mathbf{b}_k \quad [2.4]$$

When common components exist in both **X** and **Y**, d_k is equal or close to 1. Thus, when $f(\mathbf{a}_k, \mathbf{b}_k)$ is equal to 0, it indicates that the common components are found. Otherwise, the value of d_k is less than 1 and $f(\mathbf{a}_k, \mathbf{b}_k)$ would be close to 2. This spells out that no common

components exist.

The resolution of the pure spectrum of a component can be carried out by SFA method to find the common rank. The base matrix, **Y** consists of at least one common component. With the application of fixed size moving window evolving factor analysis (FSMWEFA) method, the spectra of target matrix, **X** can be scanned and matched with **Y**. Hence, the selective information can be located for the common component. The number of common rank can be acquired through scanning along the retention time axis is known as common rank analysis. In addition, the spectral correlation coefficients of the front and its followed spectra using the linear combination of orthogonal loadings, $\mathbf{E_x}$ and $\mathbf{F_y}$, $\mathbf{s}_k = \mathbf{E_x}\,\mathbf{a}_k = \mathbf{F_y}\mathbf{b}_k$ are found. Based on these, the common rank map obtained by plotting the number of common rank versus retention time and the spectral auto correlative curve obtained by plotting the correlation coefficient versus retention time could get the common component at the region with similarities close to 1.

Using this method, it aided in identifying the volatile compounds from five *Clematis* species [93], Pericarpium Citri Reticulatae Viride and Pericarpium Citri Reticulatae [95] with very limited study on these herbs in the literatures. The overlapped peak can be resolved utilizing AMWFA to get the pure spectra. By matching with the MS library, the resolved common components from the GC-MS chromatographic data of the species can be discovered. The quantification and identification can be carried out correctly by resolved the overlapped and embedded peaks via AMWFA.

2.4.2 Similarity Indices (SI)

Similarity indices (SI) reflect the sameness and difference quantitatively between the chromatographic fingerprints of different samples. It is a correlation analysis between the testing sample and the mean or median of chromatographic fingerprints under investigation. The higher value of SI represents the testing and target chromatographic profiles sharing most of the common pattern with each other. Both the separation degree and concentration distribution of all the detected components in the chromatographic fingerprint are considered for the similarity evaluation. SI is expressed as the following.

$$SI = (\mathbf{x}_1^T \mathbf{x}_2) / (\|\mathbf{x}_1\| \bullet \|\mathbf{x}_2\|) \quad [2.5]$$

$\mathbf{x}_1$ and $\mathbf{x}_2$ represent the chromatographic fingerprints.

The integrity among the chromatograms of different species could be assessed by SI. It was found that the similarity indices reflected the difference between Radix *Paeoniae lactiflora* Pallas and Radix *Paeonia veitchii* Lynch. The fingerprints of *Paeoniae lactiflora* Pallas with SI values are higher than 0.9 when compared with its common pattern while these values are less than 0.9 when compared with the chromatographic fingerprint of Radix *Paeonia veitchii* Lynch [5]. In addition to the variation in the quantity of the selected markers, other unidentified components exhibited variations among different species. Thus, the extent of the variation among different chromatographic profiles could be revealed by SI. There was not only diversity of the contents of saikosaponin a and d which are the chemical markers of Radix Buplueri, but also the other unidentified peaks which were absent in certain species [6]. This showed that the difference between target and non-target compounds should be considered. Moreover, the consistency of the samples could be found from SI with the setting of appropriate threshold value.

2.4.3 Principal Component Analysis (PCA)

Similarity index alone is not sufficient to distinguish different HM samples [92]. Principal component analysis (PCA) is another technique of discrimination analysis in finding out different species of the samples [96]. It is a method of feature extraction and dimensionality reduction with mathematical expression.

$$\mathbf{X} = \mathbf{T}\mathbf{P}^T + \mathbf{E} \quad [2.6]$$

X is the matrix of chromatographic fingerprints of the samples concerned while, **T** represents the score matrix and **P** represents the loading [97].

From the loading plot of PCA, the content variation of the HM samples can be revealed. At the same time, the score plot of PCA could tell us the discrepancy among the samples. For example, the authenticated samples of Pericarpium Citri Reticulatae and Pericarpium Citri Reticulate Viride were identified and the peaks of hesperidin, nobiletin and tangertin

were picked out to discriminate the quality of samples. These two herbs have different pharmacological activity but hersperidin was chosen as a marker compound for assessing these two herbs [92]. The differentiation of different species of HM was also carried out on Radix Paeoniae Rubra through the similarity evaluation and PCA approach as well [5]. PCA could not only aid in classification from the good to the bad samples, but also it showed its explanatory power from the chemical point of view.

2.5 Building up bioactivity-chemical fingerprint model

It is interested in studying the relationship of response and property of HM samples. There are different kinds of chemometric technique to aid in building the response-property relationship. At the same time, we can extract the relevant information from the data to predict the properties of the complex sample such as concentration as well as other chemical or physical properties [98]. The quantitative relationship can be developed via multivariate calibration technique [99]. The introduction of multivariate calibration is especially helpful for Near-Infrared (NIR) spectroscopy to determine the food composition [100, 101], gasoline property [98] and concentration of chemical components in HM [102]. It is because NIR spectra arise from overtones and combinations of vibrations of molecular bond of the organic components [103]. In our study, we are trying to establish the relationship between the chromatographic fingerprint and antioxidant activity as the chemical composition of the HM can be reflected from its chromatographic fingerprint.

Among the multivariate models, Partial Least Squares (PLS) method is the well known method in constructing the relationship between the property and variables which was proposed in 1977 by Herman Wold. PLS is a full spectrum method with the application of all variables for regression. In order to reduce the complexity of the model and facilitate interpretation, various multivariate modeling methodologies have been developed from the modification on PLS. In the following section, PLS method will be briefly described and introduced two modified PLS methods, Target Projection (TP) and Orthogonal Partial Least Squares method (O-PLS).

2.5.1 Partial Least Squares method (PLS)

PLS method is a kind of full spectrum method which involved the transformation of the instrumental response variables. The latent variables are employed instead of the real variables during prediction [104]. PLS does not require any variable selection in order to estimate the regression vector and collinearity is not a problem for this biased regression method. Instead, the other metaparameters, latent variables or principal component, must be concerned in PLS [105]. Usually, the latent variable is the linear combination of the original variables which could reduce the dimensionality of the regression space and shrink the regression vector such that the number of observations is as high as the number of predictors. For the first principal component, the largest variance among the samples is described. At the same time, the regression finds components from predictor matrix that are also relevant for response vector or response matrix. In this case, the PLS can model all the constituents from **X**, matrix of predictors variables and **Y**, matrix of response variables. Hence, the purpose of PLS is to find the small number of relevant factors that are predictive for **Y** and utilize **X** efficiently [97]. It is the most commonly applied linear multivariate calibration method, especially in NIR spectroscopy. The relationship between **X** and **Y** can be generally expressed as the follows.

$$\mathbf{Y} = \mathbf{XB} + \mathbf{F} \qquad [2.7]$$

where **B** is regression coefficient and **F** is residual.

In PLS, the decomposition of the predictor space (**X**) into uncorrelated latent variables and the calculation of the regression vector (**b**) for each response variables are involved. To begin with, the projection of the **X** and **Y** to the latent structure is performed via principal component analysis. It is carried out from the non-linear iterative partial least square algorithm. When a single **y**-variable is concerned, the algorithm is non- iterative [106].

At first, the latent variables are determined which is also known as **X**-score, **t**, they are the predictors of **Y** and also model **X**. It is estimated from the linear combination of the original variables in **X** with the coefficients, weights, **w** [106]. The weight defines the direction of the space of the predictor that has maximum covariance between the

predictor and response [107].

$$\mathbf{t} = \mathbf{Xw} \quad [2.8]$$

The similar expression was shown on **Y** variables.

$$\mathbf{u} = \mathbf{Yq} \quad [2.9]$$

The **X**-score was found through the iterative steps from the **Y**-scores, **u** such that **w** can be determined from **u** and **X**. **u** is the starting vector and determined from **Y**. When a **y**-variable is considered, **u** is equal to **y**. **w** is proportional to the vector of regression coefficients and obtained by regressing each column of **X** variables on **Y**-score, **u**. **q** which is the weight of **Y** is obtained by regressing each column of **Y** on **X**-score, **t**. At last, the updated **Y**-scores, **u** can be found from **u** = **Yq**. The convergence is tested on the **X**-score, **t**. The finding of the second component can be carried out until the new value of **t** is close to the old value of **t**. Otherwise, the algorithm is carried out successively. Cross-validation is involved for the determination of the optimal latent variables for modeling.

From the above algorithm, the latent variables with the **X**-score and **Y**-score are found to maximize the covariance. That is, the **t** on the **X** factor and the **u** on the **Y** factor should have large variance. In addition, **t** and **u** have high correlation. Then, **X**-scores is used to regress both **X** and **Y**.

For **X**,

$$\mathbf{X} = \mathbf{TP}^{T} + \mathbf{E} \quad [2.10]$$

For **Y**,

$$\mathbf{Y} = \mathbf{TQ}^{T} + \mathbf{F} \quad [2.11]$$

P is the loading matrix and contains the regression coefficients of the separate univariate regressions of individual **X** variables on **T**. **Q** contains regression coefficients of the individual variables of **Y** on **T** [97].

After the decomposition of the variables, the relationship between the reproduced predictor **X** and response **y** was found in order to establish the regression model. Regression vector, **b**, is needed by least squares sense to connect reproduced predictor matrix and response vector.

$$\mathbf{b} = \mathbf{X}^{+}\mathbf{y} \quad [2.12]$$

$\mathbf{X}^{+}$ is the generalized inverse of the reproduced **X** matrix in order to determine regression vector.

The regression coefficients, **B**, can be calculated as the following.

$$\mathbf{B} = \mathbf{W}\,(\mathbf{P}^{T}\mathbf{W})^{-1}\mathbf{Q}^{T} \qquad [2.13]$$

Regression coefficients are obtained from loadings and weights matrices.

PLS is also applied in classification or discriminant analysis besides multivariate modeling. In the classification analysis, the response would be equal to 1 or 0.

2.5.2 Target Projection (TP)

The number of predictor variables is always larger than the number of the samples as well as the response. Additionally, the predictor variables are not independent to one other resulting in rank deficiency. Hence, the latent variables are applied in modeling **X** and **Y** as mentioned above. The regression model was established between the reproduced **X** with the latent variables in the predictor space and **y**. It is usual that several components are involved in regressing to **y**. This would lead to problem in the interpretation. Because of this, target projection (TP) produces a single predictive component by projecting the latent-variable decomposition onto the response variable [108, 109]. TP included both prediction ability and show the importance of the variables to response [110].

The target model can be written as the following.

$$\mathbf{X} = \mathbf{X}_{TP} + \mathbf{E}_{TP} = \mathbf{t}_{TP}\mathbf{p}_{TP}^{T} + \mathbf{E}_{TP} \qquad [2.14]$$

It involved the reproduction of the predictor space from the original variables. Target component was generated for each response from a multi-component latent regression model [108, 110]. The direction in the multivariate predictor space most strongly related to the response is found which is defined by the regression vector, **b** with the expression stated in above. It is the best fit line to the response vector in the reproduced predictor space. As a result, **b** defines as the target rotation of the weights of the variables, **w** with the response vector as target [110].

$$\mathbf{w}_{TP} = \mathbf{b} / \|\mathbf{b}\| \qquad [2.15]$$

The projection of the variables in reproduced predictor space on the regression vector gives the target-projected scores, $\mathbf{t}_{TP}$. The target-projected score is proportional to the predicted response.

$$\mathbf{t}_{TP} = \mathbf{X}\,\mathbf{b} / \|\mathbf{b}\| \quad [2.16]$$

From the target-projected scores, the target-projected loading $\mathbf{p}_{TP}$ can be calculated as

$$\mathbf{p}_{TP} = \mathbf{X}^{T}\,\mathbf{t}_{TP} / (\mathbf{t}_{TP}^{T}\mathbf{t}_{TP}) = \mathbf{b}^{T} / \|\mathbf{b}\| * (\mathbf{X}^{T}\mathbf{X}) / \|\mathbf{t}_{TP}\|^{2} \quad [2.17]$$

Here, we have one target component for prediction and selection of the active components. Thus, the target-projected loading represents the features in the chromatographic profiles explaining and predicting the bioactivity. It is related to the regression coefficient, covariance of the **X** variables and inverse of the variance of the target projected scores. In PLS, the variable is important for modeling **Y** that has high regression coefficient. Also, the variables are important in modeling **X** which would have high loading [106]. In TP, the high loading of the variable vector represents the group of covarying variables with high regression coefficient. This could reveal the candidates of the active components [111].

A similar algorithm is carried out by TP as PLS but one target component was involved in determining the response. The loading of the target component is able to provide the useful information in the regression model simultaneously. This would enhance the interpretation of the latent variables in multivariate modeling.

2.5.3 Orthogonal Partial Least Squares (O-PLS)

Orthogonal projections to latent structure by means of partial least squares regression or also known as orthogonal partial least squares (O-PLS) was developed as the modification of partial least square method by Trygg and Wold [112]. The spectral profiles were decomposed first which is orthogonal to the response. Then, the single partial least squares component is extracted with the consideration of covarying maximally to the response. In PLS, the residual variables of **X** are unexplained by the latent variables and regress to **Y**. They contain none of the information. However, they would be included in modeling and results in over-fitting problem. Because of this, the

non-correlated systematic variation of **X** was removed that was introduced in O-PLS [112]. This would also aid in interpretation of the multivariate modeling like TP. In addition to it, this reduced the complexity of the modeling.

The decomposition of X variables through O-PLS can be expressed as the following.

$$\mathbf{X} = \mathbf{X}_{ort} + \mathbf{X}_{pred} + \mathbf{E}_{OPLS} = \mathbf{T}_{ort}\mathbf{P}_{ort}^{T} + \mathbf{t}_{pred}\mathbf{p}_{pred}^{T} + \mathbf{E}_{OPLS} \quad [2.18]$$

The algorithm of O-PLS is the same as non linear iterative PLS algorithm for correlating **X** variables to **y**. Afterwards, the non-correlated **x** variables were found for the orthogonal components. It can be accomplished from applying the unwanted property of **y** or orthogonalizing the loading vector. This could ensure orthogonality of all **y** variables from the resulting orthogonal score vector [112, 113]. Orthogonal components are extracted until orthogonal variation is exhausted in addition to the result of cross validation [111]. Therefore, the correlated variations were separated from non-correlated orthogonal variations. As a y-variable is concerned, the number of components for PLS is reduced to single principal component. It removes the systematic variation in **x**-variables and explains the predictive part of **x**-variables. This is similar to target projection. As a result, the interpretation of the model is improved.

2.6 Variable selection methodology

During establishment of the relationship between the response or property and spectroscopy variables, selection of variables is needed in order to improve the prediction of the property. The full spectrum method would introduce the noise or irrelevant information during modeling, and would increase the complexity of the model and reduce the prediction ability. To remove the systematic variation in **X** that is not corresponding to **y**, improved PLS methods such as TP and OPLS were introduced. Besides, variable selection is also one kind of means to remove this type of variation. In fact, the important variables towards the property during the variable selection procedures could be reflected.

Currently, the discovery of biomarker in metabolomics study or investigation of the most influential components on the quality of the product can be achieved through the variable selection [104]. Also, it could aid in identification of the potential active components in

HM as in our study. Indeed, there is large number of variables due to the presence of uninformative variables or redundant information from strongly correlated or collinear variables. There are different developed methods available for variable selection such as variable importance projection (VIP) [114], successive projection algorithm (SPA) [104, 115, 116], uninformative variable elimination (UVE) [104, 117, 118], selectivity ratio (SR) [109, 111], size of regression coefficient [114] or loading plot. Among them, UVE, SR and SPA are the focus of many.

2.6.1 Uninformative Variable Elimination by Partial Least Squares (UVE-PLS)

Uninformative variable elimination by partial least squares (UVE-PLS) is a selection method based on the analysis of **b** regression coefficient. During the variables elimination process, the artificial variables are added to the experimental variables. The regression between artificial and experimental variables and response is carried out. The regression coefficient can thus be obtained for each variable. For those variables with high regression coefficients, they are important and contribute significantly to the response. However, it is very hard to decide a variable is informative or not from the magnitude of the regression coefficient.

Because of this, the stability or reliability of the variables, $\mathbf{s}_j$ or $\mathbf{c}_j$, is evaluated with the consideration of the regression coefficient [117, 118].

$$\mathbf{s}_j = \text{mean}\ (\mathbf{b}_j) \,/\, \text{std}\ (\mathbf{b}_j) \qquad [2.19]$$

The ratio of the mean of regression coefficient to the standard deviation of the regression coefficient of the variable, j, is known as stability. The regression coefficient is measured for n times using leave-one-out jackknifing. The highest stability of the artificial variable is set as the threshold value for the elimination of the variables. For those variables with the stability value lower than the threshold value, they are eliminated as they are not informative variables for modeling than noise [104, 117, 118].

Another approach in retaining the informative variable was proposed by Cai et al. [117]. In this study, Monte-Carlo method was coupled with the UVE method. The Monte-Carlo

method was applied to find the stability of the variables instead of using leave-one-out jackknifing. This modification of calculating the stability of the variables would aid in reducing the over-fitting problem that may arouse in the leave-one-out approach. This kind of variable selection method is widely applied to analytical chemistry such as determination of antioxidant activity of green tea from their chromatographic fingerprints [119, 120] or identification of the potential biomarkers [121].

2.6.2 Selectivity Ratio (SR)

Selection of variables from selectivity ratio is usually corporate with target projection [109, 111]. As mentioned previously, a single predictive target component is applied in the prediction or even in discrimination. It involves the projection of the variables on this target component. Then, the variance explained by the target components for each variable can be found and compared with the residual variance for the same variable. From these, the ratio between the explained ($\mathbf{V}_{expl}$) and residual ($\mathbf{V}_{res}$) variance of the spectral variables was applied in determining the significance of the variables. It is defined as selectivity ratio (SR).

$$SR_i = \mathbf{V}_{expl,i} / \mathbf{V}_{res,i} \qquad [2.20]$$

Of course, the more significant spectral variable would have the higher SR value. The application of SR was first introduced in the discriminating the biomarker in the cerebrospinal fluid [111].

2.6.3 Successive projections algorithm (SPA)

Successive projections algorithm (SPA) is a newly developed variable selection strategy. The projection operation is carried out for the selection of variables with the minimum collinearity. It involves the selection of the new variable with the maximum projection value on the orthogonal subspace of the previously selected variable. Then, the newly selected variable is used as the new starting vector for subsequent selection. The number of optimal variables to be selected and the initial reference variables for the selection are determined from the validation set through the values of root mean square error (RMSE) [104, 115]. As the projection of the variables is transected, this would not modify the

original data vectors.

This method was applied to investigate the acetic, tartatic and lactic acids of plum vinegar using visible and near-infrared spectroscopy [116]. Also, it is useful in selecting the variables with minimal collinearity. Thus, it combined with another variable selection method, UVE, for the extraction of the NIR spectral variables [104]. Better prediction performance could be achieved in analyzing the nicotine in tobacco lamina via NIR spectroscopy. In this study, UVE removed the uninformative information and SPA further selected the variables with minimal collinearity. It is another kind of variable selection strategy for reducing complexity of the model and choosing the informative variables to the response.

The existing chemometric techniques for different quality control approaches as described above can provide information on the safety and consistency of HM. In addition, the efficacy of HM can be evaluated and identification of the potential bioactive candidates can be preformed through the established relationship between the chromatographic fingerprint and bioactivity with the help of chemometric technique.

Chapter 3

Methods of investigation on chemical analyses and antioxidant activity of herbal medicines concerned and related system

3.1 Introduction

The herbal formula, Danshen-Gegen Tang (DGT) and its two component herbs, Radix et Rhizoma Salviae Miltiorrhizae (DS) and Radix Puerariae Lobatae (YG), were studied in this work. Here, the chemical composition and the antioxidant activity of HM were correlated. Three kinds of sample preparation were involved for extracting DS, YG and DGT respectively. Ultrasound assisted extraction, maceration and boiling were used. A set of synthetic mixtures (MIX) was also prepared for in-depth study. The sample preparation was conducted following the uniform design table. Afterwards, chemical analyses of DS, YG, DGT and MIX were carried out through our hyphenated instruments. Fingerprints that are related to their chemical compositions were thus recorded. At the same time, the antioxidant activity of the HMs were evaluated by the in-vitro antioxidant assay, Ferric Reducing Antioxidant Power assay (FRAP). The experimental details were described in the following.

3.2 Sample preparation and Extraction procedures

The commonly used extraction methods for preparing the herbal extracts include reflux, ultrasound assisted extraction and maceration. The extraction efficiency of different classes of components is affected by the extraction conditions. In turn, the bioactivity of the HM extract thus obtained is affected. Different sample preparations were carried out for DS, YG and the DGT in this study. In order to obtain the optimal antioxidant activities, experimental design and response surface methodology were applied to optimize the extraction conditions of the two component herbs. The preparation of DGT was based on the optimal extraction conditions of DS and YG. Another kind of sample which is known as synthetic mixtures (MIX) was prepared by mixing twelve chemical standards with known chemical structures and properties at different concentration levels. In the following section, the extraction conditions of DS, YG and DGT in addition to the preparation of MIX were given.

3.2.1 Sample preparation of Radix et Rhizoma Salviae Miltiorrhizae (DS)

3.2.1.1 Plant materials

Twenty different DS samples were collected from different origins of P.R. China. They were authenticated by Prof. Cao Hui of National Engineering Research Center for Modernizing Traditional Chinese Medicine, Zhuhai, Guangdong, China and Prof. Wang Yali of College of Gansu Traditional Chinese Medicines, Lanzhou, China. The information of these samples is listed in the Table 3.1.

Table 3.1 Sources of the Danshen samples in P.R. China

Sample	Collection location
DS1	Meng County, Henan province
DS2	Hanhai, Jiangsu province
DS3	Gansu province
DS4	Gansu province
DS5	Lijiang city, Yunnan province
DS6	Wenshan prefecture, Yunnan province
DS7	Shangluo city, Shannxi province
DS8	Honghe, Yunnan province
DS9	Mengshan, Shandong province
DS10	Haozhou city, Anhui province
DS11	Chuxiong, Yunnan province
DS12	Henan province
DS13	Zhongjiang county, Sichun province
DS14	Yuncheng city, Shanxi province
DS15	Mengzhou city, Henan province
DS16	Rong County, Shandong province
DS17	Haozhou, Anhui province
DS18	Min County, Gansu province
DS19	Min County, Gansu province
DS20	Gansu province

3.2.1.2 Sample extraction of DS

In screening the sample extraction, ultrasound assisted extraction and reflux were carried out respectively using 1g DS herbal powder and 25ml extraction solvent for 30 minutes.

The extraction solvents included methanol, ethanol and water. The antioxidant activity levels and the contents of the reference compounds of these extracts were compared in order to select the best extraction procedures for investigating the effect of the extraction parameters as mentioned below and optimizing the extraction condition.

To study the effect of the extraction parameters on antioxidant activity, 1g of DS herbal powder was extracted by ultrasound assisted extraction according to the specific combination of the conditions listed in Table 3.2. The four extraction parameters, methanol content (X1), number of times of extraction (X2), duration (X3) and solid to liquid ratio (X4) and three levels of each extraction parameter were concerned. Re-extraction of DS was involved when two or more times of extraction was needed. After the filtration of DS, the same extraction condition was applied again to the residue. The design table was CD_2 Discrepancy -- $U_n(q^s)$ [122].

Table 3.2 Experimental conditions for investigating the effect of the extraction parameters on the antioxidant activity of DS extracts

Extract	Methanol content (%) (X1)[a]	Number of times of extraction (X2)	Duration (min) (X3)	Solid to liquid ratio (g/ml) (X4)
UM1	0 (1)	3 (3)	30 (2)	1:12.5 (1)
UM2	25 (2)	2 (2)	30 (2)	1:25 (2)
UM3	25 (2)	3 (3)	45 (3)	1:50 (3)
UM4	50 (3)	1 (1)	30 (2)	1:50 (3)
UM5	50 (3)	3 (3)	15 (1)	1:25 (2)
UM6	50 (3)	2 (2)	45 (3)	1:12.5 (1)
UM7	0 (1)	2 (2)	15 (1)	1:50 (3)
UM8	0 (1)	1 (1)	45 (3)	1:25 (2)
UM9	25 (2)	1 (1)	15 (1)	1:12.5 (1)

[a] The numerical value stated in the table represents the percentage of methanol in the solvent mixture

The optimal extraction conditions were determined by the following two extraction parameters associated with nine experimental levels. Different combinations of methanol content (X1) and solid to liquid ratio (X4) were listed in Table 3.3. Subsequently, nine extracts were prepared from the uniform design table which is Centered L_2 Discrepancy -- $U_n(n^s)$ [122].

Table 3.3 Experimental conditions for optimizing the extraction conditions

Extract	Methanol content (%) (X1)[a]	Solid to liquid ratio (g/ml) (X4)
U1	40 (5)	1:50 (5)
U2	0 (1)	1:40 (4)
U3	60 (7)	1:80 (8)
U4	10 (2)	1:70 (7)
U5	20 (3)	1:20 (2)
U6	80 (9)	1:60 (6)
U7	70 (8)	1:30 (3)
U8	50 (6)	1:10 (1)
U9	30 (4)	1:90 (9)

[a] The numerical value stated in the table represents the percentage of methanol in the solvent mixture

From the response surface methodology, the optimized extraction of DS was found. 1g of finely grinded DS powder was accurately weighted and extracted with 90ml of 80% methanol by ultrasound assisted extraction for 45minutes. The extraction was carried out once and the extract was filtered through a Watman No. 1 filter paper. The filtrate was evaporated using rotatory evaporator in order to remove the solvent, followed by freeze drying the filtrate for both antioxidant assay and chemical analysis.

3.2.1.3 Preparation of chemical reference solutions of phenolic acids and diterpenoids

Five chemical reference compounds were selected for identification and quantification. Among them, danshensu (DA), salvianolic acid B (SAB), tanshinone I (TSI) and tanshinone IIA (TSIIA) were bought from National Institute for the Control of Pharmaceutical and Biological Products while rosmarinic acid (RA) was purchased from Alexis. The stock solutions of RA, SAB and TSIIA were dissolved in ethanol. For DA and TSI, their stock solutions were prepared by dissolving in water and methanol respectively. Then, they were dissolved in 70% ethanol to give serial concentrations of DA, RA, TSI and TSIIA from 0ppm to 160ppm later. The concentration range of SAB was from 0ppm to 320ppm. The concentrations of these five compounds in the Danshen samples were determined via their corresponding calibration curves.

3.2.2 Sample preparation of Radix Puerariae Lobatae (YG) and the synthetic mixtures (MIX)

3.2.2.1 Plant materials

Seventy-nine Radix Puerariae Lobatae (YG) samples were collected from different provinces in P.R. China while one Radix Puerariae Lobatae sample was purchased in the local whole seller market in Hong Kong with voucher available. All these samples were authenticated by Dr. Chen Sibao of the State Key Laboratory of Chinese Medicine and Molecular Pharmacology, The Hong Kong Polytechnic University, Shenzhen, China. A list of all the YG samples for this study is listed in Table 3.4.

Table 3.4 Sources of Radix Puerariae Lobatae samples in P. R. China.

Sample	Collection location
YG-1	Unknown
YG-2	Huoshan, Anhui, China
YG-3	Huoshan, Anhui, China
YG-4	Huoshan, Anhui, China
YG-5	Putian, Fujian, China
YG-6	Putian, Fujian, China
YG-7	Putian, Fujian, China
YG-8	Putian, Fujian, China
YG-9	Hunan, China
YG-10	Hunan, China
YG-11	Hunan, China
YG-12	Hangzhou, Zhejian, China
YG-13	Hangzhou, Zhejian, China
YG-14	Shanghai, China
YG-15	Shenyang, Liaoning, China
YG-16	Nanning, Guangxi, China
YG-17	Nanning, Guangxi, China
YG-18	unknown
YG-19	unknown
YG-20	unknown
YG-21	unknown
YG-22	unknown
YG-23	unknown
YG-24	unknown
YG-25	unknown
YG-26	unknown

YG-27	unknown
YG-28	unknown
YG-29	Huoshan, Anhui, China
YG-30	Huoshan, Anhui, China
YG-31	Huoshan, Anhui, China
YG-32	Huoshan, Anhui, China
YG-33	Huoshan, Anhui, China
YG-34	Huoshan, Anhui, China
YG-35	Huoshan, Anhui, China
YG-36	Huoshan, Anhui, China
YG-37	Huoshan, Anhui, China
YG-38	Huoshan, Anhui, China
YG-39	Huoshan, Anhui, China
YG-40	Huoshan, Anhui, China
YG-41	Shaanxi, China
YG-42	Shaanxi, China
YG-43	Sichuan, China
YG-44	unknown
YG-45	unknown
YG-46	unknown
YG-47	unknown
YG-48	unknown
YG-49	unknown
YG-50	unknown
YG-51	unknown
YG-52	unknown
YG-53	unknown
YG-54	unknown
YG-55	unknown
YG-56	unknown
YG-57	unknown
YG-58	unknown
YG-59	unknown
YG-60	unknown
YG-61	unknown
YG-62	unknown
YG-63	unknown
YG-64	unknown
YG-65	unknown
YG-66	unknown
YG-67	unknown
YG-68	unknown
YG-69	unknown

YG-70	unknown
YG-71	unknown
YG-72	unknown
YG-73	unknown
YG-74	unknown
YG-75	unknown
YG-76	unknown
YG-77	unknown
YG-78	unknown
YG-79	unknown

3.2.2.2 Sample extraction of YG

The optimized extraction condition was determined first before using uniform design with the consideration of antioxidant activity and chemical composition. YG samples were grinded into fine powder (100mesh). 4g of YG powder was accurately weighted and 180ml double deionized water was added in the conical flask under maceration at 50℃ water bath. The duration of the extraction was 75minutes. After that, the extract was filtered through the Watman No. 1 filter paper. The same extraction conditions were applied on the filtered solid for re-extraction. The filtrates were combined together for freeze drying. The dried extract was collected for both chromatographic and antioxidant activity investigation.

3.2.2.3 Preparation of chemical reference solutions of isoflavonoids

Five isoflavonoids, puerarin (PU), daidzin (DZ), genistin (GE), daidzein (DE) and genistein (GS), were selected as chemical standards for both qualitative and quantitative analyses in this investigation. They were purchased from International Laboratory USA. The purities of these components were above 98%. Puerarin was dissolved in water and the rest of the chemical standards were dissolved in ethanol for both quantitative and qualitative analyses. Six different concentrations of each of them were prepared for quantification. Then, the calibration curve of each one was obtained by plotting the average of the corresponding chromatographic peak area against the concentration based on the data obtained.

3.2.3 Sample preparation of synthetic mixtures (MIX)

The synthetic mixtures were composed of twelve selected chemical standards in order to find out the possible bioactive candidates using the proposed chemometric methods (Chapter 6). They were also utilized to evaluate the performance of the chemometric methods in data analyses. In this investigation, sixty mixtures were prepared according to the uniform design table with CD_2 Discrepancy (Table 3.5). Different classes of compounds were chosen in making up the synthetic mixtures. Gallic acid (GA), kaempferol (KF), rutin (RT), quercetin (QT), emodin (ED), hydroxybenzoic acid (HB), ursolic acid (UA), daidzin (DZ), genistein (GS), coumaric acid (CMA), puerarin (PU), betulinic acid (BA) were chosen from several classes of herbal ingredients including phenolic acids, flavonoids, isoflavonoid, triterpenoid and anthraquinone. Different concentration ranges were selected for them and were given in Table 3.6. Levels 1 and 6 correspond to zero and the highest concentration of the interested component respectively. The concentration of each component increased in proportion with the number of level.

Table 3.5 Design table for sixty different combinations of mixtures

Sample	GA	KF	RT	QT	ED	HB	UA	DZ	GS	CMA	PU	BA
MIX 1	4	2	6	2	3	3	6	4	6	1	3	6
MIX 2	3	2	1	3	5	4	3	6	2	6	6	4
MIX 3	1	4	2	1	3	3	1	4	1	3	6	2
MIX 4	2	3	3	3	4	6	6	1	6	2	5	2
MIX 5	5	5	3	6	4	1	3	4	3	5	6	1
MIX 6	4	5	3	3	6	1	6	1	3	4	1	5
MIX 7	5	5	2	4	2	4	5	1	1	2	6	5
MIX 8	2	5	3	4	2	2	1	3	6	5	6	6
MIX 9	6	4	5	6	5	4	3	3	5	3	1	6
MIX 10	6	1	5	3	3	5	5	1	4	5	4	6
MIX 11	3	6	6	1	5	2	5	2	2	5	4	2
MIX 12	6	6	2	1	3	1	3	5	6	2	2	3
MIX 13	3	1	3	6	3	3	6	2	1	5	1	3
MIX 14	3	3	5	1	2	1	4	1	5	1	5	4
MIX 15	1	6	4	6	6	3	5	3	6	2	4	4
MIX 16	2	4	6	2	5	5	3	2	3	1	6	5
MIX 17	1	2	5	4	6	2	4	3	1	5	5	5
MIX 18	3	6	3	5	5	5	2	3	1	1	3	2
MIX 19	6	2	3	3	2	6	1	3	4	1	2	1
MIX 20	1	2	1	3	2	1	4	2	4	3	2	6
MIX 21	2	5	5	2	3	6	1	6	3	5	1	3
MIX 22	5	1	6	5	2	3	4	5	3	2	1	4
MIX 23	4	5	2	1	6	5	4	6	4	5	4	1
MIX 24	2	4	3	1	4	3	5	6	5	6	2	6
MIX 25	2	1	1	1	1	5	6	3	3	4	3	4

MIX 26	6	2	3	2	1	2	4	6	3	4	5	2
MIX 27	4	3	6	4	1	5	2	6	6	3	6	4
MIX 28	5	3	1	6	2	4	5	5	6	5	3	2
MIX 29	6	5	4	3	6	3	2	5	2	1	5	6
MIX 30	3	5	1	4	5	4	4	5	5	1	1	2
MIX 31	4	3	2	6	4	6	1	5	1	4	4	6
MIX 32	6	6	6	4	4	3	1	1	4	3	3	2
MIX 33	1	1	3	5	3	4	2	6	5	1	4	5
MIX 34	3	1	2	2	4	2	2	1	2	2	1	5
MIX 35	5	6	1	2	4	6	2	2	5	5	5	4
MIX 36	3	6	1	5	3	6	5	4	3	3	5	6
MIX 37	5	1	5	1	4	5	3	5	1	3	4	3
MIX 38	3	1	2	4	1	3	3	2	6	6	4	1
MIX 39	4	4	6	2	2	4	2	3	1	6	2	2
MIX 40	5	2	4	1	6	4	1	2	6	4	2	5
MIX 41	5	4	6	5	4	2	6	5	4	6	5	5
MIX 42	4	4	4	5	1	5	5	1	5	3	1	1
MIX 43	1	6	4	2	2	4	6	5	4	4	6	1
MIX 44	6	2	2	5	5	2	6	3	5	3	6	3
MIX 45	1	3	2	5	6	5	3	1	3	6	2	3
MIX 46	4	1	5	6	6	6	4	4	4	2	6	2
MIX 47	1	3	4	1	1	6	3	4	2	2	3	6
MIX 48	2	2	4	4	4	1	5	6	2	1	2	1
MIX 49	1	3	6	3	5	2	2	4	6	4	1	1
MIX 50	3	6	5	6	1	2	3	6	4	4	2	5
MIX 51	2	2	5	6	3	4	2	1	2	4	5	1
MIX 52	2	5	5	3	1	1	6	4	1	2	4	3
MIX 53	1	5	6	4	3	6	4	2	5	6	3	3
MIX 54	6	4	4	4	5	6	6	6	1	4	2	4
MIX 55	2	4	1	5	6	1	1	5	4	3	3	4
MIX 56	5	3	1	2	6	3	5	3	2	2	3	1
MIX 57	5	6	2	3	1	5	4	4	2	6	1	5
MIX 58	4	1	4	2	5	1	1	4	5	6	5	3
MIX 59	4	4	1	6	1	2	1	2	3	1	4	3
MIX 60	6	3	4	5	2	1	2	2	2	6	3	4

Table 3.6 Concentrations of each component at different levels

Component	**Concentration (ppm)**					
	1	**2**	**3**	**4**	**5**	**6**
GA	0	20	40	60	80	100
KF	0	12	24	36	48	60
RT	0	24	48	72	96	120
QT	0	24	48	72	96	120
ED	0	63	126	189	252	315
HB	0	63	126	189	252	315
UA	0	63	126	189	252	315
DZ	0	25	50	75	100	125
GS	0	25	50	75	100	125
CMA	0	25	50	75	100	125
PU	0	25	50	75	100	125
BA	0	63	126	189	252	315

3.2.4 Sample preparation of Danshen-Gegen Tang (DGT)

3.2.4.1 Sample extraction of DGT

The raw component herbs were mixed together with different ratios of DS to YG from the range of 1:9 to 9:1 to prepare the DGT samples with the same total mass for subsequent studies. At the ratio of DS to YG being 1:9, 0.1 g of DS was mixed with 0.9g of YG for extraction. Likewise, the DGT with different ratios were prepared in the same way. Different DGT were composed of DS to YG ratio at 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8 and 1:9.

Two extraction methods were proposed for preparing the DGT including boiling and ultrasound assisted extraction. The mixture of two herbs was extracted under 100ml boiling water for 30 minutes. For ultrasound assisted extraction, the conical flask with the herbal mixture and 100ml water was put in the ultrasound water bath for the same duration of extraction. The same extraction procedure was carried out again on the filtered solid. The extraction solvent of the combined herbal extracts was evaporated by rotary evaporation and then undergone the freeze dried process. The sample obtained was then prepared at the concentration of 1mg/ml for both antioxidant assay and chemical analysis.

3.2.4.2 Preparation of chemical reference solutions of isoflavonoids and phenolic acids

Eight chemical reference compounds were selected to analyze the DGT samples. They were PU, DZ, GE, DE, GS, DA, RA and SAB. The preparations of eight chemical reference solutions were the same as mentioned previously. Dilution of these selected chemical reference compounds were preformed by double deionized water. The calibration curve of each chemical reference compound was obtained from plotting the peak areas against the concentrations.

3.3 Chromatographic fingerprints of DS, YG, MIX and DGT

DS, YG and MIX were chemically characterized by high-performance liquid chromatography (HPLC) coupled with diode array detector (DAD) and ion trap mass

spectrometer (MS). DGT is composed of two herbal medicines which is a more complex system than a single herbal medicine. Hence, in this work, DGT was analyzed by the rapid resolution liquid chromatography (RRLC) coupled with DAD to improve the resolution and reduce the analytical time. In addition, the injection volume of the sample in RRLC system is much less than the amount of sample to be injected in the HPLC system. The same mobile phase was utilized for analyzing DS, YG and DGT. The binary gradient elution system was made up by a combination of acetonitrile (solvent A) and 0.3% acetic acid in water (solvent B). The chromatographic condition of DS, YG and DGT were stated in the following sections. For MIX, different binary gradient elution system was applied which contained methanol with 0.1% phosphoric acid (solvent A) and double deionized water with 0.1% phosphoric acid (solvent B).

For HPLC-DAD-MS analysis, the LC analysis was carried out using an Agilent 1100 system (Agilent Technologies, Inc. USA) with binary pump, a degasser, an auto-sampler, a diode array detector (DAD) and ion trap mass spectrometer (MS) using electrospray ionization source (ESI). The chromatographic separation was performed using reversed phase, ODS hypersil column with the dimension of the column being 250mm X 4.6mm and the particle size being 5μm at room temperature.

For RRLC-DAD analysis, the instrumental components of an Agilent 1200 RRLC system (Agilent Technologies, Inc. USA) are similar with Agilent 1100 series. In addition, a thermostatically controlled column apartment is equipped with. The chromatographic separation was performed using an Agilent ZorBax SB-C18 column with the dimension of the column being 100mm X 4.6mm and the particle size being 1.8μm at a temperature of 40^{o}C.

3.3.1 Chromatographic condition of DS

The complete chromatographic condition lasted for 60minutes and the flow rate was 1ml/min for analyzing DS. The detection wavelength was 280nm. The injection volume was 10μl. Details of the gradient elution were listed in Table 3.7.

Table 3.7 The gradient elution of mobile phase for analyzing DS

Time (min)	A / %	B / %
0	7	93
20	23	77
40	35	65
50	75	25
60	75	25

3.3.2 Chromatographic condition of YG

The chromatographic analysis of YG was carried out for 55min and the flow rate was 1 ml/min. An aliquot of 10μl of each sample solution was injected into HPLC system for analysis. The detection wavelength was set at 254nm. The corresponding gradient elution was summarized in Table 3.8.

Table 3.8 The gradient elution of mobile phase for analyzing YG

Time (min)	A / %	B / %
0	7	93
15	20	80
20	25	75
30	35	65
40	50	50
45	75	25
55	75	25

3.3.3 Chromatographic condition of MIX

The modified chromatographic condition reported by Conde et al. was applied in analyzing the synthetic mixtures [123]. The running time lasted for 50 minutes and the flow rate was carried out at 1ml/min. An aliquot of 20μl of each sample solution was injected and detected at 210nm. Details of the chromatographic conditions were given in Table 3.9.

Table 3.9 The gradient elution of mobile phase for analyzing MIX

Time (min)	A / %	B / %
0	20	80
6	36	64
8	49	51
15	90	10
50	90	10

3.3.4 Chromatographic condition of DGT

The analytical time was greatly reduced to 10 minutes utilizing RRLC-DAD instrument. The flow rate was 1.0ml/min. Also, the injection volume was further reduced to 2μl for each sample solution. The detection wavelengths were at 254nm and 280nm. From Table 3.10, the chromatographic conditions of RRLC utilized in analyzing DGT were summarized.

Table 3.10 The gradient elution of mobile phase for analyzing DGT

Time (min)	A / %	B / %
0	10	90
6	30	70
6.5	50	50
7.5	65	35
10	65	35

3.3.5 Mass spectrometric study on YG

Mass spectra of YG were acquired using Agilent ion trap analyzer connected to HPLC system via ESI interface. The LC effluent was introduced ESI source in the splitting ratio of 5:1. The instrument was operated in the positive ion mode with the voltage of 4 kV. The dry gas temperature was of 350°C and the dry gas flow rate was set to be 9 l/min. The nebulization was carried out by coaxial nitrogen sheath gas provided at 30psi. Mass spectra were recorded over the range of 100 and 1000m/z.

3.4 Ferric Reducing Antioxidant Power assay (FRAP)

In this research study, the antioxidant activities of DS, YG, DGT and MIX were evaluated utilizing FRAP assay. It is a fast and reliable screening method. It was preformed according to the procedure described by Benzie and Strain. COBAS FARA II spectrofluorometric centrifugal analyzer (Roche) was utilized. The experiment was conducted under 37℃ and pH 3.6 conditions. Ascorbic acid standards were tested as control. 300 μl of FRAP reagent, the mixture of 20ml of acetate buffer solution, 2ml of Fe(III) solution and 2ml of TPTZ solution, was freshly prepared. The reagent blank

reading was measured at 593nm. 10 μl of sample was added. The change of absorbance between final reading selected and the reagent blank reading was calculated for each sample after 4 minutes. The change of absorbance of Fe(II) standard solution was measured in parallel. The antioxidant activity was expressed in terms of FRAP values which is arbitrarily defined as the reduction of 1mole of Fe(III) ion to Fe(II) ion.

3.5 Fractionation of the YG extract

The bioactive components are usually not known in the herbal extract as it contains multiple constituents and very little information of this kind is available in the literature. Fractionation of herbal extract is carried out very often so as to isolate the components for bioassay. In this research study, a preparative high-performance liquid chromatography (PHPLC) was aided in fractionating the YG sample. The fractionation was conducted according to the retention time. Three fractions were acquired for both antioxidant assay and further identification of the components using LC-MS study. The first (F1), second (F2) and third fractions (F3) were collected within 2min and11min, 11min and 18min, and 18min and 40min, respectively. The same chromatographic condition was compassed with the analytical chromatographic condition of YG which was mentioned above (section 3.3.2). Details about the gradient elution were outlined in Table 3.8. It is because the working principle of PHPLC and analytical HPLC is more or less the same. This is the advantage of using PHPLC for fractionation in which the chromatographic procedures can be switched between these two types of instruments. Therefore, we could obtain the concerned fractions. However, it usually takes long time to get enough fractions for further investigation.

Our preparative HPLC instrument was equipped with an ODS hypersil column in the dimension of 250mm X 21.2mm of the particle size being 5μm. The solvent saver, Solvent Recycler Junior (Alltech, USA), diode array detector (DAD) was connected with the preparative system and the fractions of YG were collected with a Foxy® Jr. fraction collector (ISCO, Inc., USA). Here, the injection volume was 150μl and the flow rate was 20ml/min. The detection wavelength was set at 254nm.

Chapter 4

Analysis of antioxidant activities and chemical compositions in Radix et Rhizoma Salviae Miltiorrhizae (DS) using the optimized extraction conditions by experimental design

4.1 Introduction

It is well known that the growing environments and harvesting conditions are the key factors on influencing the chemical compositions of herbal medicine and the therapeutic properties as well to certain extent. In addition, the processing methodology applied to HM would induce the variation of the contents and the classes of components to be extracted [124, 125]. Since the bioactivity of HM depends on the components within, both the sources of HM and manufacturing process would affect the quality of the HM product. Generally, extraction of the components from HM is based on diffusion, mass transfer process and solubility. Several ways such as reflux, ultrasound assisted extraction, maceration or pressurized liquid extraction helped to do so. In addition to these stated methods, extraction parameters included extraction solvent, ratio of herbal powder to extraction solvent, duration and number of times of extraction should be considered also. With the selection of the appropriate extraction method and the combination of different extraction parameters, optimal outcome can be achieved.

The extraction efficiency of the HM components would depend on individual extraction parameter as well as their combined effect. If these input parameters were studied one by one, it is very time consuming. Also, it is unable to study interactions among them. Under this situation, experimental design can aid in carrying out the experiments systematically with less number of trials. Then, the relationship between them and response can be explored through the response surface methodology (RSM). Through which, the influence of the parameters can be learned. At the same time, the optimal extraction conditions can be obtained. There are various studies on optimizing the extraction process of food or HM using experimental design and RSM. Usually, the concentrations of the interested components are focused during optimization [126-131]. For example, the optimal content of flavonoids in onion and Folium eucommiae were achieved via RSM [127, 128]. It is believed that the optimal concentrations of a few selected components would result in better pharmacological activity. But they may not contribute significantly to the therapeutic effect of herbal medicine. Thus, the optimal bioactivity or therapeutic effect is the target response instead of just maximizing the concentrations of components or markers like many do during exploring the best extraction conditions.

Radix et Rhizoma Salviae Miltiorrhizae (DS), Danshen in Chinese, has been widely used in China for the treatment of the cardiovascular disease such as coronary arteriosclerosis and angina pectoris and treatment of menstrual disorder. It can promote circulation and improve blood stasis [20-22, 24, 25]. It is always applied as complementary drug in the treatment of the cardiovascular disease because of its antioxidant property [132-134]. Two classes of components in DS, phenolic acid and diterpenoid, were found to be antioxidant active [31]. As mentioned before, the biological activity of HM is due to the joint effect of multiple constituents. In this study, it is interested to get the best combination of the right components from DS in achieving the optimal antioxidant activity. Thus, the antioxidant activity was designed as the response for optimization of the extraction conditions in this work. A few studies were done on optimizing the bioactivity [135, 136]. Previously, the optimization of the extraction condition for DS was determined for having optimal recoveries of the selected tanshinones and danshensu [131]. Here, the RSM was applied in establishing the relationship between the extraction parameters and antioxidant activity of DS.

It is believed that relatively high level of antioxidant activity can be acquired from the optimal extraction conditions. In this investigation, twenty different DS samples collected from different regions of P.R. China were prepared under the optimal conditions for antioxidant activity evaluation and fingerprint analysis. Because of the cultivation conditions and others, the chemical ingredients and antioxidant properties of these samples varied among one another. In this way, we explored the relationship between antioxidant activities and compositions of DS.

Apart from that, the role of extraction parameters concerned was investigated so as to optimize the extraction conditions of DS based on the antioxidant capacity obtained. Also, the relationship between the antioxidant activity and chemical composition of twenty DS samples from different habitats was investigated under the optimal extraction conditions derived.

4.2 Background of Radix et Rhizoma Salviae Miltiorrhizae (DS)

There are over a hundred species from the genus *Salvia* which are distributed in different regions of China such as *Salvia miltiorrhiza* Bunge, *Salvia przewalskii* Maxim, *Salvia verticillata, Salvia miltiorrhiza* f. *alb*, *Salvia castanea* Diels f. *tomentosa* Stib, *Salvia bowleyana*, *Salvia deserta*, *Salvia sinica*, and *Salvia paramiltiorhiza* [21, 29, 137]. They belong to the family of Labiatae or Laminaceae. According to Chinese Pharmacopoeia 2010, *Salvia miltiorrhiza* Bunge is designated as the only source of Danshen. The other species were considered as substitute although some of them possess huge variety of activities [76]. The dried root and rhizoma of *Salvia miltiorrhiza* Bunge is commonly used to treat different diseases (Figure 4.1). Radix et Rhizoma Salviae Miltiorrhizae which is known as Danshen in China is able to promote blood circulation, possess angiogenic activity, remove stasis and lower the blood lipid level. It is important in the treatment of cardiovascular disease such as preventing and treating angina pectoris, preventing atherosclerosis and protecting against ischemia-reperfusion injury [20, 23]. Besides, it involved in anti-tumor activity, treatment of homeostasis related disease, chronic renal failure, dysmenorrheal, hepatitis, menstrual disorders, menorrhalgia and insomnia [26-31, 33, 138].

Usually, the treatments of the cardiovascular disease and human cancer are in concern. These two diseases are related to the oxidative damage caused by reactive oxygen species (ROS). Thus, antioxidant is needed in protecting the body from ROS damage. It is believed that the side effect of the natural source in healing is less than that of the western drug. There are more and more studies focusing on screening the antioxidant activity from different natural sources. The cardioprotective effect of DS is comparable to the commonly used western drug, ramipril. It was found that the cardio-protective effect was contributed by the antioxidant activity through enhancing the antioxidant enzymes and reducing the free radical production [26]. Also, it prevented the myocardial infarction injury, decreased the lipid peroxidation, enhanced the antioxidant enzyme activities, scavenged free radical, lowered the endothelin release and improved the local circulation

[133, 139, 140]. Because of these, cardiovascular system is protected from the oxidative damage and thus reducing atherosclerosis.

Apart from that, the aqueous extract of DS was found to have antitumor activity. It prevented the proliferation of human hepatoma HepG2 cells and caused the apoptotic cell death [31]. Both phenolic acid and diterpenoid especially tanshinone are essential in causing the apoptosis of cancer cell. A study showed that tanshinone IIA could induce the apoptosis on leukemic cell line also [141]. A recent finding showed that the newly discovered compound, neo-tanshinlactone, in DS was active against the estrogen receptor positive human breast cancer cell lines [31]. Many other tanshinones possess the cytotoxicity against tumor cell lines. These studies suggested that Danshen was potential anti-cancer agent.

Figure 4.1 Appearance of Radix et Rhizoma Salviae Miltiorrhizae

4.2.1 Chemical composition of DS

Two classes of components, diterpenoid and phenolic acid, are the major constituents in DS. The former class of compounds is lipid soluble while the latter one is water soluble. They were selected as the reference compounds for authentication of our DS sample. Tanshinone IIA (TSIIA) and salvianolic acid B (SAB) are recommended as chemical makers for identification of DS as stated in the Chinese Pharmacopoeia 2010 edition. Their respective contents should not be less than 0.20% and 3.0% [142]. Besides these two compounds, rosmarinic acid (RA) and cryptotanshinone are the additional markers

for quality control of DS regarding to Hong Kong Chinese Materia Medica Standards (HKCMMS). The limits of TSIIA, RA and SAB in DS sample are set at 0.12%, 0.17% and 4.4% correspondingly [143].

In this work, five chemical reference compounds were selected for identification and quantification of DS. They were tanshinone I (TSI), tanshinone IIA (TSIIA), danshensu (DA), rosmarinic acid (RA) and salvianolic acid B (SAB). Among them, TSI and TSIIA are diterpenoids. The other three belong to phenolic acids. The phenolic acids in DS are mostly caffeic acid derivative. DA (3-(3,4-dihydroxyphenyl)-2-hydroxypropanoic acid) is the monomeric caffeic acid. It is hydrated form of caffeic acid. RA (3-(3,4-dihydroxyphenyl)-2-[3-(3,4-dihydroxyphenyl)prop-2-enoyloxy]propanoic acid) is composed of the one caffeic acid and one danshensu unit via the ester linkage and it is the dimmer of caffeic acid. Salvianolic acid B (Lithospermic acid B or 3 - Benzofurancarboxylic acid) is the tetramer of caffeic acid which is the condensate of two rosmarinic acid units [60, 82, 144]. Except RA, most of the phenolic acid can be found in DS only. Therefore, they are named as salvianolic acid [27]. All these compounds from DS are main constituents and show beneficial effects on the circulatory system. The chemical structures of the five studied reference compounds are given in the following two tables (Table 4.1 and Table 4.2).

Table 4.1 Pharmacological activities and chemical structures of two diterpenoid compounds studied

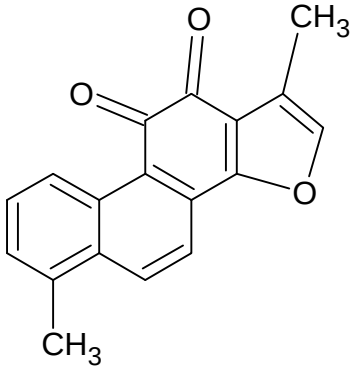
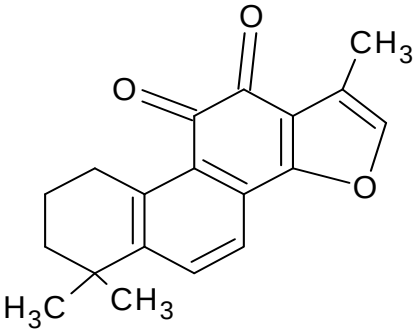
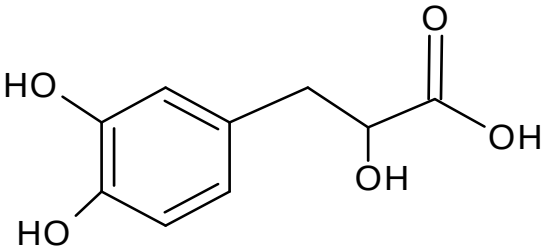
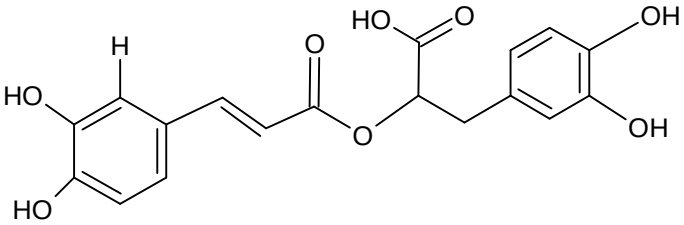
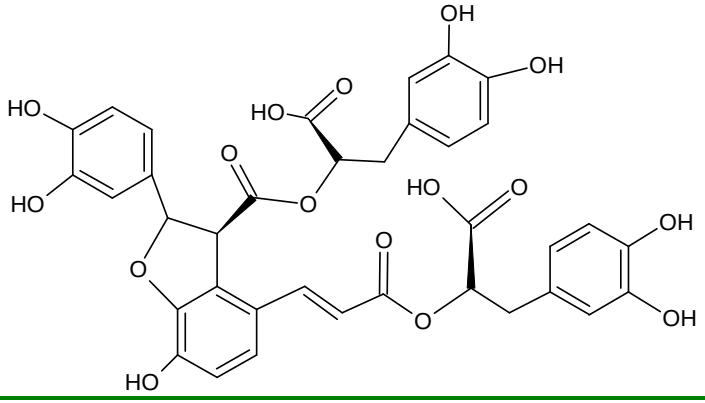
Compound	Chemical structure	Pharmacological activity
Tanshinone I (TSI)		1. Usage for therapy of angina pectoris [131, 145] 2. Protection against myocardial ischemia [31] 3. Effective in coronary dilators [31] 4. Treatment of the immunological diseases [31]
Tanshinone IIA (TSII)		1. Improvement of the blood circulation [131, 146] 2. Possessing antioxidant activity [31, 131] 3. Prevention of myocardial infarction [31, 131] 4. Effective in coronary dilators [31] 5. Possessing anti-inflammatory activity [31] 6. Possessing anti-platelet aggregation activity [31]

Table 4.2 Pharmacological activities and chemical structures of the phenol acids studied

Compound	Chemical structure	Pharmacological activity
Danshensu (DA)		1. Protection of the cardiac muscle [71, 147] 2. Inhibition of the peroxidative damage [71] 3. Prevention of the plate aggregation and calcium antagonizing [71, 139] 4. Scavenging the free radical [140] 5. Inhibition of myocardial cell apoptosis [139]
Rosmarinic acid (RA)		1. Anti-inflammatory activity [148] 2. Acting as antimutagen, antibacterial and antiviral agent [148] 3. Possessing anti-cancer activity [148] 4. Protecting against HIV-1 virus [31, 149]
Salvianolic acid B(SAB)		1. Possessing strong antioxidant activity [33, 150] 2. Scavenging the free radical [150] 3. Protecting renal dysfunction, hepatitis, uremia and lung fibrosis [150] 4. Improving blood circulation [150] 5. Treatment of hepatocirrhosis [33] 6. Restraining the lipid peroxidation of brain, liver and kidney [147] 7. Lowering blood pressure [31] 8. Protecting against HIV-1 virus [31]

4.2.2 Pharmacological activity of DS constituents

Several studies have been done on the pharmacological activity of the constituents of DS. Each one has its own activities and the details can be found in Table 4.1 and Table 4.2. Generally, diterpenoid compounds exhibit anti-ischemic, antioxidant, antineoplastic, antibacterial and antitumor activities [31]. In Lin et al. study, the cytotoxicity against human tumor cell line such as laryngeal epidermoid carcinoma (Hep-2), colon adrenocarcinoma, cervical carcinoma, etc. were demonstrated by this class of compounds [151]. Also, TSI and TSIIA were active against the human breast cancer cell lines [31].

Phenolic acids show antioxidant and anticoagulant activities. DA, RA and SAB could prevent lipid-peroxidation and scavenge the free radicals [31]. Hence, the protective effect was done by these compounds on endothelial cells and cardiovascular system. Eventually, they could reduce atherosclerosis [132-134]. In addition, the phenolic acids were found to be responsible for protecting the myocardium from ischemia-induced derangement and neural cells, inhibiting the platelet aggregation and the activities of HIV-1, and reducing hepatic fibrosis [31, 144].

4.3 Methods of investigation

Experimental design is a kind of strategy to conduct minimal number of experiments but still obtaining the maximum amount of information from them [152]. It is desirable to get the optimal yield of the desire components like bioactive compounds in order to have the optimal biological activities of HM. The optimization is usually carried out by changing one variable at a time via taking the so called trial and error approach. But it is time consuming and there is no guarantee of success in achieving the desire response [153]. Furthermore, interaction effect between different input parameters should not be ignored also. As a result, the relationship between the response and input parameters is usually expressed by response surface methodology. From this, the optimal experimental condition can be determined. Through which, the relationship between extraction parameters and antioxidant activity was investigated here. The relatively high level of antioxidant activity of the DS extract in this work was achieved under the optimal

extraction conditions found.

4.3.1 Response Surface Methodology (RSM)

RSM has been applied to optimize the extraction or other manufacturing processes. Quite often, the interaction effect among the variables involved is ignored during the optimizing process using only one variable at a time. But the independence and interactions between the process parameters could be learned from RSM through building up the relationship between the process parameters and response making use of statistical and mathematical techniques [154]. Thus, the effects of the input parameters and the optimal experimental conditions can be determined. The linear or quadratic models were commonly employed to describe the relationship. For example, the linear model for two factors, X1 and X2, can be expressed as the following.

$$Y_i = \beta_o + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_{12} X_{1i}{}^{*} X_{2i} + r \qquad [4.1]$$

The product of X_1 and X_2 linked with the interaction effect of these two factors. Also, different level of the factors can be investigated with i representing the level studied. r is the random error.

The quadratic model is shown as the following.

$$Y_i = \beta_o + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_{12} X_{1i}{}^{*} X_{2i} + \beta_{11} X_{1i}{}^{2} + \ldots + r \qquad [4.2]$$

Second order character in the model provided by the square terms of factor X1 is due to self-interaction.

The regression coefficients obtained from computation reflect the extent of the input parameters on affecting the characteristics of the response [155]. As the number of experiments depends on the number of parameters, it is necessary to determine a suitable number of experiments to get good results [156]. There are different kinds of experimental design and they differ from one other on the selection of the experimental points, number of runs and blocks. Through performing the specific combination of experimental parameters at the destined level, the relationship between the input parameters and response can be studied.

4.3.2 Uniform Design

The aim of experimental design is to carry out a small number of experiments and obtain the maximum information without losing the important one. Thus, it requires the experimental points to be orderly and efficiently mapped out the experimental domain. Various experimental design methods are available such as factorial design, orthogonal design, central composite rotatable design and uniform design. They are different from each other based on the selection of the experimental points and number of experiments.

In this work, uniform design was selected for investigating the effect of the experimental parameters and optimizing the experimental conditions. The characteristic of uniform design is that the experimental points scattered uniformly in the domain [122, 152, 156]. The uniformity of the space filling in the experimental domain is emphasized. Using this design, the most representative points in the experimental domain are chosen. Through which, information can be obtained as many as possible by a relatively few experiments with more number of levels considered for each parameter. Besides, there is no assumption on the model.

The uniform design is based on the orthogonal test and applied the experiments with many factors and many levels. There are multiple experimental design tables available in the literature which is convenient to the users. The major characteristic of the design table is to ensure the minimal discrepancy and the maximal uniformity. The uniform design table offers the number of experiments that is equal to the number of levels. Thus, it allows the largest possible amounts of levels to be studied for each parameter and denoted as $U_n(n^s)$. The data matrix is $X_{n,s}$. It has n points on the experimental domains and in unit cube, C^s. Another kind of uniform design is denoted as $U_n(q^s)$. The number of experiments is the multiple of the levels. In Factorial design, the number of experiments increased with the number of level exponentially. Instead, it is possible to study the parameters in multiple levels associated with it and the interaction among the parameters through the application of uniform design [130].

4.4 Results and Discussion

4.4.1 Identification and quantification of the diterpenoids and phenolic acids in Radix et Rhizoma Salviae Miltiorrhizae

Five chemical reference standards from two classes of components were selected for both identification and quantification of the extracts of Radix et Rhizoma Salviae Miltiorrhizae (DS extracts) according to the analytical procedure stated in Chapter 3. The HPLC-UV chromatogram of these reference compounds was given in Figure 4.2. Their identities were confirmed through their UV spectra and retention times (Table 4.3). Quantifications of these chemical markers were carried out using the calibration curves based on their regression equations having correlation coefficients above 0.99 (Table 4.4). These curves had their own specific linear range.

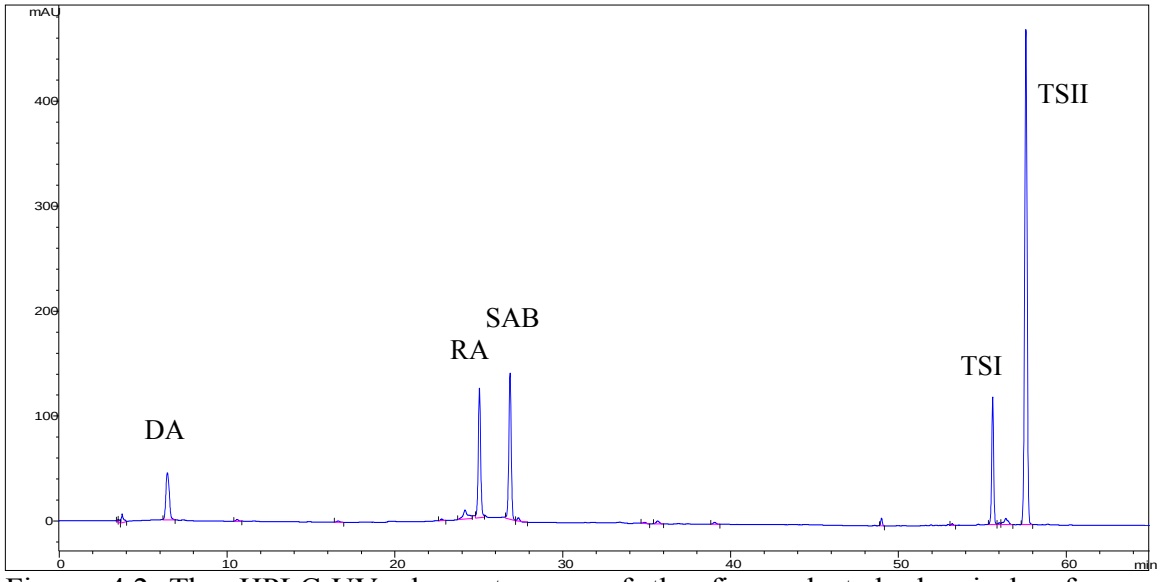
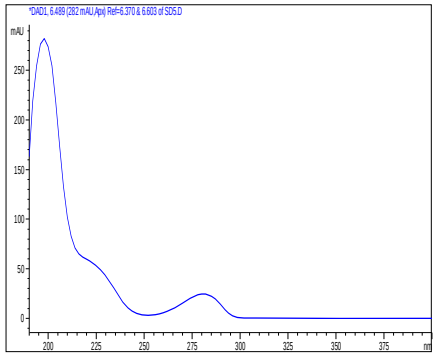
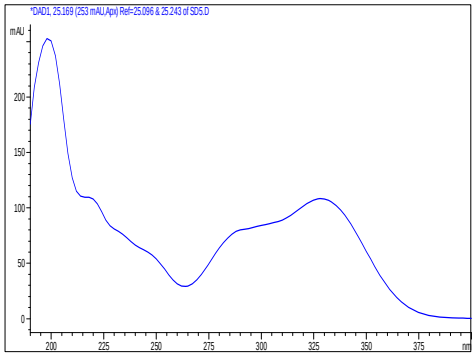
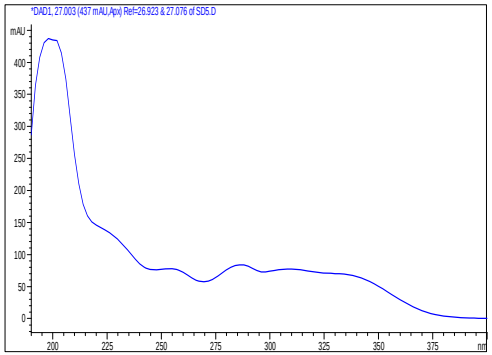


Figure 4.2 The HPLC-UV chromatogram of the five selected chemical reference compounds of Danshen at 280nm

Table 4.3 The retention times and UV spectra of the five selected chemical reference compounds (See Figure 4.1)

Component	Danshensu (DA)	Rosmarinic acid (RA)	Salvianolic acid B(SAB)
Retention time (min)	**6.4min**	**25.0min**	**26.9min**
UV spectrum			

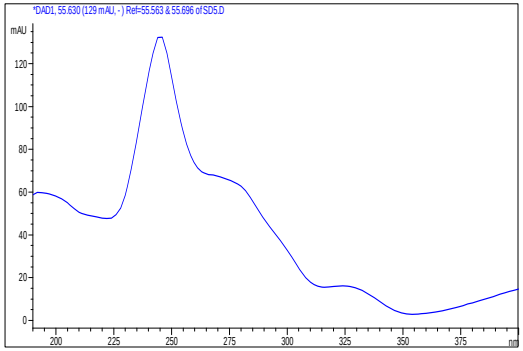
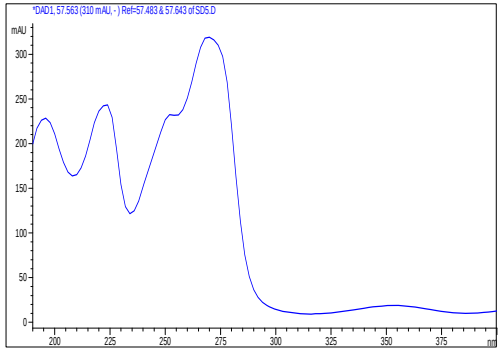
Tanshinone I (TSI)	Tanshinone IIA (TSII)
55.6min	**57.6min**
	

Table 4.4 The regression equations, related correlation coefficients and linear ranges of the five chemical reference compounds

Reference compound	Regression Equation	Correlation coefficient	Linear range (ppm)
DA	y = 3.68x - 27.39	0.9976	3.01-160
RA	y = 5.94x - 25.94	0.9948	1.50-80
SAB	y = 3.01x - 72.04	0.9953	2.00-320
TSI	y = 12.67x + 24.06	0.9927	0.75-80
TSIIA	y = 24.91x - 61.40	0.9990	0.75-160

4.4.2 Validation of the chemical analysis

The adequacy of the analytical method in this part of experimentation is often evaluated by its linearity and precision. Referring to Table 4.4, a good linear relationship between the peak area and the concentration of the reference compounds was obtained with the correlation coefficient being higher than 0.99. Apart from this, both inter-day and intra-day variations were monitored by the relative standard deviation of the peak areas within the same day and between five consecutive days respectively. The sample, DS 10, and the solution of all reference compounds were selected for assessing the variability within and between days. The deviation of the selected sample and the standard solution was found to be less than 5% and 11% respectively (Table 4.5).

In addition to using the conventional validation procedure as mentioned above, we also did this through evaluating the similarity between related chromatographic profiles and expressed it quantitatively in terms of similarity index (SI). Thus, the assessment is not only based on the selected chemical compounds but also the whole chromatographic fingerprint with all constituents detected. When the SI value is close to 1, this denotes that the two chromatographic profiles are identical to each other. Then, the analytical method used is perfect. In this investigation, the average chromatographic profile of sample or mixture of the selected compounds on Day 1 was utilized as the reference to compare with the average one on Day 5 during the assessment of inter-day variability. Besides, the chromatographic profile of the first injection was acted as reference. Then, the similarity evaluation was carried out by comparing the chromatograms of each of the three injections respect to reference one for intra-day variability. Table 4.6 listed the

ranges of similarity indices for two samples. The results showed that there was no significant change in all characterized components in both sample and mixture of chemical reference compounds. Because of these, the analytical method of DS is valid from the RSD value and similarity indices. Furthermore, the repeatability of the sample preparation was assessed. The deviations in the concentrations of the identified components were determined and expressed in terms of the RSD values. All the RSD values obtained was less than 6% for all the identified components.

Table 4.5 RSD values of the five chemical reference compounds to the selected sample and their mixture for evaluating both intra-day and inter-day variability

Reference compound	**Intra-day variability (m=3)** [a]		**Inter-day variability (n=5)** [b]	
	Sample [c]	**Mixture**	**Sample** [c]	**Mixture**
DA	-	1.75	-	1.89
RA	3.50	2.78	1.32	1.54
SAB	3.63	3.66	10.08	1.28
TSI	-	0.64	-	0.56
TSIIA	1.61	0.47	0.28	0.03

[a] m represents the number of injections
[b] n represents the number of days
[c] "–" denotes that the compound was not detected

Table 4.6 Similarity evaluation between the chromatographic profiles of the selected sample and mixture of the reference compounds for evaluating both intra-day and inter-day variability

Chromatographic profile	**Intra-day variability (m=3)** [a]	**Inter-day variability (n=5)** [b]
Sample	0.9947 – 0.9957	0.9689
Mixture of reference compounds	0.9811 – 0.9928	0.9425

[a] m represents the number of injections
[b] n represents the number of days

4.4.3 Screening of the extraction methods

Reflux, ultrasound assisted extraction and maceration are common procedures for preparing herbal extracts. During reflux, the viscosity of the solvent is reduced and the mass transfer rate is enhanced when high temperature is applied to the system. However, the heat liable components would be easily destroyed [157]. In dealing with this problem, ultrasound assisted extraction is an alternative. The cavitation is produced in the

extraction solvent when the ultrasonic wave passed through so that the penetration of solvent to the sample matrix can be improved [158]. Thus, it is widely utilized in the extraction of DS samples [131, 159]. Maceration is another traditional extraction method. Here, the extraction of components mainly depends on the diffusion or mass transfer process leading to significant reduction in the destruction of the components. But it requires a longer duration of the extraction. In this investigation, reflux and ultrasound assisted extraction were focused since these two methods were found to be able to improve our extraction efficiency.

For ultrasound assisted extraction, the extracts were labeled here as DS-UW, DS-UM and DS-UE. For those labeled as DS-RW, DS-RM and DS-RE, they were extracted by reflux. Three kinds of extraction solvents, water ('W'), methanol ('M') and ethanol ('E') were applied accordingly. The contents of the five identified reference compounds were summarized in Table 4.7. The highest amounts of these reference compounds can be obtained under different extraction conditions. DA can be obtained using water only. The highest concentrations of RA and TSIIA can be found in DS-RE extract. The maximal amount of SAB can be obtained in DS-UW. Apart from this, the two chromatographic profiles as shown in Figure 4.3 were similar to each other although different amounts of identified components were extracted under two different extraction conditions. As the chromatographic profile of an extract displayed all the detectable components, this indicated that the two extraction methods caused mainly the variation in contents of the compounds.

The chromatographic profiles of DS-UW, DS-UM and DS-UE (Figure 4.4) were different within the region of 45min and 60min. In general, relatively non-polar compounds elute out later with respect to retention time. In this region, TSI and TSIIA were identified at 55.6min and 57.6min respectively. Hence, this group of compounds may relate to diterpenoids. Water is unable to extract the non-polar compounds while ethanol is unable to get the phenolic acids. As no phenolic components found, this leads to its extremely low antioxidant activity levels of DS-UE and DS-RE (see discussion later).

We also compared the antioxidant activities of the DS extracts prepared by ultrasound assisted extraction and reflux. Their antioxidant activities were depicted in Figure 4.5. Usually, extracts prepared by the two methods used give similar antioxidant activities except the ethanol extracts. Among them, DS-UW had the strongest antioxidant activity (Figure 4.5). This indicated that ultrasound assisted extraction is preferred in this work. Recently, ultrasound assisted extraction method is widely accepted because it is a convenient method and can enhance the extraction efficiency [131, 158, 160]. In addition, destruction of compounds is less likely to occur. The mechanical action of the ultrasound on the cell walls would increase accessibility and extractability of the bioactive components [161]. Because of these, the optimization of extraction conditions utilizing ultrasound assisted extraction was focused here.

The antioxidant activity of DS was found to be affected by the extraction solvent from the results of the preliminary experiments. Water extract was preferred here for different types of extraction method utilized because it was able to get the phenolic acids, not diterpenoids. Both phenolic acids and diterpenoids can be acquired using the methanol solvent despite antioxidant activity of the resulting extract was not as strong as that of the water extract. Concerning the contents and antioxidant activities of DS, the combinations of methanol and water in different ratios were considered for further study.

Table 4.7 Contents of the identified reference compounds and antioxidant activities of the extracts prepared by the two screening extraction methods[a]

Extract	Content of the reference compound (ppm)				
	DA	**RA**	**SAB**	**TSI**	**TSIIA**
DS-UW	9.39	9.16	100.35	-	-
DS-UM	-	7.69	66.29	0.43	14.12
DS-UE	-	-	-	1.04	29.40
DS-RW	10.63	9.35	93.52	-	-
DS-RM	-	8.01	63.91	-	10.00
DS-RE	-	10.40	70.40	3.72	31.73

[a] "-" denotes that the compound was not detected

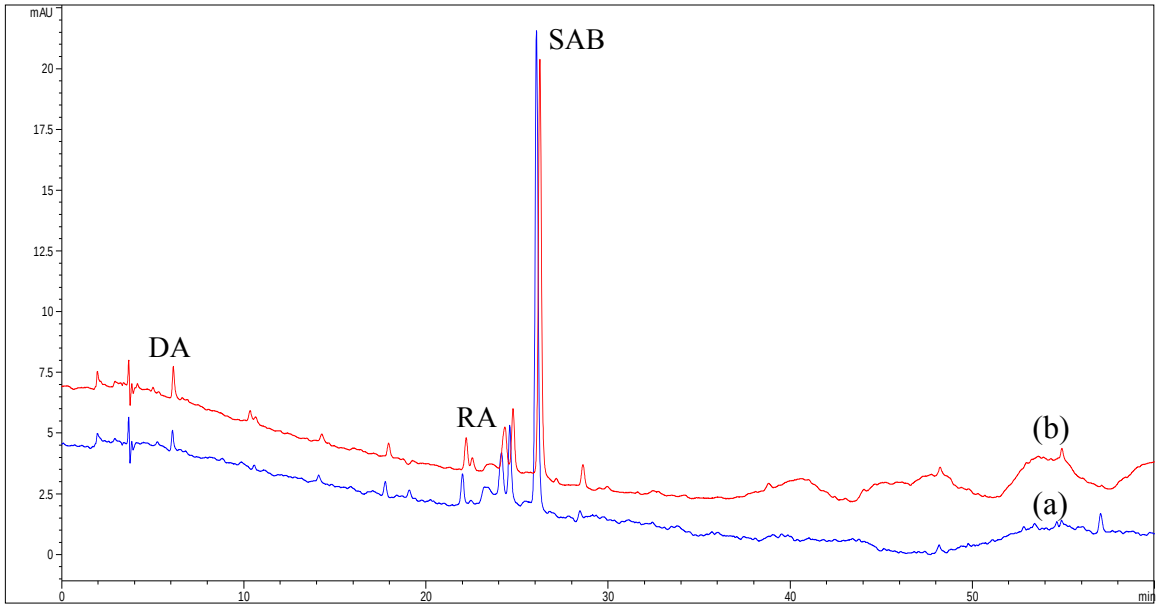


Figure 4.3 The HPLC-UV chromatograms of (a) DS-UW and (b) DS-RW at 280nm

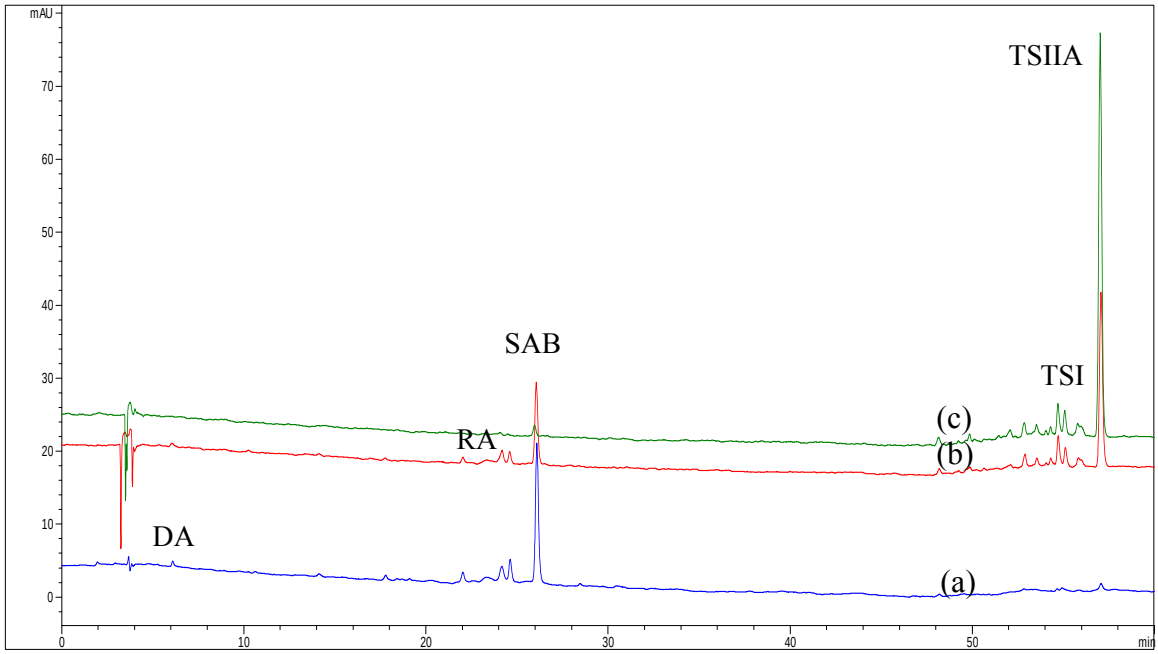


Figure 4.4 The HPLC-UV chromatograms of (a) DS-UW, (b) DS-UM, (c) DS-UE that were extracted by using water, methanol and ethanol respectively at 280nm

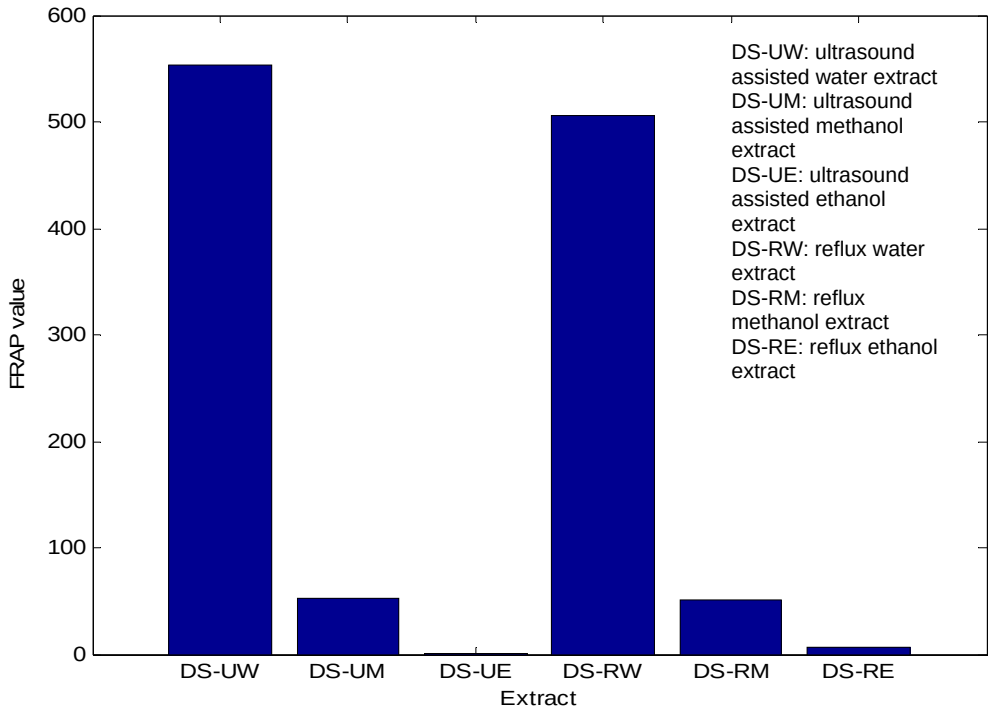


Figure 4.5 Antioxidant activities of the DS extracts prepared by the two screening extraction methods as mentioned

4.4.4 Studying the effect of extraction parameters on the antioxidant capacity

Uniform design was selected to study the influence of the extraction parameters on the antioxidant activity of DS. These included methanol content (X1), number of times of extraction (X2), duration (X3) and solid to liquid ratio (X4). These are important parameters for extractions. An appropriate extraction solvent is needed as the components to be obtained which is based on the principle of 'like dissolve like'. The polar components are extracted by water while the less polar ones can be obtained with the addition of methanol and ethanol. Since the diffusion or mass transfer process is involved between the secondary metabolites in the plant cell and extraction solvent, the proper volume of solvent used and duration of extraction are required. To enhance the diffusion, the extraction solvent may be required to refresh for repeating the extraction. Otherwise, saturation may result and the amount of components to be extracted is greatly reduced.

The active components extracted from HM are responsible for different pharmacological activities. Thus, attention was focused on obtaining the right contents of the interested components using RSM to optimize the extraction conditions [127, 161]. By screening the methods concerned, it is not necessary to have optimal antioxidant activity when gaining the highest amount of identified components under the conditions studied. It is because the efficacy of the HM usually comes from multiple components with appropriate amounts. Here, the relationship between the extraction parameters and the bioactivity was cared in order to find the optimal extraction conditions. In the following, the effect of the extraction parameters on the antioxidant activity was discussed first.

Table 3.2 in Chapter 3 gives how the nine UM extracts were obtained. They showed different antioxidant capacities and contained diverse contents of the chemical reference compounds obtained under different experimental conditions. The phenolic acids of DS were quantified in all the extracts. No TSI was identified in any one under the extraction conditions used (Table 4.8). In addition, TSIIA can be obtained using 50% methanol (Figure 4.10). This means that it is not able to obtain TSIIA when the proportion of methanol is too low. Also, the amount of SAB was the highest among the five chemical reference compounds in Danshen while the contents of DA and RA were close to each other (Figures 4.7 and 4.8).

UM3 was the most active extract. It was prepared by 50ml of 25% methanol for 45 minutes. On the other hand, UM1 had the weakest antioxidant activity among all and it was extracted by 12.5ml water for 30 minutes. Yet, both extracts required triplicate extractions. But distinct antioxidant activity level was observed (Figure 4.6). For those with the relatively strong antioxidant activities, they were extracted by 25% methanol. But UM1 and UM8 exhibited the relatively low antioxidant activity and water was utilized. Thus, it is obvious that the extraction solvent affects the antioxidant activity of the extract concerned.

UM2 and UM3 were on the top rank with regard to antioxidant activity (Figure 4.6) but

they did not contain the highest amounts of the reference components. UM4 had the highest amount of RA, SAB and TSIIA and exhibited the third highest antioxidant activity (Figures 4.8, 4.9 and 4.10). It was extracted by 50% methanol for 30minutes once only. Although the highest amount of DA can be found in UM7, it had relatively low concentration of RA at the same time. That is, the extraction conditions studied maybe suitable for obtaining a specific component with higher amount. UM1 consisted of the lowest concentrations of DA and SAB and none of RA and TSIIA. As UM1 and UM4 were extracted for 30minutes, there was a significant difference in their contents of the four selected components. This illustrated that the duration of extraction is not an important factor on the antioxidant activity of DS in our study. Various extraction conditions were required to get the optimal amount of the specific compound. From the information acquired above, it is not necessary to have the highest amount of the four chosen components in order to have the strongest antioxidant activity. But they do contribute to the DS antioxidant activity to certain extent. In addition, the efficacy of the HM may be based on compounds other than the target components. Therefore, the bioactivity of HM should be the main target in optimizing the extraction conditions.

To gain more information about the compositions of the extracts, their chromatographic fingerprints can help. Similar chromatographic pattern can be observed from UM1 to UM9 (Figure 4.11). The major difference among them can be observed on the peak heights of the detected components. Moreover, tanshinone IIA can be found in UM4, UM5 and UM6 only.

Table 4.8 Contents of the chemical reference compounds in UM extracts using HPLC-DAD instrument[a]

Extract	Content of the reference compound (ppm)			
	DA	**RA**	**SAB**	**TSIIA**
UM1	12.75	-	71.66	-
UM2	13.53	9.79	95.80	-
UM3	13.48	10.35	101.47	-
UM4	13.42	11.38	112.47	4.69
UM5	13.06	10.87	104.21	3.83
UM6	13.00	10.71	98.57	3.37

UM7	13.82	6.42	95.60	-
UM8	13.44	7.63	93.86	-
UM9	13.76	11.18	104.53	-

[a] "-" denotes that the compound was not detected

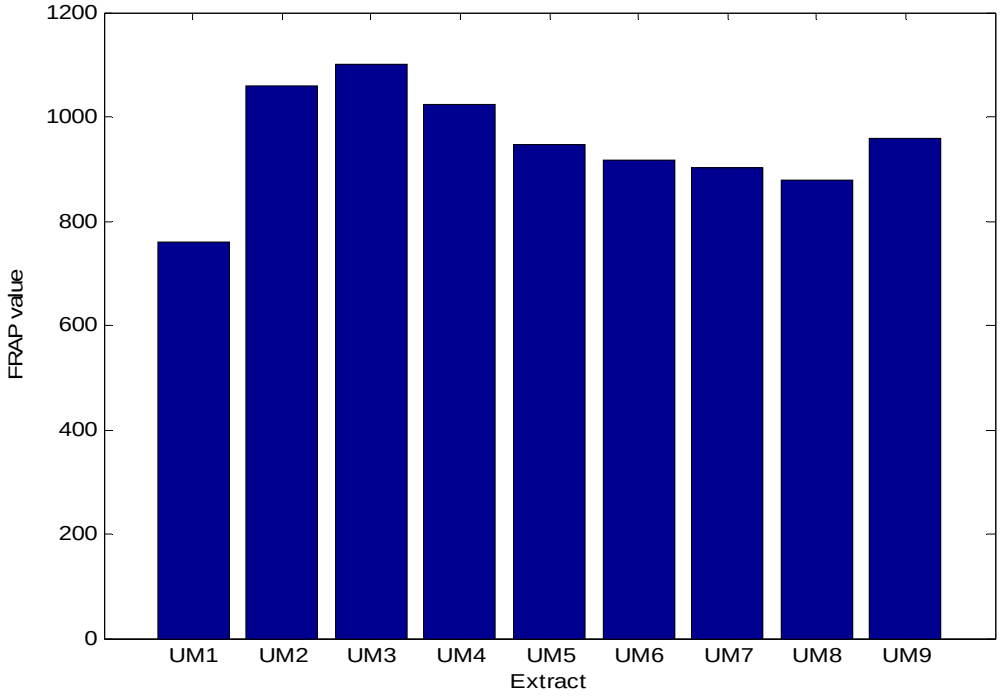


Figure 4.6 Antioxidant activities of UM extracts according to the $U_n(q^s)$ design table

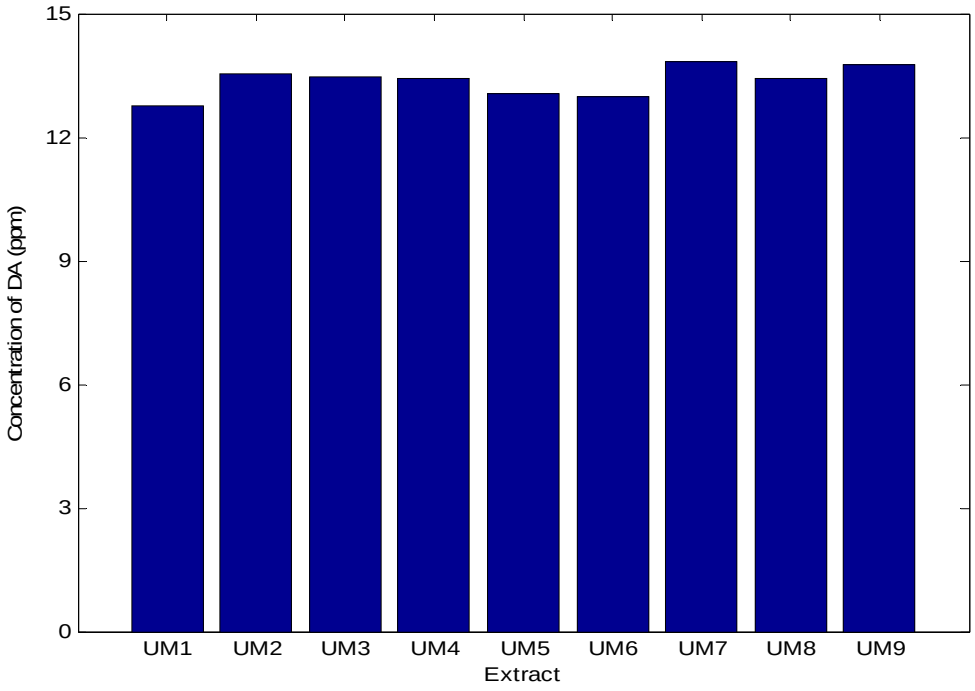


Figure 4.7 The DA content in different UM extracts

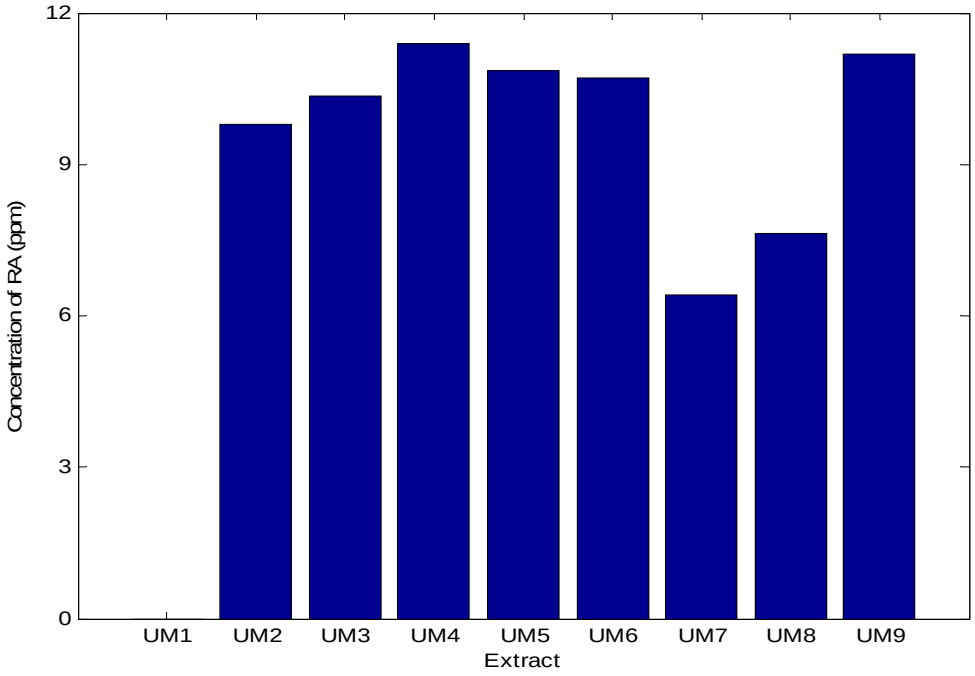


Figure 4.8 The RA content in different UM extracts

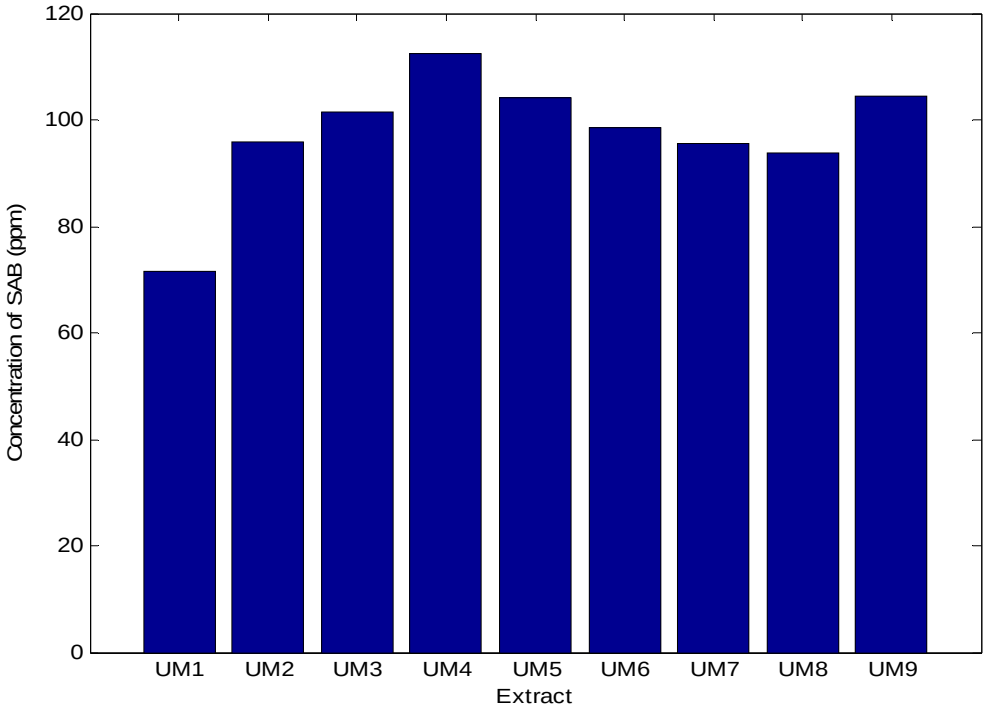


Figure 4.9 The SAB content in different UM extracts

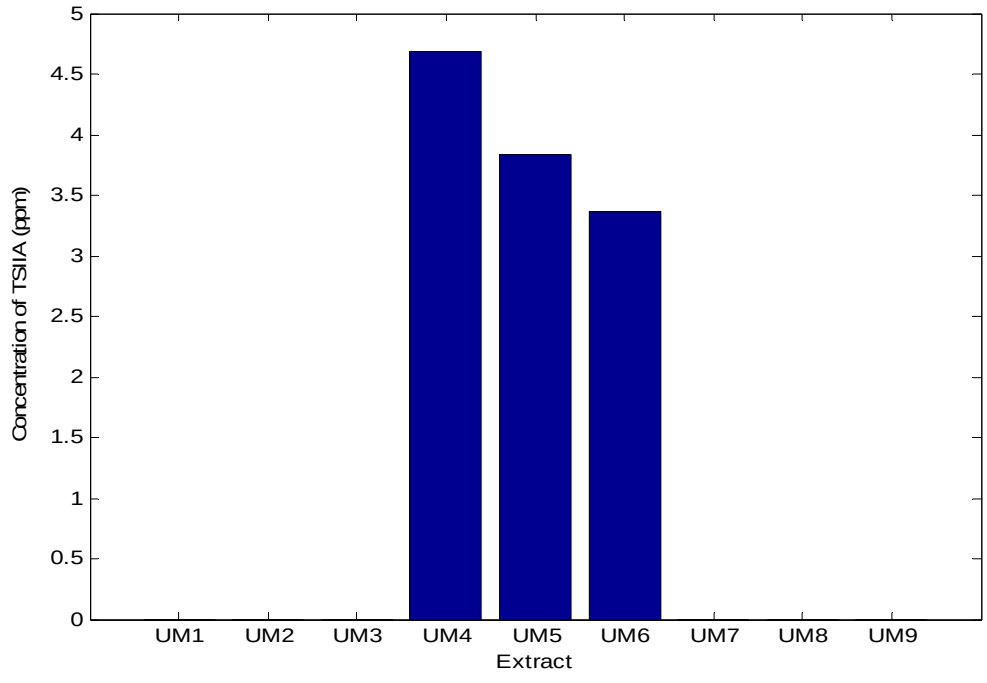


Figure 4.10 The TSIIA content in different UM extracts

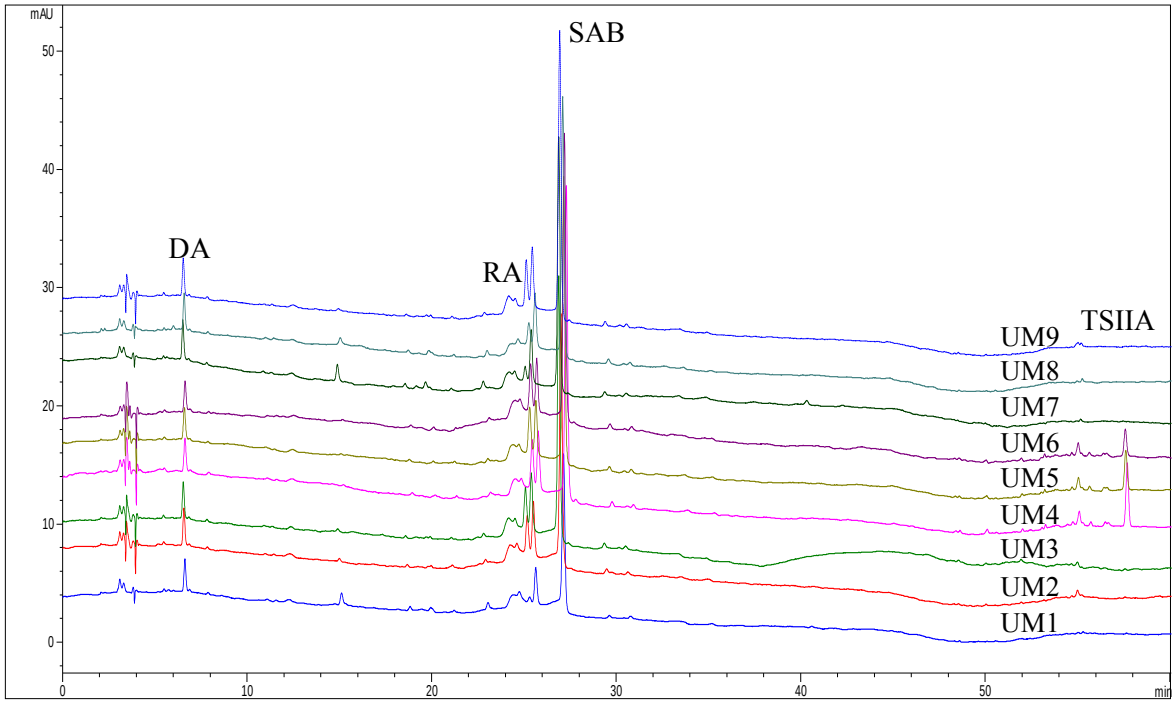


Figure 4.11 The HPLC-UV chromatograms of the UM extracts that were prepared according to the experimental design table (Table 3.2) at 280nm

As mentioned before, RSM is helpful to guide the study of the influence of each extraction parameter more systematically. In order to determine the importance of the parameters, stepwise selection was adopted. Our statistical analysis on the data obtained showed that methanol content (X1) and solid to liquid ratio (X4) were important on the antioxidant activity of the extracts and had the positive influence. Their p-values were less than 0.01 and this implied that they are significant parameters. From this piece of information, the methanol content in the extraction solvent (X1) was found to be important in affecting the antioxidant activity. The activity increased when the methanol content in the extraction solvent increased. Water was not an appropriate option for improving the antioxidant activity in this study. Extraction solvent is one of essential parameters because the physical properties of the solvent such as vapour pressure, viscosity and surface tension are crucial to the sonication efficacy [127].

With the consideration of the extracts that were prepared by the same methanol content,

the antioxidant activity was found to increase when the solid to liquid ratio (X4) increased. Since mass transfer occurred between herbal powder and extraction solvent, the mass transfer rate increased when more extraction solvent was applied in the process. This extraction parameter was always considered seriously in optimizing the extraction conditions [127, 130, 161]. Similar result was reported by Huang et al. study [127].

The influence of the two other extraction parameters, number of times of extraction (X2) and duration (X3), was not clearly shown here. From our quadratic model, the associated regression coefficients had relatively low values with high p-values. As the regression coefficient reflected the significance of the variables, they were found not so important in the DS antioxidant activities. Furthermore, there were no dramatically changes in the activities when X2 or X3 varied while other parameters were kept unchanged. They were also not so influential compared to other factors in this study. This may be due to their effects are not directly influencing the response. The number of times of extraction and duration depended on the extraction solvent, volume of solvent used and the type of the extraction method applied. There was a slight increase in the response study with the number of extractions at first. Then, it decreased slightly when three times of extractions was needed. This showed that the efficiency decreased when large number of extractions involved. On the other hand, the antioxidant activity increased only slightly with duration of extraction.

4.4.5 Optimization of extraction condition of DS extracts based on the two extraction parameters

The optimization strategy was adopted here to find the best combination of the experimental conditions to the antioxidant activity of DS extracts. From the above investigation, the two extraction parameters, methanol content (X1) and the solid to liquid ratio (X4), were found to play important role in this aspect. Hence, the other two parameters, number of times of extraction (X2) and extraction duration (X3), were set at a fixed level with only one extraction being carried out for 45minutes. It is because the antioxidant activity of the extract would be further reduced when the number of extraction increased in our study. On the contrary, the duration of extraction increased

with the antioxidant activity. Thus, these two parameters were set at these levels to get the desirable performance. In the following, discussion of the optimal extraction conditions was looked into based on X1 and X4. Nine levels associated with each factor were examined. As stated in the above section, the antioxidant activity of DS increased with methanol content in the extraction solvent and solid to liquid ratio. Then, the ranges of these two covered from 0% methanol to 80% methanol and 10ml to 90ml of extraction solvent respectively in optimizing the conditions. The combinations of these two factors and the associated experimental levels were given in Table 3.3. Their respective contents of the four identified chemical reference compounds and the antioxidant activities were determined and summarized in Table 4.9 and depicted from Figure 4.12 to Figure 4.16.

Among the nine extracts, U3 and U6 had the strongest antioxidant activity (Figure 4.12). Referred to Table 3.3, U3 was prepared by 80ml of 60% methanol and U6 was prepared by 60ml of 80% methanol. U7 and U8 gave the lowest level of antioxidant activity. They were extracted by 30ml of 70% methanol and 10ml of 50% methanol respectively. More extraction solvent in volume was needed in preparing U3 and U6 than U7 and U8. But they were extracted with similar methanol content. This indicated that the volume of extraction solvent used influenced the level of antioxidant activity. Despite that, U9 was acquired by using large volume of solvent but did not have high level of the activity as U6 did. The major reason is that relatively less methanol content was utilized in the preparation of U9. This suggested that these two factors were important to the response under study.

In addition, the amounts of the selected components in these extracts were examined (Figures 4.13-4.16). U3, U6 and U7 consisted of all (Table 4.9). They were extracted using 60% methanol or more in the solvent. Generally, the methanol solution containing some water could extract the polyphenolic compound more efficiently [162]. With regard to U6, it had the maximal amount of all four components compared to the others and exhibited relatively strong antioxidant activity. Although U3 had the lower amounts of SAB and TSIIA when compared with U6 and U7, its antioxidant activity level was the highest. U7 contained less amounts of DA and RA than U3. These two components were

found to be antioxidant active when their activities were studied individually. Thus, the lower amounts of DA and RA in the samples resulted in lower antioxidant activity.

Similar compositions of the four selected compounds and antioxidant properties were attained for U5, U7 and U8 (Table 4.9). They were extracted by 20ml of 20% methanol, 30ml of 70% methanol and 10ml of 50% methanol accordingly. U2 had the minimal amount of DA, RA and SAB with the relatively low level of antioxidant activity. It was extracted using 40ml of water. This showed that less volume of extraction solvent was related to the lower level of antioxidant property. For those extracts with high level of antioxidant activity, the contents of the identified components in U1 and U3 were not the highest. From these, it can be seen that it is not necessary to have the highest concentration of all the identified compounds in the extracts in order to have high level of antioxidant activity. But, definitely, too low a concentration of phenolic acids would induce the level of antioxidant activity.

Figure 4.17 showed the chromatographic fingerprints of the U1-U9 extracts at 280nm for determining the optimal extraction conditions. They looked similar to each other at this wavelength except those of U3, U6 and U7. The relatively non-polar compounds were detected in these three extracts because of high methanol content used in extraction. Despite this, their variations in the overall compositions are little. Therefore, different combination of extraction solvent and solid to liquid ratio can obtain similar antioxidant activity level and similar composition. Still, the optimal extraction condition towards the antioxidant activity can be found from RSM.

Table 4.9 Contents of the reference compounds in extracts U1-U9 as determined by HPLC-DAD instrument[a]

Extract	Content of the reference compound (ppm)			
	DA	RA	SAB	TSIIA
U1	12.63	10.61	67.12	-
U2	12.22	9.50	62.71	-
U3	12.95	10.93	74.98	4.61
U4	13.01	10.60	72.00	-

U5	12.66	10.53	81.32	-
U6	14.00	11.94	102.29	5.13
U7	12.68	10.26	82.16	4.83
U8	12.95	10.69	82.06	-
U9	13.01	11.36	83.36	-

[a] "-" denotes that the compound was not detected

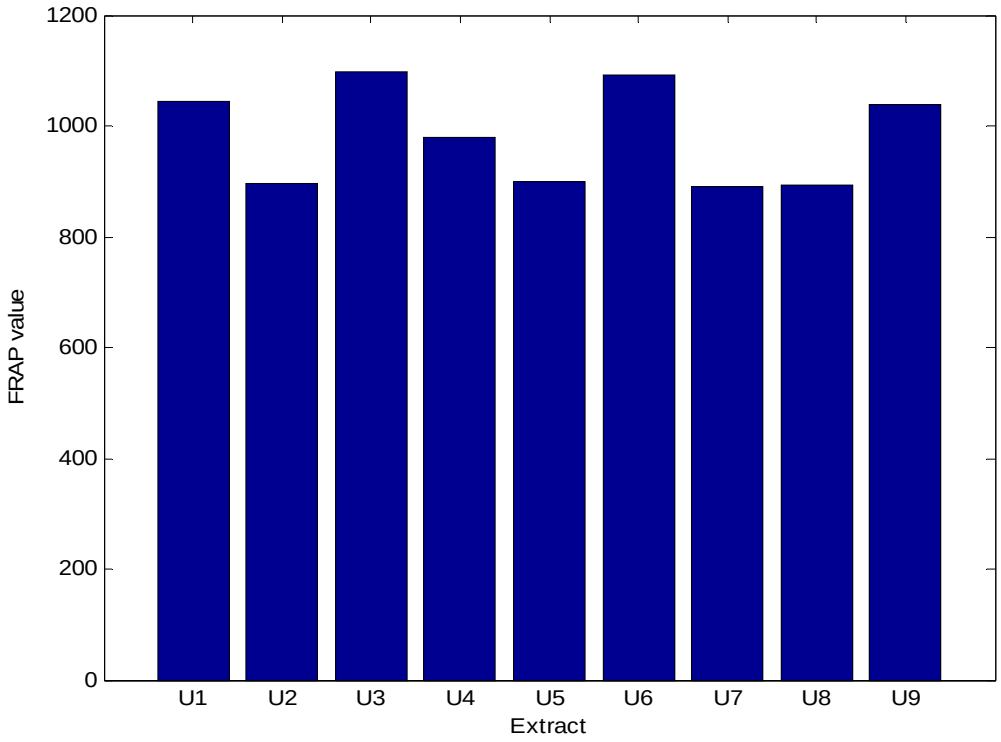


Figure 4.12 Comparison of the antioxidant capacities of the U1-U9 extracts for optimizing the extraction condition

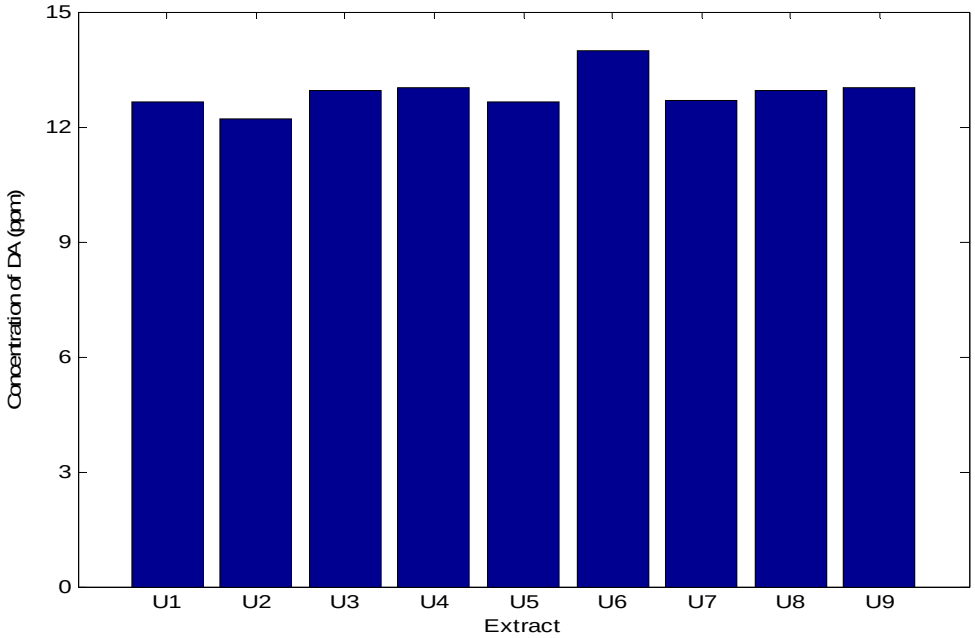


Figure 4.13 The content of DA in different DS extracts for optimizing the extraction conditions

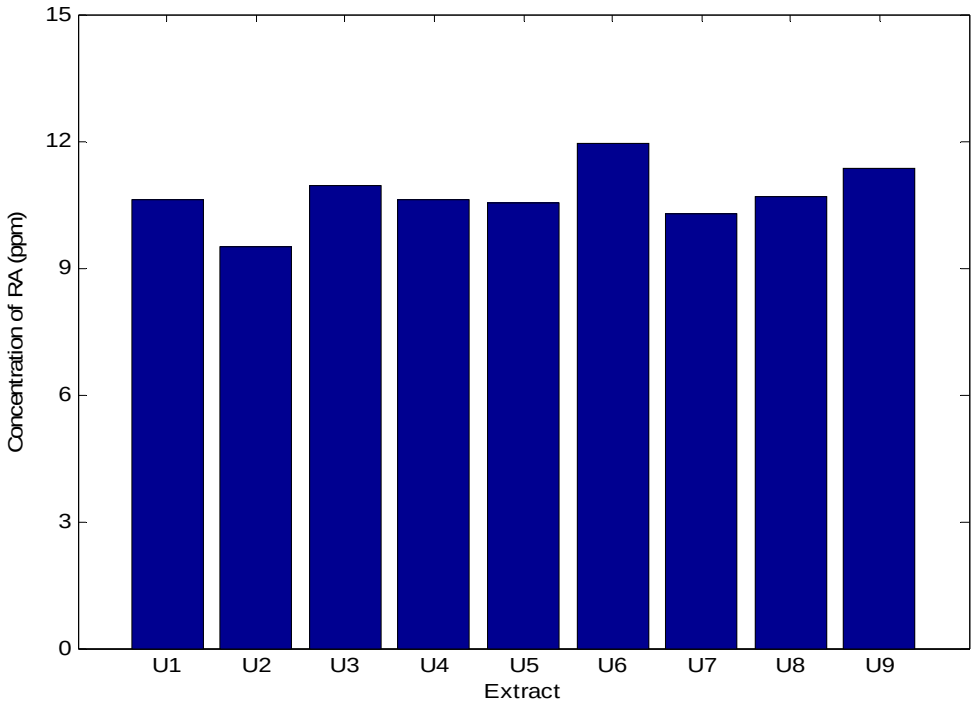


Figure 4.14 The content of RA in different DS extracts for optimizing the extraction conditions

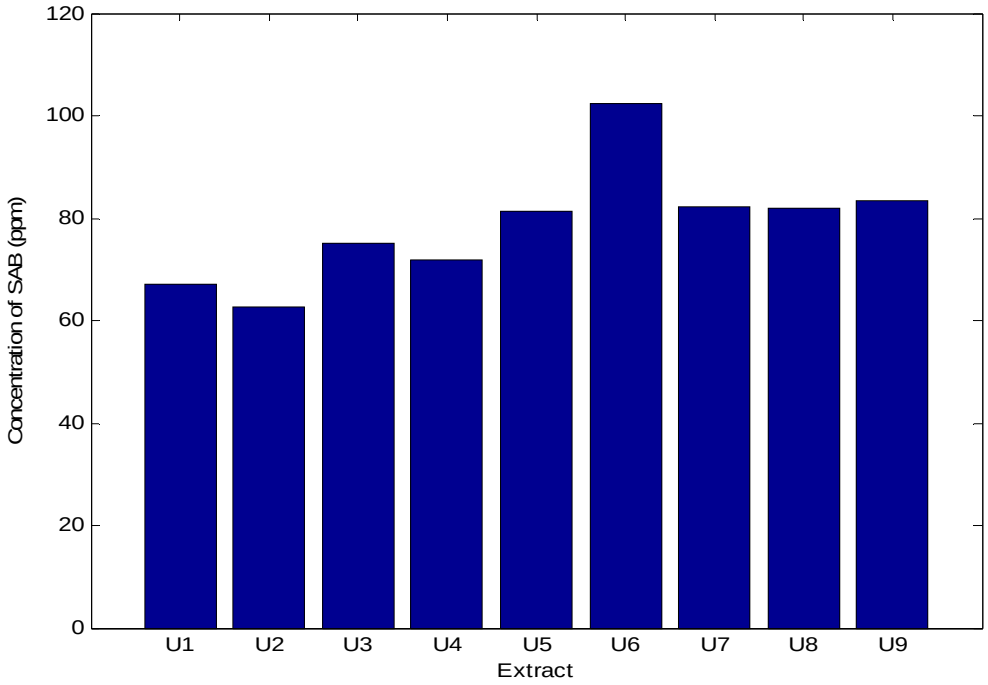


Figure 4.15 The content of SAB in different DS extracts for optimizing the extraction conditions

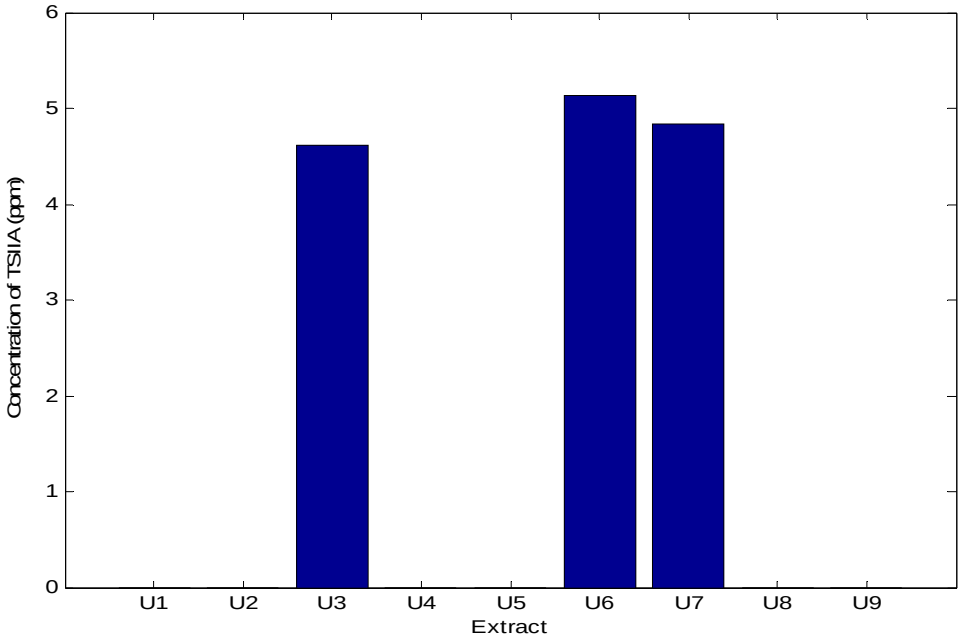


Figure 4.16 The content of TSIIA in different DS extracts for optimizing the extraction conditions

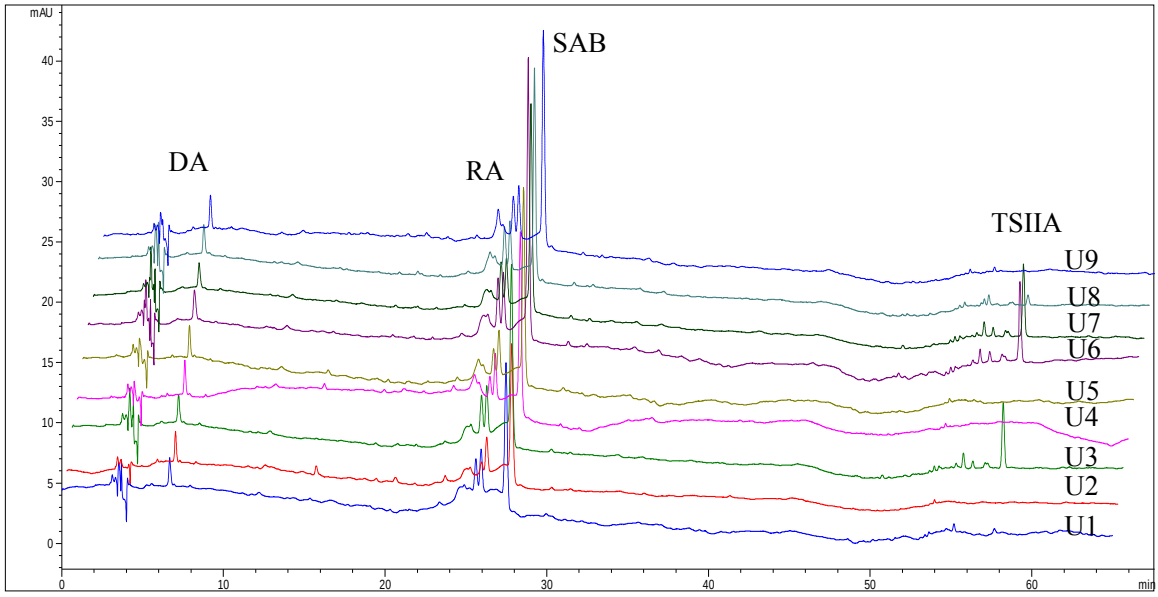


Figure 4.17 The HPLC-UV chromatograms of the DS extracts (U1-U9) for optimizing the extraction conditions at 280nm

Based on the data obtained, the empirical relationship derived in this work between the antioxidant capacity and the test variables relating to the extraction parameters used is given as below.

$$FRAP=895.028-1.410*X1^2+5.271*\ X1*\ X4$$

The coefficient of correlation, R, was equal to 0.936. Moreover, the p-value was less than 0.01 for the corresponding coefficient of interaction effect of methanol content ($X1^2$) and solid to liquid ratio (X1X4). At the same time, the optimal response can be achieved when the methanol content was at 80% and the solid to liquid ratio was at 1:90 (Figure 4.18). Under this optimized conditions, the average total antioxidant capacity of the extract obtained was 1138.36 with the RSD value of 4.49% in triplicate extractions. The deviation from the predicted value from the RSM was 4.18%. This represents that the optimized extraction condition can be determined efficiently from the model. Furthermore, the recovery of the four reference compounds under the optimized extraction conditions ranged from 94.94% to 109.51%. This implied that this condition was a suitable one for DS in this investigation.

It should be noted that the concentrations of reference compounds and antioxidant activity were at low level when extracts were prepared from the low solid to liquid ratio (X4). But the effect of another parameter (X1) alone on the antioxidant activity of the extracts obtained is not clearly known. As observed from the outcomes of U3, U6, U7 and U8, they were extracted by 50% methanol or above. A larger volume of extraction solvent was utilized for U3 and U6 that leads to higher antioxidant activity level. Besides, the low methanol content in addition to low solid to liquid ratio would result in low contents and antioxidant activities. For example, U2 being extracted in water led to not so good results in chemical compositions and antioxidant activity. Hence, this required the higher levels of these two extraction parameters, X1 and X4, in optimizing the antioxidant activity output. As a consequence, the interaction among these two extraction parameters affected most on the response.

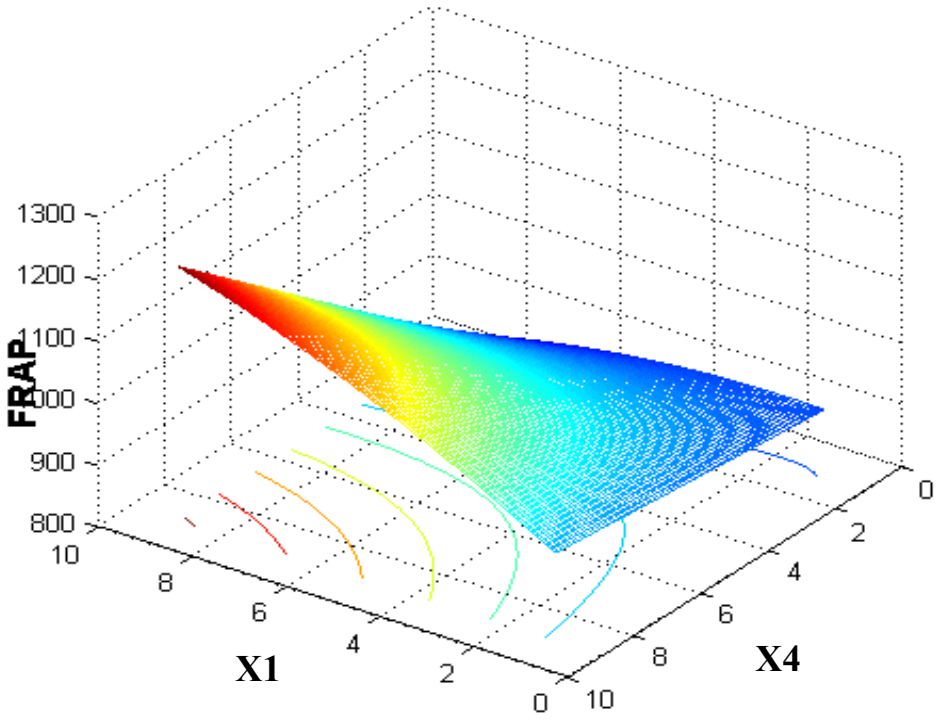


Figure 4.18 Response surface curve for the antioxidant activity with the interaction of the two extraction parameters methanol content (X1) and the solid to liquid ratio (X4)

4.4.6 Investigation of the relationship between the chemical composition and the antioxidant activity of Radix et Rhizoma Salviae Miltiorrhizae from various sources under optimal extraction condition

The antioxidant activity of DS is the result of multiple compounds. The extraction condition for DS was thus determined here from RSM to produce extract with the optimal antioxidant activity. Based on this finding, twenty Radix et Rhizoma Salviae Miltiorrhizae herbal samples (DS1 to DS20) which were collected from different regions of P.R. China were utilized to prepare their extracts. At the same time, their chemical compositions were characterized by the chromatographic fingerprints acquired. From these, the relationship between the composition and antioxidant activity was explored.

Since there are different *Salvia* species in addition to different cultivation environments, the chemical composition and the antioxidant activities level varied quite significantly among them. From the Kasimu et al. study, the compositional variation between *Salvia* species is the major reason for different aldose reductase inhibitory activities [21]. In this work, the variation of the contents was found to be quite significant among these twenty samples (Table 4.10). Also, they had their own characteristic chromatographic fingerprints (Figure 4.19). Consequently, variation in antioxidant activities was observed (Figure 4.20 and Figure 4.21). In the following, the antioxidant activity and composition of twenty DS would be discussed.

Table 4.10 Contents of the five selected chemical reference compounds in the twenty Danshen samples from HPLC-DAD study [a]

Sample	Content of the reference compound (ppm)					Similarity Index
	DA	RA	SAB	TSI	TSIIA	
DS1	-	12.69	103.81	0.05	4.54	0.9920
DS2	-	12.06	112.16	-	3.51	0.9831
DS3	-	29.41	29.89	7.15	26.97	0.9078
DS4	-	68.24	-	0.75	2.47	0.9402
DS5	-	9.28	81.98	0.62	5.01	0.9907

DS6	-	14.12	138.69	-	3.42	0.9918
DS7	-	11.70	98.88	-	4.22	0.9864
DS8	-	8.24	67.72	0.91	3.30	0.9767
DS9	-	12.89	84.07	0.09	4.45	0.9948
DS10	-	9.29	78.52	-	3.93	0.9667
DS11	-	9.46	65.85	-	4.15	0.9763
DS12	-	13.47	74.83	1.30	8.25	0.9302
DS13	13.16	10.58	94.54	-	4.81	0.9817
DS14	-	10.64	67.77	-	3.96	0.9760
DS15	-	7.33	62.31	0.05	5.34	0.9686
DS16	-	10.07	77.95	1.54	7.96	0.9016
DS17	-	-	48.57	0.14	5.37	0.9883
DS18	-	41.69	30.35	2.53	14.26	0.6853
DS19	-	34.76	-	1.74	12.96	0.7684
DS20	-	41.02	-	-	5.03	0.7382
Mean	13.16	18.35	65.89	0.84	6.70	0.9322

[a] "-" denotes that the compound was not detected

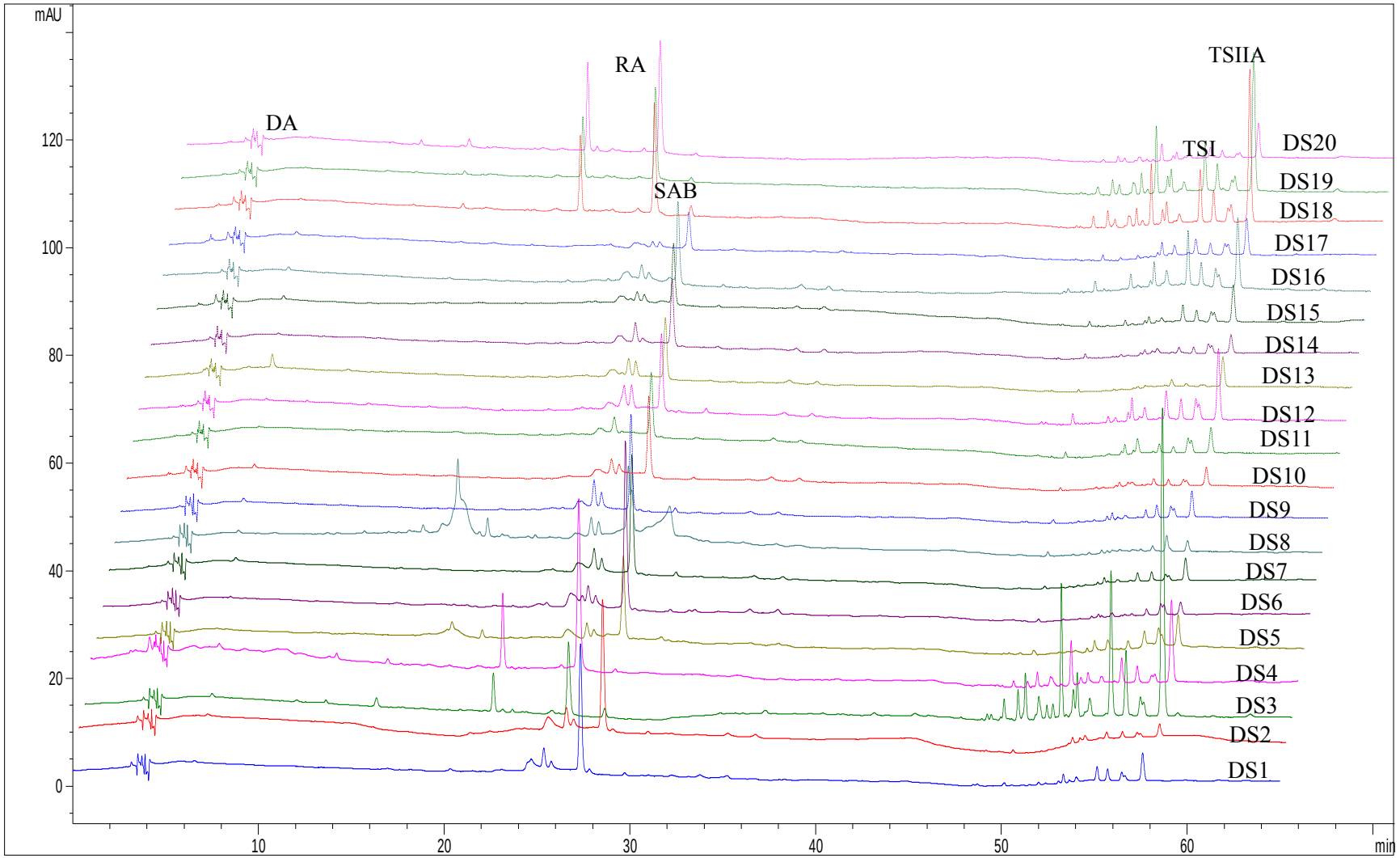


Figure 4.19 HPLC-UV chromatograms of twenty DS samples at 280nm

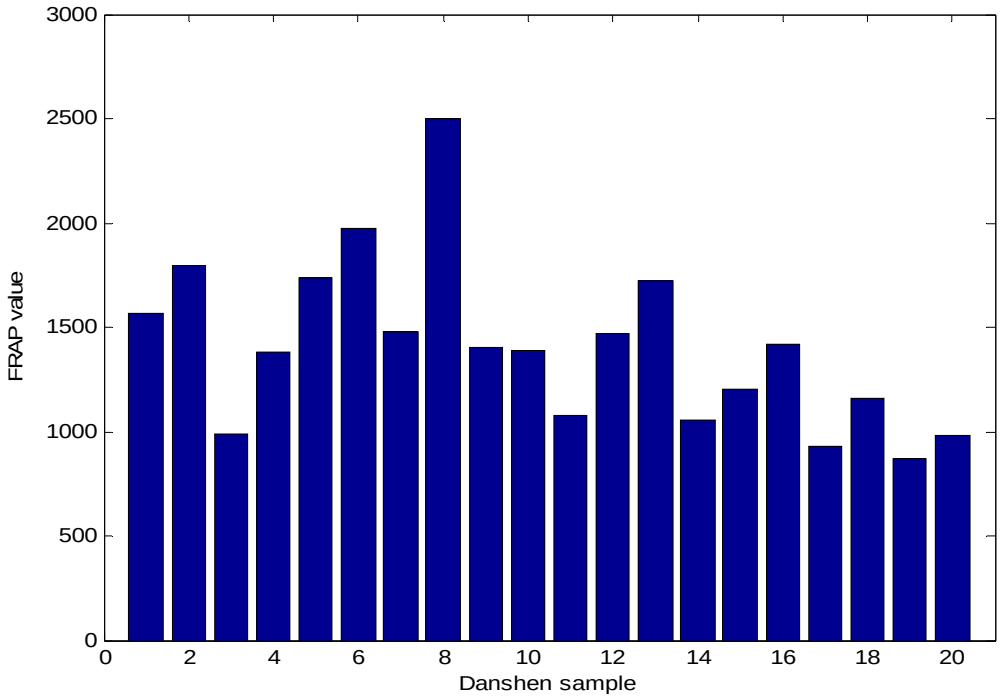


Figure 4.20 Antioxidant capacities of the twenty DS samples

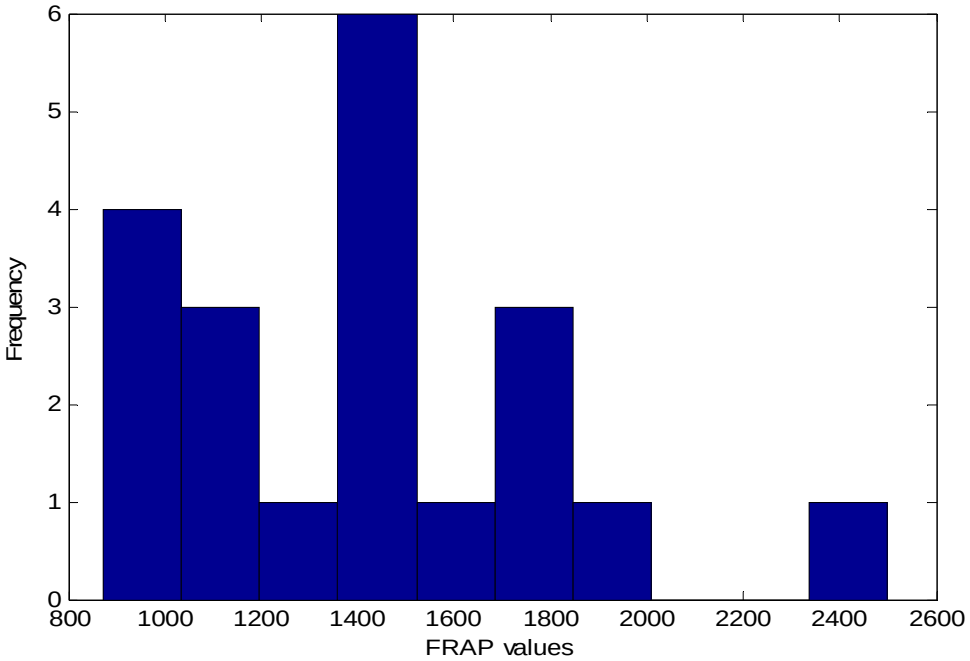


Figure 4.21 Distribution of the antioxidant capacities of twenty DS samples

4.4.6.1 Assessment of the antioxidant activity of the DS samples

The twenty DS samples had a variety of antioxidant activities in the range of 850 to 2500 with the mean around 1400 (Figure 4.20). Four DS samples, DS3, DS17, DS19 and DS20 had the lowest FRAP values below 1000 while nine samples had values higher than the mean. Relatively strong antioxidant activities were found in DS1, DS2, DS5, DS6, DS7, DS8, DS12, DS13 and DS16. Among them, DS8 was the most active one while DS19 was the least active one (Figure 4.20). For those samples with relatively high level of antioxidant activity, DS5, DS6 and DS8 were from Yunnan province (Table 3.1). Besides, those from Gansu province such as DS3, DS19 and DS20 had relatively low activity level. DS9 and DS16 from Shandong province had similar antioxidant activity. But it is not always true that samples from the same region would have the same antioxidant activity level like DS1, DS12 and DS15. It is because the quality of HM is affected by different factors such as cultivation, harvesting and storage conditions.

4.4.6.2 Quantitative analysis of the five chemical reference compounds in the DS samples

In general, DA was absent in all of the samples except DS13. On the other hand, most of the samples consisted of the other four target components (Table 4.10). The mean concentrations of RA, SAB, TSI and TSIIA were 18.35ppm, 65.89ppm, 0.84ppm and 6.70ppm respectively (Table 4.10). SAB was the dominant compound in all the DS samples. In addition, the concentrations of phenolic acids were relatively higher than those of diterpenoids. This indicates that the environment and conditions of HM cultivation affect the content of the reference chemical components.

Comparing the phenolic acids in twenty Danshen samples, the quantities of RA and SAB were ranged from 0ppm to 68.24ppm and 0ppm to 138.69ppm respectively (Table 4.10). The samples from Gansu province (Table 3.1) had high content of RA. The highest amount of RA can be found in DS4. Also, DS3, DS18, DS19 and DS20 contained the maximal amount of RA among all. Furthermore, DS5 and DS8 were from Yunnan province which had similar quantity of RA as well. Their contents were not as high as those from Gansu province. DS17 had none of RA so it had relatively low antioxidant

activity level. This hints that the presence of RA is needed to maintain the high level of antioxidant activity. But it is not needed to obtain the highest amount of RA.

As mentioned before, most of the Danshen samples contained relatively high concentration of SAB when compared with other reference compounds. Hence, it was assigned as the chemical markers of DS by Chinese Pharmacopoeia (2010) and Hong Kong Chinese Materia Medica Standards. When we compared the amount of SAB solely, DS6 had the highest quantity and DS3 and DS18 had the lowest amount. Also, it cannot be found in DS4, DS19 and DS20. The samples from Gansu province had the lowest amount of SAB while their contents of RA were the highest among the samples. DS9 and DS16 had similar amount of SAB while their contents of RA varied a lot. Although RA and SAB are referring to the same class of compounds, their quantities may vary significantly from each other even in the same sample. For instance, DS4 had the highest amount of RA while no SAB can be detected. Because of this, its antioxidant activity level was lower than the average value. DS3, DS19 and DS20 had no SAB and their antioxidant activities were weak. This showed that SAB contributes to the antioxidant activity of the DS samples to certain extent. But it seems that the antioxidant activities of the samples did not increase with concentration of SAB. From the SAB amount in DS8 and DS6, SAB in DS8 was half of that in DS6. Still, DS8 had the strongest antioxidant activity. Similar result was got with RA. The antioxidant activity is related to the presence of RA and SAB but does not depend on their concentrations. It can be explained in part that the other unidentified components with similar chemical properties contributes antioxidant activity as well.

Another class of compound in DS, diterpenoids, presented at lower concentrations than the phenolic acids. The amount of Tanshinone I in twenty samples was close to each other with the range of 0.24ppm to 5.93ppm. Apart from that, the quantities of TSIIA were less than 10ppm in most samples. Gansu samples except DS4 and DS20 (Table 3.1) had both high concentrations of TSI and TSIIA. DS1 and DS15 from Yunnan province contained very low TSI quantity. DS6 and DS8 from the same region had low concentration of TSIIA. TSI cannot be identified in DS6 as well. Since antioxidant activity of DS1, DS6, DS8 were above the average, the effect of diterpenoids on antioxidant activity was

different from phenolic acids. The absence of TSI or TSIIA did not affect much on the antioxidant activity, in contrast to RA and SAB. DS6 had relatively high level of antioxidant activity but it consisted of none or low level of the reference diterpenoids. Also, the samples with small amount of TSI and TSIIA exhibited moderate antioxidant activity.

Based on the above observation, the presence of RA and SAB are more important than that of TSI and TSIIA to the antioxidant capacity. But the activity of the samples did not increase with the concentrations of all the components studied. Similar result was obtained before. For example, DS8 did not contain the highest concentration of the five selected chemical markers among the rest of the DS samples even though its antioxidant activity was the strongest. It contained quite low amount of TSIIA among all. Because of this, it is not necessary that any sample consisting of high concentration of the selected chemical markers exhibits strong antioxidant activity. This is somewhat expected as there may be multiple antioxidant related components in DS sample.

4.4.6.3 Relationship between the chemical reference component and antioxidant activity of DS samples

As mentioned previously, the antioxidant activity of the DS sample depends on the phenolic acids rather than the diterpenoids. The sample with the highest concentrations of the reference components would not have the strongest antioxidant activity. Similar result was obtained by Matkowski et al. study [29]. The main characteristic phenolic acids were responsible for the scavenging of hydrogen peroxide in another study [10]. Furthermore, the presence of lipophilic compounds caused major difference between samples with distinct antioxidant activities. The effect of individual reference components in DS samples on the antioxidant activity was explored here which would aid in digging out the bioactive components.

In order to study the effect of individual chemical reference compounds in DS, the relationship between the content of the selected chemical markers in the samples and their antioxidant activities were found. It turns out that the antioxidant activities of the

samples do not strongly depend on the concentration of these compounds. The correlation coefficients of the relationship between RA, SAB, TSI and TSIIA and the antioxidant activities of DS were -0.2896, 0.6197, -0.2588 and -0.4427 respectively. This is contradictory to the outcome of Tepe et al. study in which the strong correlation between the RA and antioxidant activity potential was found [148]. The comparatively linear relationship was found between SAB and antioxidant activity of DS. Still, the amount of the reference compound is not the key factor in affecting the antioxidant activities.

Besides studying the individual effect of the reference component, the relationships between the antioxidant activities of DS and the total concentrations of all the target markers, all target phenolic acids (DA, RA and SAB) and all target diterpenoids (TSI and TSIIA) were investigated. Better linear relationship of all phenolic acids with antioxidant activity than that of all the target markers was got. The correlation coefficients of these two relationships were 0.6605 and 0.6036 respectively. When all reference lipophilic components were considered, no linear relationship can be obtained and a very poor correlation coefficient value of -0.3885 was obtained. Based on these, the conclusion is that the antioxidant activity of DS sample is related mainly to the concentration of phenolic acids.

4.4.6.4 Fingerprint analysis of DS samples

Partial information on DS chemical composition can be got from focusing on the identification and quantification of a few selected chemical markers as most do. As the antioxidant activity of DS may be due to other unidentified compounds, the chromatographic fingerprint that display all the detectable components. Some may have contribution to the bioactivity and may not be the known markers. The similarity between the chromatographic fingerprints of samples and the median of all under study was evaluated using CASE in this work. By comparing the chemical composition of the samples, they are close to each other. Generally, the similarity index of the sample was above 0.90 except DS18, DS19 and DS20 (Table 4.10). These samples had the index values of 0.6852-0.7684 compared to their median and this indicated that they had significant difference in chemical composition. Additionally, their FRAP values were less

than the average values of the samples. Thus, this further illustrates that the antioxidant activity of the DS sample associates with the known and unknown components other than the selected target compounds.

From Figure 4.22, the chromatographic profiles of the samples with the FRAP values at the bottom four and top four were shown. When similarity evaluation was performed using DS8 as the reference, the samples with the FRAP values below 1000 were dissimilar to DS8 with the similarity index value of 0.7086-0.9638. For those samples in the top ranking, they are much resembled to each other with similarity indices above 0.97. Thus, there is significant variation among the samples with distinct antioxidant activities. From the DS chromatographic fingerprints, the major difference was observed in the regions between 48 min and 60 min. The concentrations of TSI and TSIIA in DS3, DS17, DS19 and DS20 were relatively high while none or very low content of SAB was found which was mentioned previously. Also, there were other unidentified diterpenoid compounds in these samples. These may account for the low antioxidant activity.

Another variation between the samples with high and low antioxidant activity capacity was demonstrated in the early eluted compounds. The UV spectra obtained from the corresponding peaks showed that the related components were possibly phenolic acids. Compounds at 18.4min and 20min were found in DS5 and DS8 only (Figure 4.22). They may contribute significantly to the antioxidant activities because they were found in samples with high level of antioxidant activity. Even though the contents of RA and SAB in DS5 were higher than DS8, the activity of DS5 was lower than that of DS8. In addition, more diterpenoids can be found in DS5 instead of DS8. This suggested that the large amount of reference compounds would not yield the higher level of antioxidant activity. Apart from this, the component at 22.1min was found in DS3, DS19 and DS20 samples. It is unlikely that this kind of compound play a significant role to the antioxidant activity. Both target and non-target compounds which may be responsible for the antioxidant activity can be detected from the whole chromatographic fingerprints. This would aid in sorting out the components that would exert the pharmacological effect of DS.

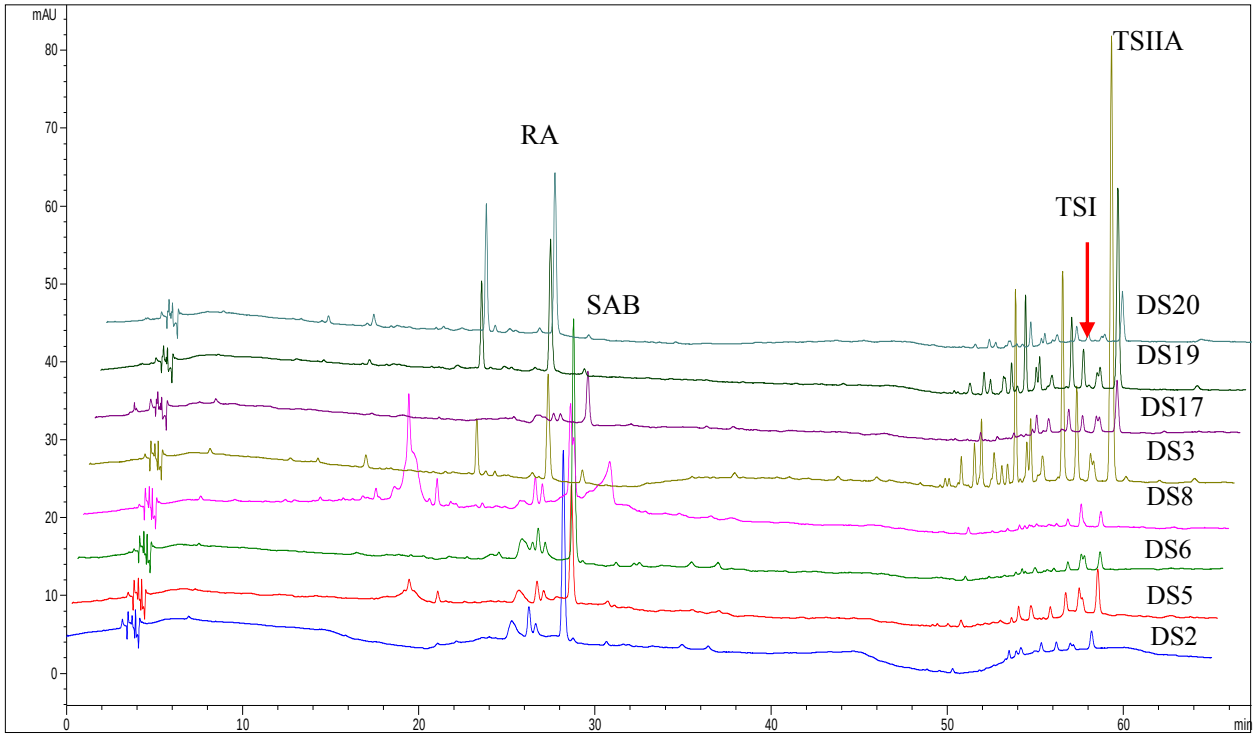


Figure 4.22 The HPLC-UV chromatograms of the DS samples with the highest (DS2, DS5, DS6 and DS8) and lowest antioxidant activity (DS3, DS17, DS19 and DS20) with content 1mg/ml at 280nm

4.5 Conclusion

Radix et Rhizoma Salviae Miltiorrhizae (DS) was composed mainly of phenolic acids and diterpenoids. Because of its antioxidant activity, it is very useful in protecting the cardiovascular system from the oxidative damage. As a result, it is commonly used to prevent and treat coronary arteriosclerosis, angina pectoris and hyperlipaemia. In addition, it involved in anti-tumor activity, treatment of menstrual disorder and hepatitis.

In this study, the effect of the extraction parameters and the optimal extraction conditions towards antioxidant activity of Radix et Rhizoma Salviae Miltiorrhizae (DS) were investigated using response surface methodology (RSM). Four extraction parameters were studied during the screening procedures and their combinations were based on uniform design table. They are methanol content (X1), number of times of extraction (X2), duration (X3) and solid to liquid ratio (X4). Before studying their effects on antioxidant activity, the extraction method was selected. Ultrasound assisted extraction

was preferred because it showed the advantages over reflux when comparing antioxidant activity level. From the screening procedure, methanol solvent (X1) and solid to liquid ratio (X4) played significant role to the antioxidant activity of DS extract. Then, the optimal extraction condition of DS was determined from these two parameters. Higher antioxidant activity level of DS extract can be obtained using 90ml 80% methanol for 45 minutes.

Based on the optimal extraction conditions, the antioxidant activities and chemical compositions of twenty DS samples from different sources were compared. At the same time, the relationship between these two pieces of information was investigated. It is well known that the quality of HM could be affected by the cultivated environment and conditions. Hence, the contents of the target components as well as the antioxidant activities are expected to vary among them. Also, the low level of antioxidant activity of DS is caused by the low amount of phenolic acids. But it is not necessary to have high contents of the reference compounds as selected in this work in order to have high level of antioxidant activity. Also, the poor linear relationship between the concentrations of diterpenoids compounds and antioxidant activity with the correlation coefficient being -0.3885 was found. That is, the antioxidant activity did not increase with amounts of diterpenoids. The correlation coefficient of relationship between the total identified phenolic acids and antioxidant activity was found to be as high as 0.6605. Thus, the influence of diterpenoid compound on antioxidant activity is less than phenolic acids. Besides, similarity evaluation between the chromatographic profiles of different DS samples was performed. Variation between the detected chemical constituents of the samples with the distinct antioxidant activities was reflected from the measured SI value. From the chromatographic fingerprints, it was observed that the presence of the diterpenoids compounds would not enhance the antioxidant activity dramatically.

To conclude, the antioxidant capacity of a Danshen extract was affected by both extraction conditions and its instinct properties such as its growing conditions or harvesting conditions. Furthermore, both phenolic acids and diterpenoids in Danshen are responsible for the antioxidant activities. But the contribution of phenolic acids was more

significant than diterpenoids under the Ferric Reducing Power Antioxidant (FRAP) assay.

Chapter 5

Establishment of the Quantified Pattern-Activity Relationship between the chromatographic fingerprint and antioxidant activity of Radix Puerariae Lobatae (YG)

5.1 Introduction

The current quality control of HM is based on the "compound-oriented approach" and "pattern-oriented approach". These measures are used to ensure the safety and consistency of HM materials. However, the efficacy of HM has not been considered. In addition, it is well known that the therapeutic effect of HM is holistic by nature. The pharmacological activity of HM is attributed to usually more than one kind of bioactive components [5]. But the variations of only a few selected chemical reference compounds were monitored in compound-oriented approach. This limits the applicability of this approach in quality evaluation. Alternatively, the whole chromatographic fingerprint of HM has been utilized for confirming the identity and consistency which contains more information of chemical constituents. The variations of all these detectable components could be observed from the chemical fingerprints for assessment.

In these years, more and more scientific researches were preformed on the therapeutic effect of HM. There is a long history in applying the HM in treatment of various diseases. However, it is not widely accepted in healing as it is lack of scientific proof on its efficacy [2]. The therapeutic effect of the western drug targets on individual part of the human body. This leads to the problems in curing certain diseases. Cancer treatment is a typical example of this kind. HM could be an alternative in this aspect as it aims at the whole principal. Yet, it is necessary to guarantee the quality of HM beforehand. To deal with the limitations in the current quality control measures as mentioned previously, it is desirable to establish the relationship between the chemical compositions and bioactivity. Both biological activity and the identity of the HM could be learned from the chromatographic fingerprint once this kind of relationship is set up.

It was mentioned before that the better proof of HM required the correlation of chemical composition and the medication of HM [1, 8]. However, the correlation between the chromatographic profiles and bioactivity of the HM is rarely known. Because of this, chemometric technique can act as the bridge to achieve this to greater extent. All the components including all the active and inactive components that can be detected by the hyphenated instruments would be considered and related to the efficacy of HM. A good

example is the Quantitative Pattern-Activity Relationship (QPAR) approach [163]. Through which, the biological activity can be estimated from the chemical profile once the HM QPAR model has been developed. The multivariate modeling techniques such as Partial Least Squares (PLS) or Uninformative Variable Elimination by PLS (UVE-PLS) can serve the purpose. Recently, there are studies on the application of multivariate modeling techniques in predicting the antioxidant property of green tea [119, 120, 164] and gegen, a Chinese medicine [163]. It could be the new strategy in the quality control of herbal medicine.

In this work, the quality assurance of Radix Puerariae Lobatae (YG) was carried out through "component-oriented approach", "pattern-oriented approach" and the new QPAR approach as well. YG contained rich sources of isoflavonoid and displayed antioxidant activity [158]. The antioxidant activity has received increasing attention as many diseases are related to the oxidant stress such as cardiovascular disease, atherosclerosis, cancer, diabetes disease and Alzheimer's disease. In this investigation, the antioxidant property of YG was measured by Ferric Reducing Antioxidant Power assay (FRAP). At the same time, chemical study of YG was carried out by high-performance liquid chromatography coupled with diode array detector (HPLC-DAD). Five chemical reference isoflavonoids were selected for quantification and identification. They were puerarin (PU), daidzin (DZ), genistin (GE), daidzein (DE) and genistein (GS). Once the QPAR model is available through connecting the chromatographic profiles and antioxidant activity levels, the total antioxidant activity of YG can be determined from the chromatographic fingerprints alone with no bioactivity measurement required. Our results showed that it is able to predict the antioxidant activity level of YG from its chromatographic profile through the QPAR model. Hence, one can see that the chromatographic fingerprint of YG provides valuable information on chemical composition and biological activity. There is no doubt that more thorough proof of quality of HM can be performed in this way.

5.2 Background of Radix Puerariae Lobatae (YG)

There are over 35 *Pueraria* species all around the world such as Japan, Korea, Thailand and China. They belong to the family of Leguminosae. *Pueraria lobata* (Willd.) Ohwi is

also known as Gegen or Yege in Chinese (YG) which is commonly employed to treat different diseases. *Pueraria thomsonii* Benth. and Fenge (FG) in Chinese, is also commonly used in southern China. But they referred to different species and the contents of puerarin and antioxidant activities of FG and YG varied from each other [36]. FG can be found commonly in the commercial market but YG is not. Besides, the species cultivated in Thailand which is known as *Pueraria mirifica* contained their own specific isoflavonoids, miroestrol and deoxymiroestrol, that have strong estrogenic effects [165, 166].

Radix Puerariae Lobatae (Figure 5.1) has been applied in various treatments for a long period of time in China. It was first described in the Shen Nong Ben Cao Jing which had antidipsotropic (anti-alcohol abuse), amethystic (anti-alcohol intoxication), antidiarrhetic, diaphoretic, anti-emetic activities. It was applied in releasing the stiffness and pain of the neck, eyes, induction of early measle eruption in Huang Di Nei Jing [34, 167-169]. In addition to the antiviral, sedative and antipyretic actions, YG has been used to manage the upper respiratory airway diseases like curing the flu and headache. It could also relieve fever and dysentery [35, 36, 170]. Because of these, YG was involved in the prevention of EV71 infection from a recent study [170]. Besides, it was useful in the treatment of cardiovascular disease, hypertension and liver disease, promoting the production of body fluid, possessing prokinetic and antihyperglycemic properties which increase the insulin sensitivity for the treatment of the diabetes mellitus [35-38, 167, 170, 171]. Additionally, YG is a potent anti-cancer agent. The serum metabolite in the rat after administration of YG extracts was able to inhibit the growth of MDC-7 cells [172].

YG has different pharmacological activities, particular its action on preventing oxidant stress related diseases including cancer, cardiovascular disease and diabetes. It is because there is increasing number of people around the world that suffers from these killing diseases. The oxidative modification of low density lipoproteins with reactive oxygen species plays a fundamental role in atherogenesis that leads to atheroma. Atheroma is due to fatty degeneration of inner coat of arteries or abnormal fatty deposit in an artery [173]. The antioxidants in YG exerted the protective effect against the oxidative damage. Not

only this, but also the antioxidant activity of YG was shown by the in-vivo system. The treatment of YG extract could reduce the antioxidant stress on the diabetes mellitus animal model [167]. In these studies, the antioxidant activity of YG was related to the abundance of isoflavonoids.

Figure 5.1 Appearance of Radix Puerariae Lobatae

5.2.1 Chemical composition of YG

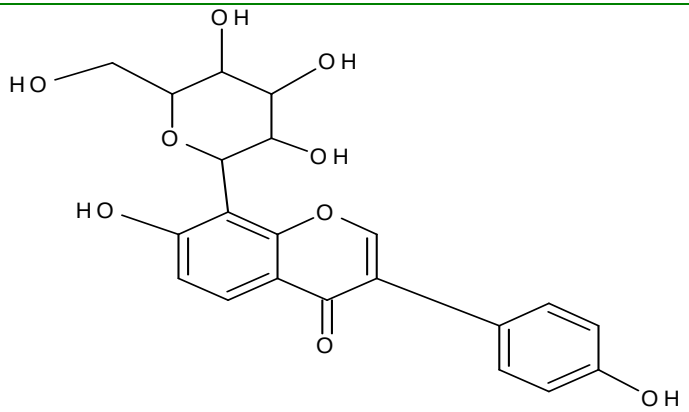
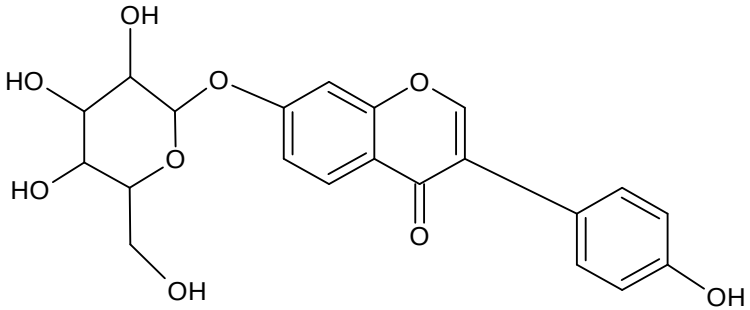
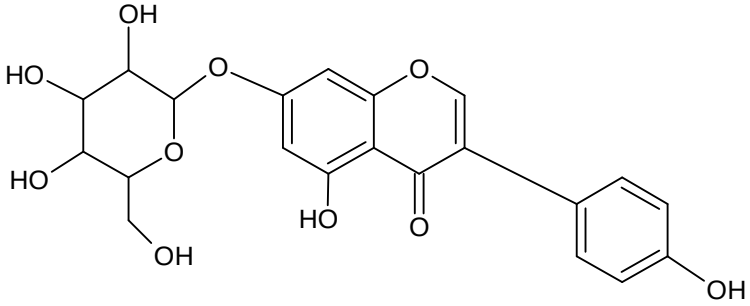
Apart from soybean, YG is another natural source that is rich in isoflavonoids. It is a subclass of flavonoid compound [172]. The active ingredients of YG are referred to isoflavone. According to Chinese Pharmacopoeia (2010 edition), the identity of YG is confirmed by the content of puerarin (PU) which should be higher than or equal to 2.4% in YG [142]. It is because PU is the characteristic isoflavonoid that can be found in YG only. In addition to this, four other renowned isoflavonoids were selected as chemical reference compounds for both identification and quantification in this work. They were daidzin (DZ), genistin (GE), daidzein (DE) and genistein (GS). These five isoflavonoids were identified at the high level in the *Pueraria lobata* tuber [42, 166]. Their chemical structures are given in Table 5.1.

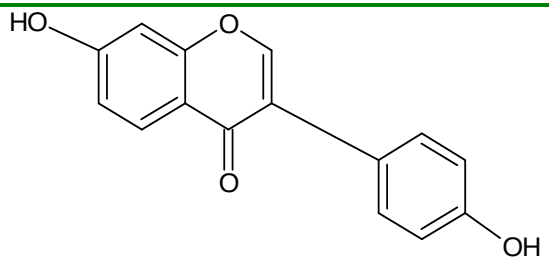
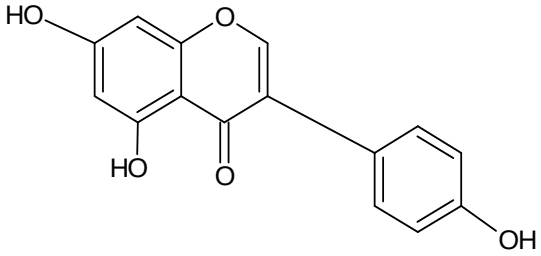
Daidzein (4',7-dihyroxy-isoflavone) and genistein (4',5,7-trihydroxy-isoflavone) are major soy isoflavones in soybean and can be found in YG as well. Many isoflavonoid glycosides are derived from daidzein and genistein. Puerarin (daidzein-8-C-glycoside) and daidzin (daidzein 7-O-glucoside) are glycoside of daidzein while genistin (genistein

7-O-glycoside) is glycoside of genistein [174]. The chemical structures of genistin and daidzin are similar to each other but differ in the hydroxyl group present in the aglycone. Hence, they exhibited different biological activity and the mechanism in disease treatment is not the same as their corresponding aglycones (See Table 5.1) [43].

Since PU could donate hydrogen and form monoanion, fluorescence is emitted when it acts as an anion. It can be utilized to study the antioxidant property in biological system. Furthermore, it is more polar than DE because of the sugar moiety in PU [175]. The polarity of daidzin is similar to that of puerarin because their chemical structures are quite similar to each other with different position of the sugar moiety only. Usually, the degree of glycoslyation is directly linked to the water solubility. Thus, glycoside is more soluble than aglycone. Additionally, the solubility of genistin is relatively low leading to the reduction of its biological availability in the in-vivo system [176].

Table 5.1 Pharmacological activities and chemical structures of the five reference isoflavonoids for studying the YG samples

Compound	Chemical structure	Pharmacological activity
Puerarin (PU)		1. Improvement of blood circulation [158, 177] 2. Preventing cardiovascular diseases [158, 177] 3. Treatment of arrhythmia [158, 177] 4. Possessing antioxidant and anticancer effect [158, 177] 5. Improving the memory impairment [158, 177] 6. Possessing antithrombotic and anti-allergic activities [178, 179]
Daidzin (DZ)		1. Acting as antidipsotropic agent [168] 2. Treatment of alcoholism [177, 178] 3. Protection against oxidative DNA damage [43]
Genistin (GE)		1. Acting as anti-cancer agent against hormone related cancer diseases [37, 178] 2. Protection against oxidative DNA damage [43, 176] 3. Scavenging superoxide anion [176] 4. Reduction of low density lipoprotein [176]

Daidzein (DE)		1. Protective effect against DNA oxidative damage [41, 180] 2. Preventing the oxidative damage on the low density lipoprotein particles [181, 182] 3. Treatment of alcoholism [177] 4. Processing antidipsotropic effect [158]
Genistein (GS)		1. Protective effect against DNA oxidative damage [41, 183] 2. Preventing the oxidative damage on the low density lipoprotein particles [181, 182] 3. Exhibiting anti-cancer property [165]

5.2.2 Pharmacological activity of YG constituents

As mentioned before, the therapeutic effect of YG is related to the abundance of isoflavonoids. Different isoflavonoids displayed their own specific biological activities. Generally, isoflavonoids exhibit anti-inflammatory activity, anti-thrombotic activity, anti-hypertensive activity and cancer chemopreventive activity [184]. Since their chemical structures are similar to the estrogen in human, they are known as phytoestrogen. Phytoestrogen is important in preventing certain cancers, reducing the risk of osteoporosis, lowering plasma cholesterol and decreasing the hazard of coronary heart disease [39-42]. Particularly, the risk of cardiovascular disease would dramatically increase under estrogen deficiency [40]. For instance, puerarin has chemical structure similar to the estrogen which relates to cardiovascular protective effect [185]. The isoflavonoids have positive effect through inducing the cell growth and differentiation in the skeletal, cardiovascular, central nervous system in the menopausal woman. It would reduce the chance in inducing hormone-related cancers because of the antioxidant property, anti-estrogenic and antiproliferation effects of isoflavonoids [42].

Details of the pharmacological activities of the five selected reference isoflavonoids in this investigation are given in Table 5.1. They have different capabilities on the same biological assays. For example, the aglycone, genistein and daidzein, showed the relatively stronger estrogenic activity while puerarin exhibited the weakest activity in Zhang et al. study [42]. Similar results were obtained when comparing the antioxidant properties among the isoflavonoids [14, 182].

This class of compound is able to attenuate the oxidative damage on protein or cellular system in the in-vivo study [41]. Genistin and daidzin could protect DNA from the oxidative damage through scavenging hydrogen peroxide and reducing the superoxide anion formation [41, 43]. Apart from that, genistin could up-regulate the antioxidant metallothionein expression [176]. In addition, puerarin could prevent the low density lipoprotein oxidation which accounts for the anti-atherogenic activity and hypocholesterolemic effect. Since hypercholesteremia would modify cholesterol metabolism, it is the major risk factor for the development of atherosclerosis [38]. Based

on these, puerarin could prevent atherosclerosis and related cardiovascular disease [38, 186]. Moreover, puerarin is important in antidiabetic activity owning to its antioxidant activity. It inhibited the free radical production induced by hydrogen peroxide and increased the superoxide dismutase activity [171, 186].

Owning to antioxidant activities, isoflavonoids could prevent the cardiovascular diseases and diabetes. Also, they have anti-cancer effect through scavenging the free radical or inducing the endogenous antioxidants [183]. Generally, the glycosides are less effective than their aglycones. Genistein was more potent than daidzein in inhibiting the growth on the stomach cancer cell line, esophageal cancer cell line and colon cancer cell line [187, 188]. These two compounds were also effective in inhibitory of the growth of the breast cancer cells [172, 187]. Although puerarin was less competent in the prevention of the gastrointestinal related cancers, it could induce apoptosis of breast cancer cells. It was also reported that genistin and its aglycone were capable of inhibiting the proliferation of human ovarian cell. But genistin exhibited less potent activity than its aglycone, genistein [189].

5.3 Methods of investigation

Multivariate calibration has been utilized in establishing the relationship between the variables and response. It is commonly applied in determining the chemical and physical properties from the NIR spectra [98, 100-102]. Partial least squares method (PLS) is a well developed multivariate method which considers all the variables for regression. In this investigation, this methodology was applied in building up the quantitative relationship between the chromatographic fingerprint and bioactivity of HM. Instead of utilizing all the chromatographic variables, variable selection was also involved before building up the relationship. Uninformative variable elimination by partial least squares method (UVE-PLS) was employed for estimating the antioxidant activity of YG as well. Details about PLS and UVE-PLS were discussed in Chapter 2. Only brief description related to this work is provided here.

5.3.1 Partial Least Squares method (PLS)

In brief, PLS is a well established methodology in correlating the response (**Y**) and variables (**X**). Different multivariate calibration methods were developed based on PLS such as target projection (TP) or orthogonal PLS (O-PLS). There is no variable selection in PLS and employed the latent variables for multivariate modeling. All the variables were first decomposed into different latent variables. Hence, the relationship between the chromatographic fingerprint (**X**) and antioxidant activity (**y**) can be generally expressed as the following

$$\mathbf{y} = \mathbf{Xb} + \mathbf{f} \qquad [5.1]$$

where **b** is regression coefficient vector and **f** is residual vector.

From the computed regression coefficients of the variables, the antioxidant activities of YG can be predicted from the chromatographic fingerprint.

Alternatively, another multivariate modeling method, UVE-PLS, is applied in this study with variable selection prior to the modeling. The major difference between these two multivariate methods is that UVE-PLS removes the variables that are not correlated to the response in the estimation of the response. It is believed that better prediction performance can be achieved based on the variables that are closely related to the response. Additionally, this would provide the explanatory power of the multivariate model.

5.3.2 Uninformative Variable Elimination by Partial Least Squares method (UVE-PLS)

The selection of variables in UVE-PLS is based on their stability. This was determined by the ratio of mean of regression coefficient to the standard deviation of the regression coefficient of the variable j through jackknifing [117, 118].

$$\mathbf{s}_j = \text{mean}\ (\mathbf{b}_j) \,/\, \text{std}\ (\mathbf{b}_j) \qquad [5.2]$$

A set of artificial variables is included in finding the stability. The highest stability found from the artificial variables was set as the threshold value. Hence, the variables from the chromatographic fingerprint which is related to the bioactivity can be picked [104, 117, 118]. The chosen variables are included in multivariate regression using PLS method.

In order to evaluate the adequacy of the multivariate model, the samples are classified into the training set and the validation set. The multivariate model is developed based on the samples in the training set. The established relationship is further confirmed by the validation set, an independent group of sample. In order to characterize the model fits and assess how good the prediction is, all comparison is based on the root mean square error of cross validation (RMSECV) and root mean square error of prediction (RMSEP) that are obtained from the training set and validation set respectively [107]. The prediction ability of the model can be evaluated by these two parameters. Also, the correlation coefficient (R) between the predicted and experimental values is calculated.

Root mean square error of cross validation is also known as leave-one-out root mean square error of cross validation. It is used to make decision on the optimal number of latent variables in the model so that it could avoid the overfitting or underfitting problem. The RMSECV should be minimal when the model consists of the optimal number of latent variables.

$$RMSECV = \sqrt{\sum(yi - y\hat{}i)^2 / N} \qquad [5.3]$$

y_i is experimental value of the i^{th} sample and y^i is the predicted value of the i^{th} sample. N is the number of samples in the training set.

The measure of RMSECV in training set characterized the model fit in order to find the optimal number of latent variables. A similar procedure is also applied to the measurement of RMSEP. RMSEP evaluated the deviation between the experimental values of the samples and their predicted values from the model in the validation set.

The correlation of the predicted and experimental values can be determined by the correlation coefficient (R). The square of it is the proportion of the variation of response that is explained by regression. The finding of R is expressed in the following.

$$R = \sum (y\hat{}i - y_m)(yi - y_m) / [\sqrt{\sum (yi - y_m)^2 \sum (y\hat{}i - y_m)^2}] \qquad [5.4]$$

y^i and yi are the predicted and experimental value of the i^{th} sample respectively while y_m is the mean of the experimental results for all samples in either training set and validation

set. When the value of correlation coefficient is close to 1, it represents a close relationship between the experimental and predicted values under the multivariate model studied. Although the model with the value of correlation coefficient close to 1 is preferred, it cannot be considered alone in validating the multivariate model. When the model is overfitting, the correlation coefficient would also be close to one. Then, the overfitting problem will be neglected based on this parameter.

5.4 Results and discussion

5.4.1 Authentication of Radix Puerariae Lobatae (YG) through "compound-oriented approach" and "pattern-oriented approach"

5.4.1.1 Identification and quantification of the reference isoflavonoids in YG

Authentication of Chinese medicinal herb is mainly based on a few chemical reference compounds with known chemical structures taking the so called "compound-oriented approach" [51]. As mentioned before, isoflavonoid is the major class of compounds in YG and responsible for the therapeutic effect. Therefore, five chemical reference compounds of this class were selected here for both quantification and identification. They are puerarin (PU), daidzin (DZ), genistin (GE), daidzein (DE) and genistein (GS). The chromatographic fingerprint of their mixture solution at 254nm was shown in Figure 5.2. Their identities in the YG samples were confirmed based on their retention times and UV spectra (Table 5.2). As HM is a complex multi-component system, overlapped peak clusters are commonly observed in its chromatographic profile. The spectral information in addition to the retention time would aid in verifying the identities of the components.

Based on the extraction conditions stated in Chapter 3, seventy nine aqueous extracts of the YG samples were prepared for chemical analyses. By matching retention time and UV spectrum, PU can be found in all YG samples while GS can be identified in YG-18, YG-20, YG-21 and YG22 only. The other chemical standards can be found in most of the YG samples studied. Figure 5.3 depiced the HPLC-UV chromatographic fingerprints of the seven samples YG-1, YG-2, YG-5, YG-6, YG-7, YG-43, YG-69 and the median chromatogram at 254nm with the same concentration of 1mg/ml. It can be seen that

except GS, the remaining four selected isoflavonoids present in some of these samples.

In order to study the contents of these reference isoflavonoids in YG, calibration curve of each reference isoflavonoid was set up with the related chromatographic peak area against concentration. All these curves were found to be linear with correlation coefficients greater than 0.99 (Table 5.3). Based on the regression equations of the five chemical reference compounds, their concentrations in all the 79 YG samples were found and listed in Table 5.4. The average values of puerarin, daidzin, genistin, daidzein and genistein are 67ppm, 8.56ppm, 5.23ppm, 3.14ppm and 0.94ppm respectively.

Among them, puerarin was present in all the YG samples at the highest concentration compared to other reference compounds. It is the most abundant isoflavonoid glycoside in YG. As for genistein, the average amount in the YG samples is less than 1ppm and can be found in a few YG samples. Compared with the average contents of PU, GS was present in extreme low amount. The main reason may be that PU, DZ and GE are more polar while DE and GS are less polar compound as indicated from their retention time. Additionally, PU, DZ and GE are glycosides and more soluble in water compared to DE and GS. Hence, the extraction of DE and GS is not as efficient as their glycosides using procedures proposed in this study. Apart from this, they are metabolites of the herb and their quantities in different YG are affected by the genetic and environment factors [166].

Comparing the content of each chemical reference compound in all the samples, YG-41, YG-38, YG-29, YG-9 and YG-18 have the highest content of puerarin, daidzin, genistin, daidzein and genistein respectively. On the contrary, the lowest content of puerarin was found in YG-5. Also, YG-1, YG-6 and YG-69 consisted of less than 15ppm PU. The amounts of the other reference isoflavonoids were also less than the average values in these samples. Although PU and DZ are the glycoside of daidzein, DZ was presented at lower concentration than that of PU in our YG samples. Unlike PU, all except YG-18 and YG-32 contained DZ with the content eight times less than that of PU. Another glycoside, genistin (GE), was chosen as the reference chemical compounds of YG. The glycoside of genistein was present at lower concentrations when compared with the glycoside of

daidzein. The average content of PU is 12 times to that of GE. Additionally, GE was absent in more YG samples such as from YG-5 to YG-14, YG-18, YG-20, YG-32 and YG-33. The significant difference in the contents of these glycosides was shown. It is because the contents of the secondary metabolites are related to the growing, harvesting and storage conditions.

Besides, the aglycones were concerned in chemical analyses of YG. Daidzein was discovered in most of YG samples except YG-1, YG-21 and YG-24. Similar result applied to its glycosides, PU and DZ, which were found in nearly all samples. Despite that, the average content of DE was not as high as their glycosides. As mentioned previously, the polarity of DE is lower than its glycoside such that the content was determined to be quite low in YG samples. On the other hand, genistein was detected in four samples only. They are YG-18, YG-20, YG-21 and YG-22. YG-18 and YG-20 had relatively high concentrations of GS than the other two samples. But no GE could be observed in these two. The YG samples were constituted by the glycosides majority instead of aglycone. It is possible that genistein and daidzein were present in the form of glycosides in herbal medicine [182]. Based on this, the contents of the reference isoflavonoids of YG samples varied from each other. Subsequently, YG samples had distinct biological activities.

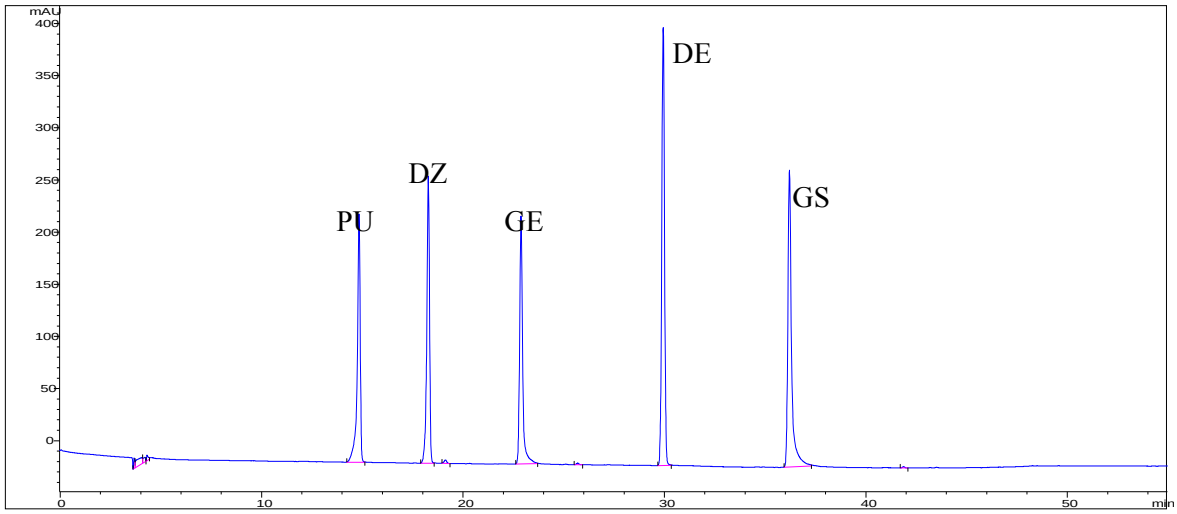
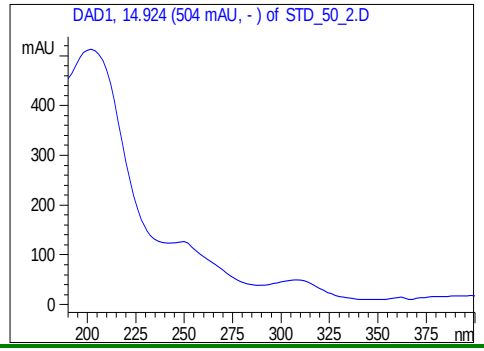
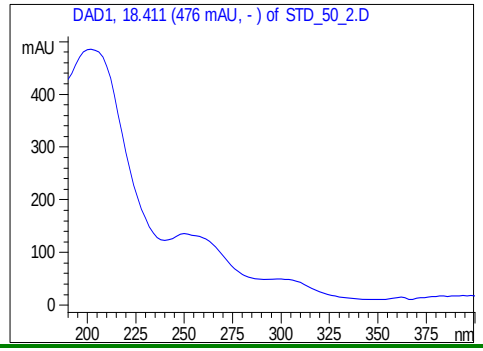
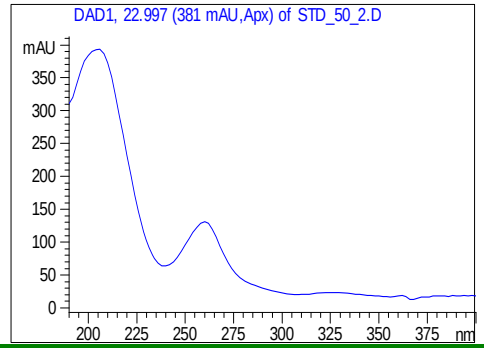


Figure 5.2 The HPLC-UV chromatogram of the five reference isoflavonoids at 254nm

Table 5.2 The retention times and UV spectra of the five chemical reference isoflavonoids (see Figure 5.1 also)

Compound	**Puerarin (PU)**	**Daidzin (DZ)**	**Genistin (GE)**
Retention time (min)	**14.4min**	**17.6 min**	**22.3min**
UV spectrum			

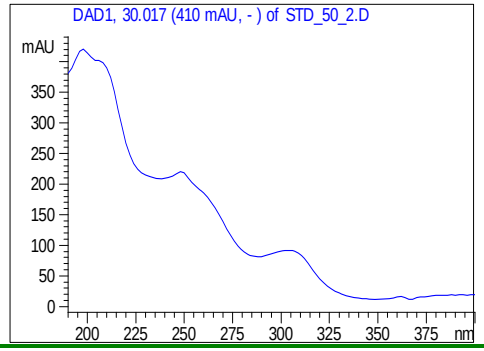
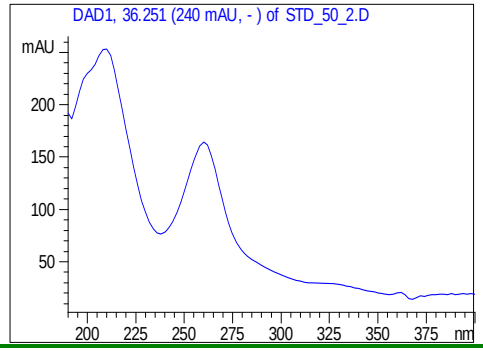
Compound	**Daidzein (DE)**	**Genistein (GS)**
Retention time (min)	**29.4 min**	**35.7min**
UV spectrum		

Table 5.3 The regression equations, related correlation coefficients and linear ranges of the five reference isoflavonoids

Reference Compound	Regression Equation	Correlation coefficient	Linear range (ppm)
PU	y=24.12x – 7.53	0.9996	1-100
DZ	y =25.87 x – 1.12	0.9996	1-100
GE	y =24.54 x – 0.13	0.9994	1-100
DE	y =39.88 x + 14.60	0.9995	1-100
GS	y =36.1 x - 17.50	0.9996	1-100

Table 5.4 The concentrations of the five reference isoflavonoids and the similarity indices of all the YG samples studied [a]

Sample	Concentration of reference isoflavonoid (ppm)					Similarity Index
	PU	DZ	GE	DE	GS	
YG-1	14.08	2.55	1.04	–	–	0.9730
YG-2	61.84	3.71	2.02	0.41	–	0.8745
YG-3	84.64	5.45	6.04	3.42	–	0.9884
YG-4	87.47	3.98	3.00	2.18	–	0.9709
YG-5	9.19	1.37	–	1.18	–	0.9501
YG-6	9.90	1.48	–	0.67	–	0.9417
YG-7	21.25	4.12	–	0.72	–	0.9730
YG-8	80.61	7.40	–	3.39	–	0.9867
YG-9	111.90	1.51	–	10.81	–	0.8251
YG-10	85.68	10.84	–	1.79	–	0.9273
YG-11	100.45	7.19	–	2.44	–	0.9103
YG-12	89.35	11.16	–	5.09	–	0.9294
YG-13	94.86	8.20	–	2.07	–	0.9236
YG-14	95.22	11.04	–	2.27	–	0.9273
YG-15	112.28	11.77	1.40	2.19	–	0.9411
YG-16	94.70	8.48	0.70	2.78	–	0.9611
YG-17	107.6	10.79	1.78	2.74	–	0.9753
YG-18	22.18	–	–	7.94	1.07	0.8825
YG-19	124.77	11.22	3.39	1.01	–	0.9657
YG-20	59.24	1.20	–	2.79	1.01	0.7131
YG-21	94.56	6.94	2.78	–	0.82	0.9421
YG-22	112.85	10.37	3.45	0.77	0.87	0.9719
YG-23	50.70	2.78	1.44	0.23	–	0.9204
YG-24	54.43	3.30	2.09	–	–	0.9285

YG-25	53.74	2.00	3.95	5.12	–	0.9829
YG-26	85.52	4.68	4.31	3.86	–	0.9795
YG-27	89.19	3.57	4.73	4.99	–	0.9794
YG-28	47.63	7.72	9.32	3.29	–	0.9660
YG-29	76.93	14.86	16.03	1.38	–	0.9803
YG-30	87.77	8.35	4.53	1.65	–	0.9853
YG-31	67.22	7.62	3.28	4.72	–	0.9867
YG-32	69.33	–	–	10.67	–	0.9513
YG-33	93.34	4.03	–	8.36	–	0.9864
YG-34	72.06	8.72	1.80	0.76	–	0.8908
YG-35	72.61	11.30	4.35	2.53	–	0.9914
YG-36	80.51	9.55	3.90	5.21	–	0.9938
YG-37	79.27	13.56	3.91	1.38	–	0.9437
YG-38	107.08	22.31	10.97	3.21	–	0.9638
YG-39	72.83	9.52	3.91	3.47	–	0.9952
YG-40	74.44	11.56	3.99	3.84	–	0.9834
YG-41	125.96	17.95	3.45	2.43	–	0.9700
YG-42	118.59	17.02	3.23	1.97	–	0.9858
YG-43	106.53	14.04	10.43	1.48	–	0.9657
YG-44	37.07	9.91	5.20	2.74	–	0.9778
YG-45	48.99	11.96	4.69	3.09	–	0.9854
YG-46	50.53	10.93	3.57	4.13	–	0.9692
YG-47	48.42	10.41	8.07	2.83	–	0.9573
YG-48	59.15	15.84	9.15	2.86	–	0.9795
YG-49	59.43	15.22	10.31	2.93	–	0.9789
YG-50	45.66	8.25	5.98	2.98	–	0.9781
YG-51	38.11	6.81	4.57	2.93	–	0.9850
YG-52	69.93	9.75	6.98	2.90	–	0.9918
YG-53	55.15	8.61	5.86	2.03	–	0.9925
YG-54	91.90	7.80	6.12	2.30	–	0.9946
YG-55	46.31	9.11	4.71	4.31	–	0.9704
YG-56	98.43	13.01	8.25	2.73	–	0.9812
YG-57	66.10	9.77	4.48	4.25	–	0.9767
YG-58	60.57	10.10	7.71	4.27	–	0.9550
YG-59	56.35	3.29	6.50	4.84	–	0.9615
YG-60	44.21	2.25	1.88	2.84	–	0.9452
YG-61	49.63	3.61	4.71	3.68	–	0.9788
YG-62	42.28	10.55	6.66	2.15	–	0.9371
YG-63	29.24	6.37	4.52	2.23	–	0.9403

YG-64	21.48	6.46	3.50	1.95	–	0.9597
YG-65	31.71	7.67	4.85	2.21	–	0.9631
YG-66	63.56	15.45	7.82	4.39	–	0.9749
YG-67	48.86	12.84	6.17	4.14	–	0.9797
YG-68	37.98	16.87	8.75	4.31	–	0.9213
YG-69	10.25	3.42	2.33	1.85	–	0.9059
YG-70	26.89	12.84	6.09	5.92	–	0.9038
YG-71	65.32	17.91	11.10	2.88	–	0.9785
YG-72	60.86	16.35	10.12	2.44	–	0.9811
YG-73	61.75	15.06	12.48	2.73	–	0.9764
YG-74	43.39	6.97	4.10	3.33	–	0.9284
YG-75	53.77	4.40	3.17	4.76	–	0.9422
YG-76	60.72	7.88	5.55	3.47	–	0.9871
YG-77	78.49	6.66	3.07	1.31	–	0.9756
YG-78	82.22	6.29	3.12	1.54	–	0.9781
YG-79	88.37	6.30	2.28	1.48	–	0.9786
Mean[b]	67.00	8.86	5.23	3.14	0.94	0.9564

[a] "-"denotes that the compound was not detected

[b] Mean= (Sum of the concentrations of the markers in all the samples)/ (number of samples with non-zero concentration)

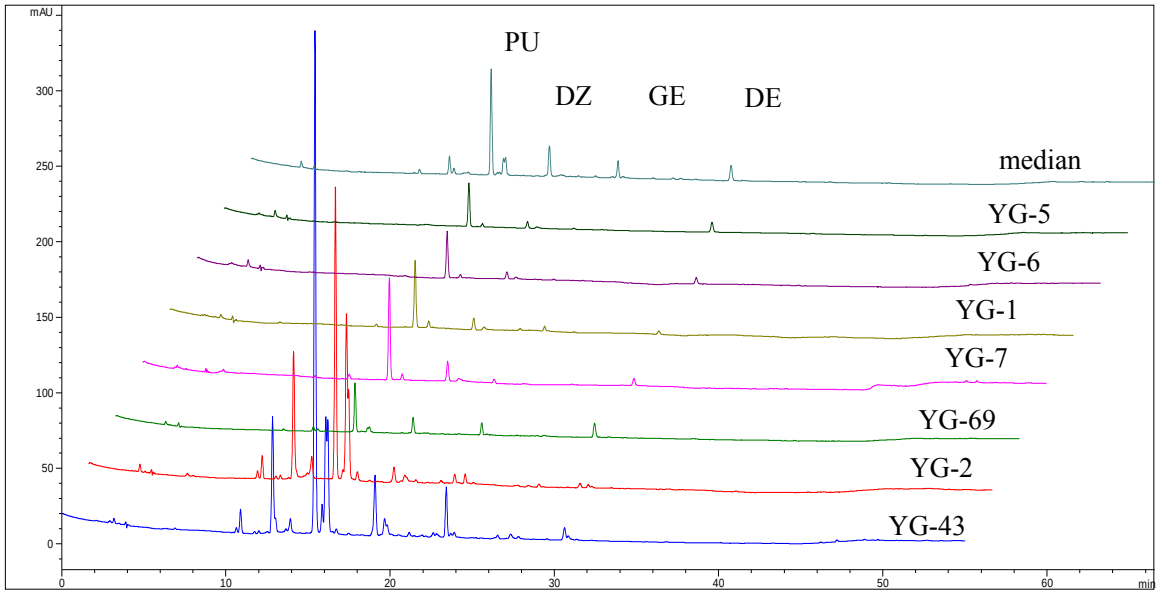


Figure 5.3 The HPLC-UV chromatograms of seven YG samples and their median at 254nm.

5.4.1.2 Comparison of the compositions in YG samples from their chromatographic fingerprint

As reported in different studies, the variation between different species of HM is not only due to the content of a few selected compounds or markers but also other unidentified components. Usually, differentiation of HM species is carried out by comparing chemical contents of some markers and sometimes even the chromatographic fingerprints [4, 5, 184]. The whole chromatographic fingerprint patterns of YG and FG varied from each other in addition to their puerarin content [184]. In the previous section, it was mentioned that the contents of the target isoflavonoids in YG samples were not the same. This applies also to those unidentified isoflavonoids. But it is unable to quantify them due to the lack of reference compounds. Therefore, comparison of the YG chemical composition was done by means of their chromatographic fingerprint with respect to their median chromatogram here using Computer Aided Similarity Evaluation (CASE). All the detectable components in YG samples were involved in the similarity evaluation and expressed in terms of similarity index (SI). This provides a quantitative measure in the variation of their chemical compositions. In Table 5.4, it can be seen that most of the samples were resemble with one other with the average SI value of 0.95. In general, the chromatographic patterns of YG samples were close to one other irrespective to their origin.

It is a common practice to compare the chromatographic fingerprints of different samples with their median. There was one sample, YG-20 with the SI value (0.7131) less than 0.8 (Figure 5.4). It indicated that the chemical ingredient of this sample is differed from the contents of others. The elution compounds can be found at 6min, 11min and 17min in YG-20 but not in the median chromatogram. On the other hand, the elution compounds in the range of retention time from 22min to 28min were found in the median chromatogram instead of YG-20 (Figure 5.5). This observation may explain why YG-20 had extremely low SI value. On the contrary, the chromatographic patterns of those samples with SI values above 0.99 were much or less identical (Figure 5.6). YG-35, YG-36, YG-39, YG-52, YG-53 and YG-54 had similar constituents as observed from the retention time and UV spectra. Among them, YG-35, YG-36 and YG-39 were from Anhui province and their

chemical compositions were close to one other.

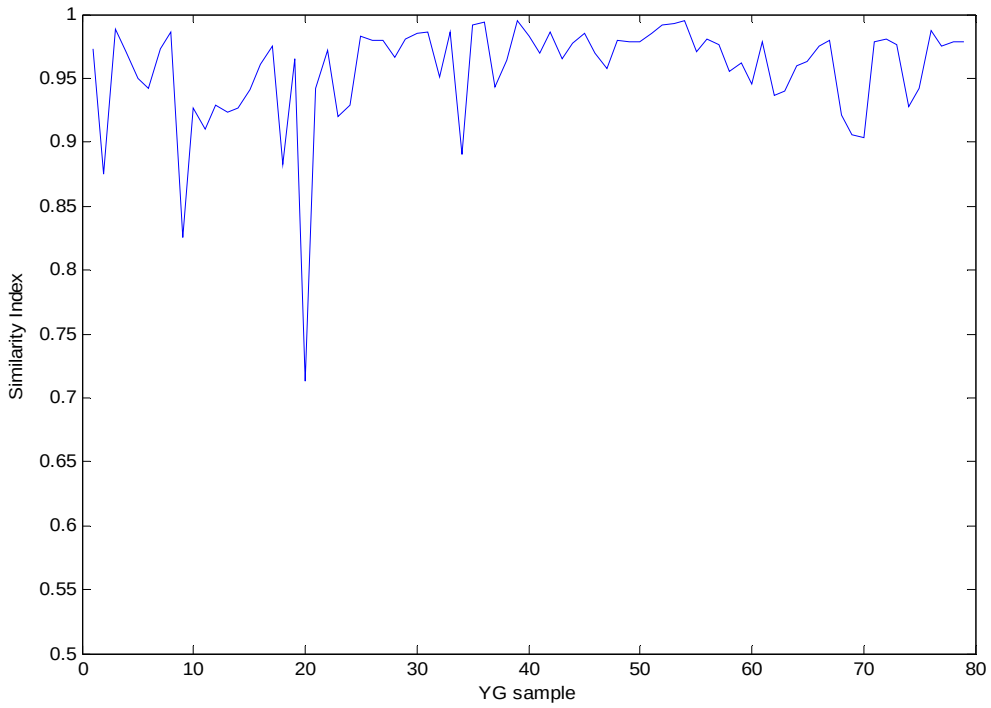


Figure 5.4 The variations of similarity index of 79 YG samples when they are compared with the median chromatographic profile

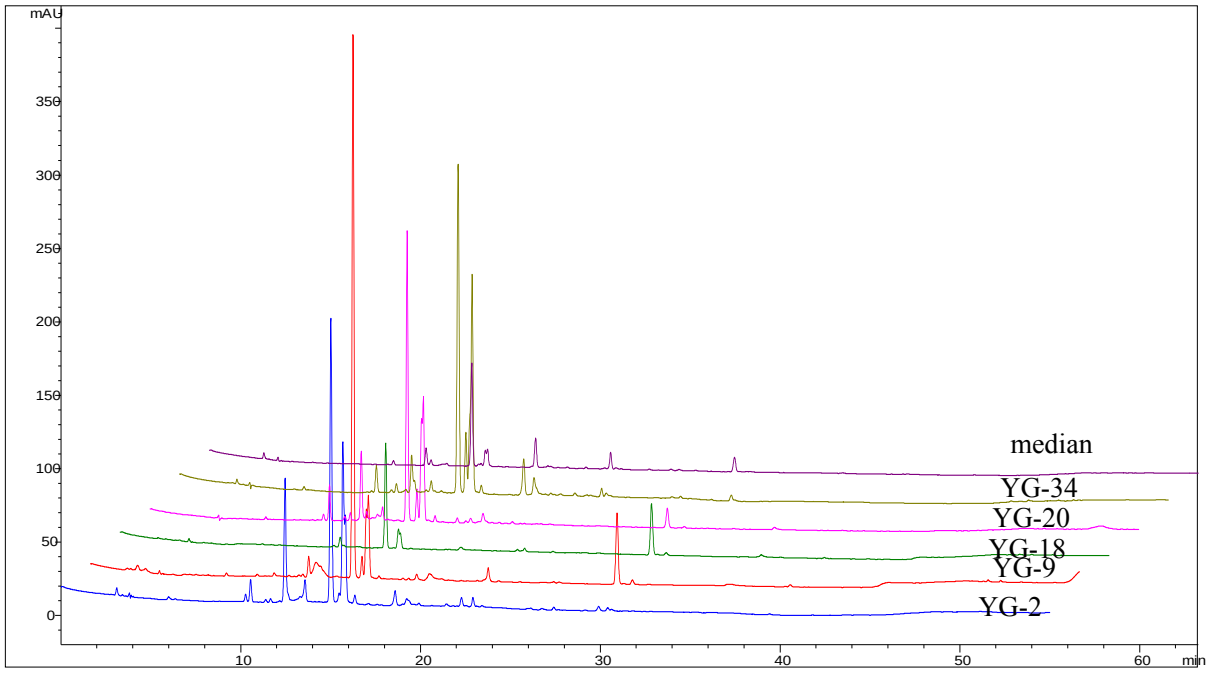


Figure 5.5 The HPLC-UV chromatograms of YG samples with SI values below 0.9 when

compared with the median chromatographic profile at 254nm

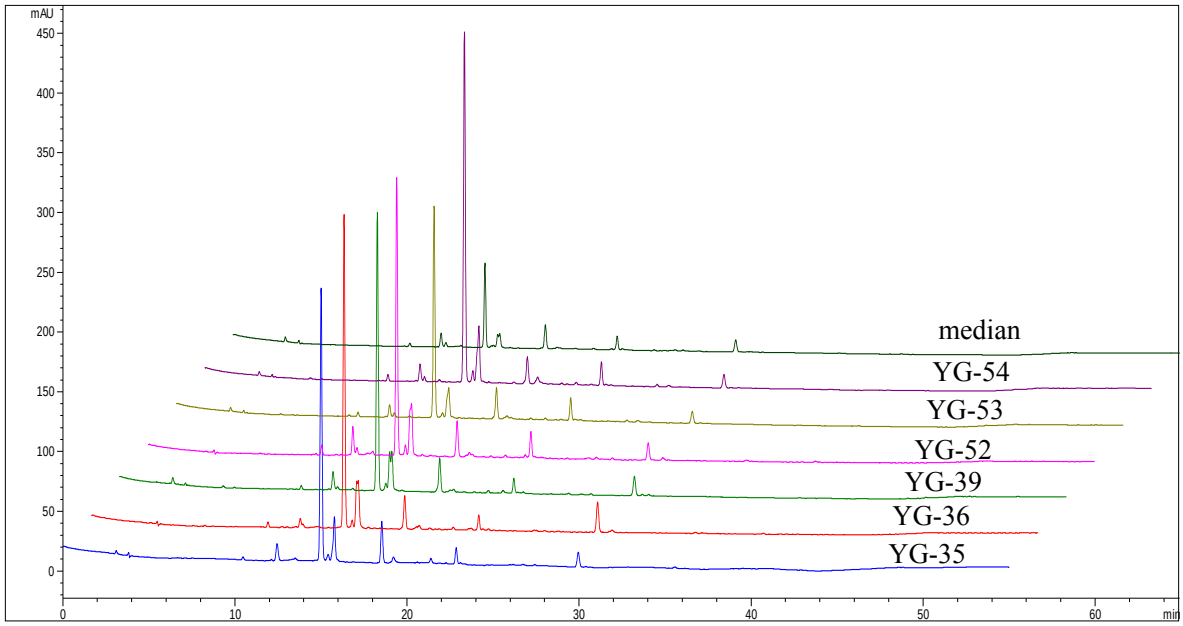


Figure 5.6 The HPLC-UV chromatograms of YG samples with SI values close to 1 when compared with the median chromatographic profile at 254nm

5.4.1.3 Validation of the chemical analyses of YG

As mentioned in Chapter 4, the adequacy of the analytical method could be evaluated from linearity and precision. Referring to Table 5.3, very good linear relationship between the peak area and the concentration of the solution of every chemical reference compounds was found with their correlation coefficients higher than 0.99. Apart from that, the reproducibility and repeatability of our chromatographic method were evaluated by the relative standard deviation (RSD) of the peak area of these selected compounds observed in the HPLC-UV chromatograms concerned. As mentioned before, herbal medicine is a complicated multi-component system, monitoring only the change of selected chemical reference compounds is insufficient for the purpose. Consequently, we carried out the validation by comparing the sameness and difference between the entire chromatographic profiles of the samples with all the detectable components being monitored. The measurements of the intra-day and inter-day variabilities were carried out according to procedures stated in Chapter 4.

For method validation, two YG samples were chosen randomly among the 79 samples. The RSD values of the identified isoflavonoids found in these samples for the intra-day variability ranged from 0.33% to 2.52%. Their SI values were greater than 0.98 among the successive injections of the same YG sample. For inter-day variability, the RSD values were less than 10% and the SI values were greater than 0.97. All these indicated that our chromatographic results were precise and valid (Tables 5.5 and 5.6). Besides, the repeatability of the sample preparation was evaluated via measuring the deviations in the concentrations of the identified components. The RSD values of all the identified components were less than 2%.

Table 5.5 The RSD values of the chemical reference compounds in the selected YG samples for evaluating both the intra-day and inter-day variability

Reference compound	**Intra-day variability (m=3)** [a]		**Inter-day variability (n=5)** [b]	
	YG-27	**YG-29**	**YG-27**	**YG-29**
PU	0.33	0.32	1.39	2.57
DZ	1.79	0.66	9.73	2.75
GE	2.52	1.72	5.96	4.98
DE	1.68	1.79	8.99	2.33

[a] m represents the number of injections
[b] n represents the number of days

Table 5.6 Similarity indices between chromatographic profiles of selected samples for evaluating both the intra-day and inter-day variability

Chromatographic profile	**Intra-day variability (m=3)** [a]	**Inter-day variability (n=5)** [b]
YG-27	0.9820 – 0.9949	0.9951
YG-29	0.9921 – 0.9989	0.9798

[a] m represents the number of injections
[b] n represents the number of days

5.4.2 Quantitative Pattern-Activity Relationship (QPAR) of YG

5.4.2.1 Comparing the antioxidant activity of YG

The antioxidant activity of YG was evaluated and studied by Jian et al. using a model of free radicals mediated damage of red blood cells. The aqueous extract of YG exhibited significant potent antioxidant activity which is due to the high content of isoflavonoid [36]. This antioxidant activity can prevent the cardiovascular disease through the

protection of the cellular system from the oxidative damage. Additionally, the antioxidant activity of YG is contributed by various isoflavonoids instead of a single compound. It was found that the ability in scavenging free radical of YG is stronger than that of puerarin alone [190].

The strength of antioxidant activity of 79 samples was ranged from 39.36 to 2816.73 while the mean antioxidant activity was around 525.79 (Figure 5.7). The antioxidant activity of YG-43 had the highest FRAP value of 2816.73 and was abnormally high compared to the other samples. Yet, its concentrations of the five reference isoflavonoids and the SI value being 0.9657 were not unusual to others also. There may be some unknown reasons (e.g. the sample was contaminated) that led to the extremely high FRAP value. Hence, YG-43 was considered as an outlier and was not included in building up the QPAR modeling in this study. Another sample, YG-2, had the second highest FRAP value of 1246.41. With the removal of YG-43, YG-2 showed distinct antioxidant activity but was not too different from the rest of the samples (Figure 5.8) Therefore, YG-2 was not considered as the outlier.

YG-5 exhibited the weakest antioxidant activity with a FRAP value of 39.63. However, its chemical composition is not significantly different to others as its SI value was 0.9501. But the chromatographic fingerprints of YG-2 and YG-5 were obviously dissimilar each other (Figure 5.3). Less chemical constituents in YG-5 were found rather than in YG-2. Using the chromatographic fingerprint of YG-2 as the reference point, the SI value of the YG-2 and YG-5 pair was 0.7657. This further supports the observation. From the chemical components point of view, the concentrations of all chemical reference compounds except daidzein are relatively lower in YG-5 than those of YG-2 as stated in Table 5.4. The amounts of PU and DZ in YG-2 were about 7 times and 3 times more than those of YG-5. But the antioxidant activity of YG-2 was stronger than that of YG-5 in more than 30 times. This might imply that the antioxidant activity of YG is attributed by various constituents.

Although YG-2 was the most antioxidant active one, it did not contain the highest amount of the five selected chemical reference compounds. The highest concentration of puerarin,

daidzin, genistin, daidzein and genistein were found in YG-41, YG-38, YG-29, YG-9 and YG-18 respectively. But their antioxidant activities were moderate with the FRAP values ranged from 245.41 to 680.68. This clearly showed that the strongest antioxidant activity of YG sample was not induced by the highest amounts of target compounds. Similar result from Spogno et al.'s study supported that the antioxidant power is not directly related to the concentration of the antioxidant in certain way [191]. Although genistein was found to exhibit stronger biological activity than the other isoflavonoids in different studies, the distinct antioxidant property could not be shown for those samples with the highest genistein concentration in this investigation. Comparing the antioxidant activities of those samples with GS, the more effective one is YG-21 but not YG-18. It is interesting to note that YG-21 had the lowest concentration of GS (Table 5.4). This further suggests that the biological activity of YG in this case is based on a group of components instead of a single compound. In a research study, the overall antioxidant effect of a sample was reported to be dependent on interaction of the phenolic compounds and the ratios of the components within [192].

YG-5 had the lowest antioxidant activity level than the rest of YG samples. YG-1, YG-6 and YG-7 also show weak antioxidant activity and their FRAP values were less than 60. Figure 5.3 showed that they had similar chemical profiles when compared with the median chromatographic fingerprint. When considering all the detectable components in these samples, they had similar chromatographic pattern to one other. Despite that, they had very low total contents of all the reference isoflavonoids. Moreover, GE and GS were absent in YG-5, YG-6 and YG-7. Both reference aglycones were not identified in YG-1 as well.

For those samples with similarity indices of 0.99, their antioxidant activities strength ranged from 256.89 to 448.46. Not only their chemical compositions were close to each other, but also they had similar antioxidant properties. On the contrary, distinct differences in the antioxidant strength were observed in the samples in which their chemical compositions were not equivalent to their median sample. For those samples with the SI values less than 0.9, their FRAP values ranged from 367.03 to 1246.41. Based

on these data, the antioxidant activity seems to be strongly correlated to the constituents of the sample. The specific antioxidant activity level is ascribed to the specific combinations of the chemical constituents. This further supported that the antioxidant property did not rely on single or a few compounds.

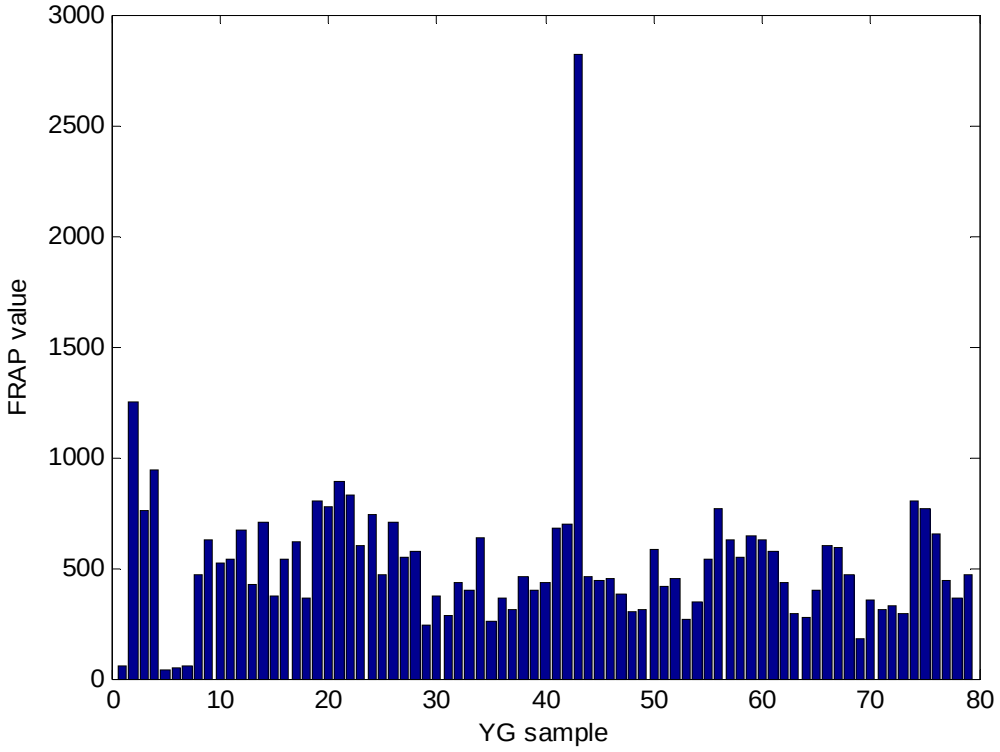


Figure 5.7 The antioxidant activities of all the 79 YG samples

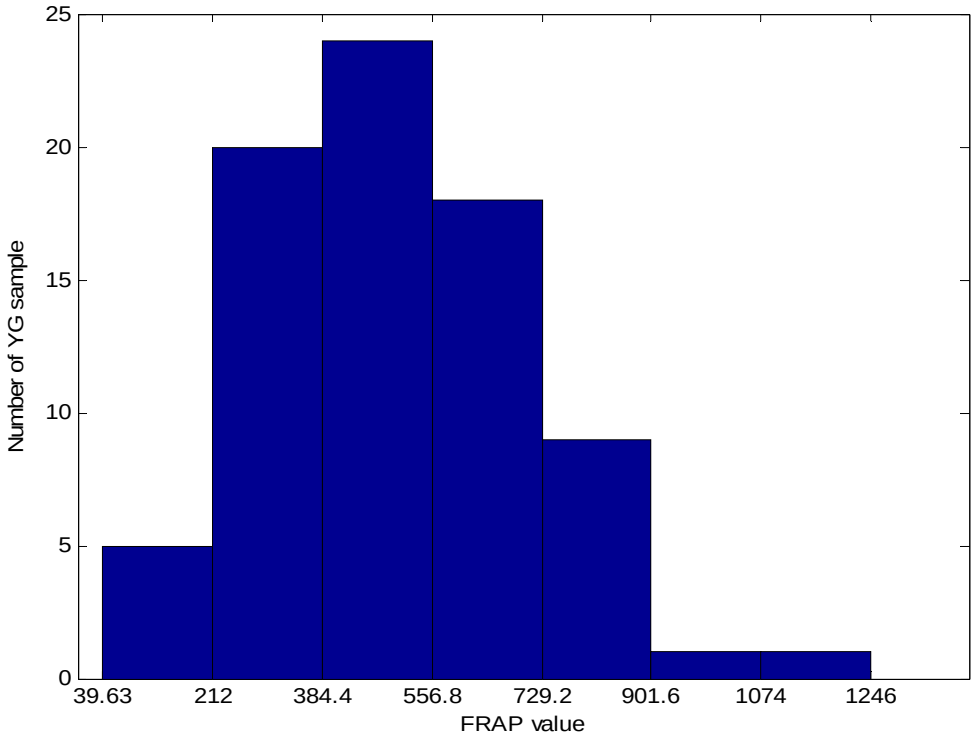


Figure 5.8 Histogram of the FRAP values of the 78 YG samples excluding YG-43

5.4.2.2 Establishment of the Quantitative Pattern-Activity Relationship (QPAR) of YG

It is not necessary that the antioxidant properties of YG samples depend only on the selected reference isoflavonoids as stated above. The antioxidant activity of YG samples also did not increase with the concentrations of our selected reference isoflavonoids. Instead, the combination of both identified and unidentified constituents is significantly related to the strength of antioxidant activity of YG. Based on these, quantifications of a few selected reference isoflavonoids in YG are insufficient for the quality control purpose with bioactivity of the HM included also. In addition, the pharmacological information of HM as well as their respective active ingredients cannot be learned from its chromatographic fingerprint only [10]. It is unable to determine the efficacy of HM based on the quality control approaches that are widely used at present. Consequently, the Quantitative Pattern-Activity Relationship (QPAR) was established in this work through linking the antioxidant activity and chromatographic profiles with the help of multivariate modeling. Partial least squares method (PLS) and uninformative variable elimination by

partial least squares method (UVE-PLS) were applied to attain the goal. Once the relationship was established, the antioxidant activity of a YG sample could be predicted from its chromatographic fingerprint.

Data preprocessing treatment of the chromatographic fingerprint obtained was needed before establishing the QPAR relationship. It involved retention time alignment and background correction. The retention time alignment was done here by correlation optimized warping (COW) as mentioned before [193]. In so doing, the chromatograms were divided into twenty sections for alignment and the maximal shifting of the end point in each section was 10. Also, asymmetric least squares method was applied for the background correction. The apexes of the related peaks from different chromatographic fingerprint were arranged in the same position and the influence of the mobile phase could be reduced after the correction.

The relative standard deviation (RSD) in percentage between the replicate measurements of the antioxidant activity was evaluated with the value less than 5% for all YG samples. Also, the analytical method of YG sample was validated through the RSD of peak area and similarity indices for the intra-day and inter-day variabilities. Thus, the data obtained from both FRAP assay and chemical analyses of YG samples were confirmed to be good for building up the relationship. As mentioned previously, YG-43 was considered as outlier and only seventy eight YG samples were involved in QPAR modeling. They were divided into two groups of the training set and the validation set with 52 and 26 samples respectively according to the Kennard stone method.

PLS is known as all spectrum method which does not require the selection of variables. But the reduction of the dimensionality of the regression space from the predictor variables is needed. That is, the variables of chromatographic profiles are decomposed in different latent variables with maximal variance. At the same time, the regression finds components from predictor matrix that are also correlated to the matrix of dependent variables. In this work, the features of the chromatogram with 1120 variables were reduced into ten latent variables which was determined from the leave-one-out cross

validation. These coefficients were found by least square mean in order to correlating **X** and **y**. It reflected the contribution of the variables to the final model [119, 120]. The plot of the regression coefficient using ten factors in PLS model was shown in Figure 5.9.

All the variables were included in building up the PLS model and gave different contribution to the final model. Based on their computed regression coefficients, the regions located around the retention time of 12.5min, 15.6min, 15.8min and 30.02min in addition to the background absorption showed the largest values. Their values were quite significant when compared with their peak heights. The regression coefficients of daidzin and genistin were relatively high in magnitude but they were not as significant as the other unidentified components. On the other hand, puerarin and daidzein were close to zero. The amount of puerarin in YG sample was quite high but it had relatively low value of regression coefficient. Thus, this meant that the regression coefficient was not directly proportional to the abundance of the variables.

Alternatively, the prediction of the total bioactivity of YG, FRAP in this case, was carried out by using the retained variables in UVE-PLS. In this investigation, the complexity of the model in UVE-PLS was the same as PLS. It is because there is no further improvement of RMSEP in the model using the retained variables and reducing the number of principal component. Twenty random variables were added in choosing the variables that were related to the response. The cut-off value was set at 0.99 in the selection of the informative variables. The variables would be eliminated when the stability of the variable was lower than this threshold value. Here, the PLS model was built up using ninety four retained variables (Figure 5.10) which included background absorption, puerarin and daidzin as well as other unidentified components eluted at 10.6min, 11.6min, 12.5min, 13.2min, 15.4min, 15.6min and 16.1 min. As the random variables added are different, the variables retained will be slightly different for every repeated calculation. In repeating the UVE-PLS procedures, the common variables to be retained would be puerarin, daidzin and the unknown components eluted at 10.6min, 12.5min and 15.6min. Of course, it is unavoidable to select the background variables. Yet, the uninformative variables from chromatograms can be removed.

The regression coefficient of PLS and stability value of UVE-PLS give information about common variables that are significant in modeling and strongly correlated to the antioxidant activity. The compounds eluted at around 12.5min and 15.6min were found from these two models. Among the identified isoflavonoids, daidzin showed relatively high correlation coefficient comparing with the peak height and was selected as retained variable in PLS and UVE-PLS respectively. It could prevent the DNA from the oxidative damage as stated previously and it is significant to the modeling. The corresponding regression coefficient of puerarin was not significant in the PLS model. But it was found to be more important than the random variables as determined from the stability. It was retained for building the relationship. This illustrated the computed stability in UVE-PLS could assist in choosing the variables for the modeling. Particularly, the threshold value of regression coefficient is not clearly defined. Moreover, the antioxidant property was predicted from the multiple variables using PLS and UVE-PLS. It may be referred to the holistic approach of HM in treatment of diseases.

In order to evaluate the prediction performance of the two proposed methodologies, PLS and UVE-PLS, root mean square error (RMSE) for the training set and all samples, root mean square error of prediction for the validation set (RMSEP) and correlation coefficient were determined (Table 5.7). The overall performance of UVE-PLS was better than PLS from the values of these evaluation parameters. The RMSE of the training set of PLS model was 88.07 while that of the training set of UVE-PLS model was 76.88. They were 7.30% and 6.37% to the total range of the FRAP values respectively. Besides, a set of independent samples were applied in validating the established multivariate model. The RMSEP of UVE-PLS was slightly lower than the RMSEP of PLS. Their corresponding values were 79.59 and 79.72. The possibility of overfitting was low although the RMSEP was slightly higher than the value of RMSE using UVE-PLS. It was found that there is no improvement in the RMSEP when the complexity of the model was further reduced.

Besides, the correlation between the estimated values through the two models and

experimental values for both training set and validation set were shown in Figures 5.11-5.14. In general, the values of correlation coefficient for the samples in both training set and validation set were close to one for the UVE-PLS model. Because of these, the establishment of relationship between chromatographic fingerprint and antioxidant activity of YG via UVE-PLS was preferred. The informative variables that were correlated to the antioxidant activity were included in the prediction of the bioactivity from UVE-PLS. Hence, better prediction ability and explanatory power can be demonstrated in this kind of multivariate modeling.

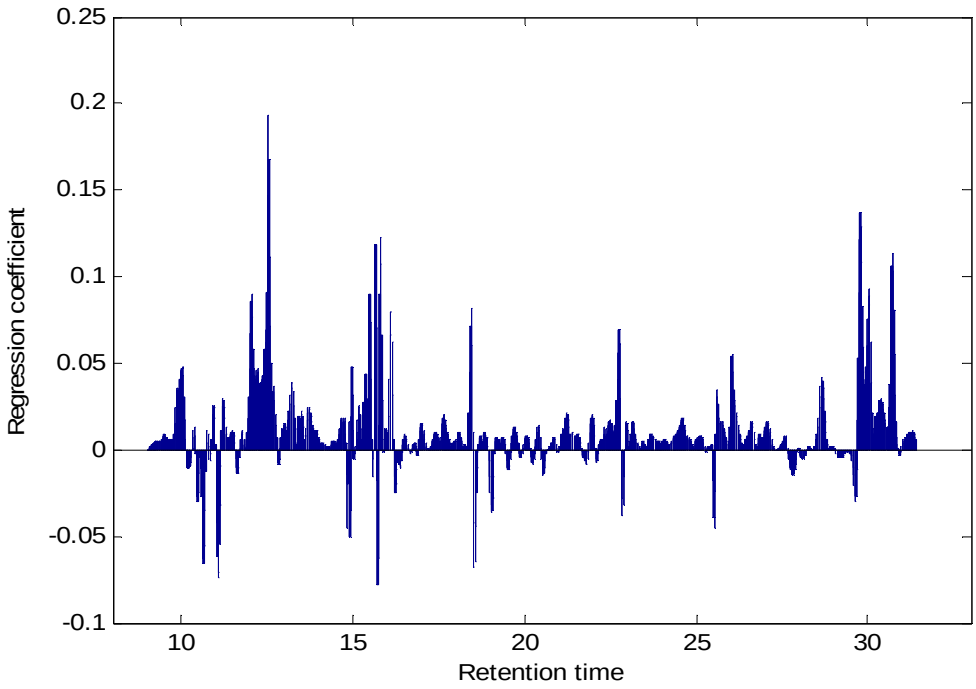


Figure 5.9 The regression coefficients of PLS method obtained from the chromatograms of YG samples with ten latent variables considered in the model

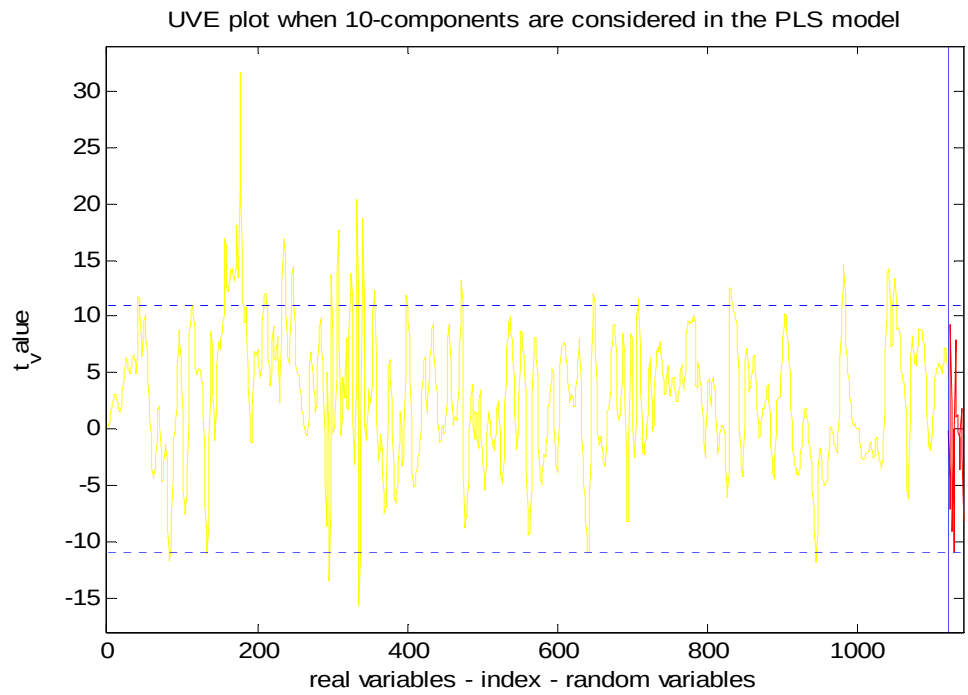


Figure 5.10 The selection of variables with the stability higher than the threshold values using UVE-PLS

Table 5.7 The performance evaluation of PLS and UVE-PLS for both the training and validation sets

Model	**PLS**	**UVE-PLS**
RMSECV	88.07(7.30%)[a]	76.88(6.37%)
RMSE-training	88.07(7.30%)	76.88(6.37%)
RMSEP	79.72(6.61%)	79.59(6.60%)
RMSE-sample	85.38(7.07%)	77.80(6.44%)
correlation coefficient (training set)	0.9112	0.9318
correlation coefficient (validation set)	0.8857	0.8926

[a]Percentage error = (RMSE / total range of FRAP values) X 100%

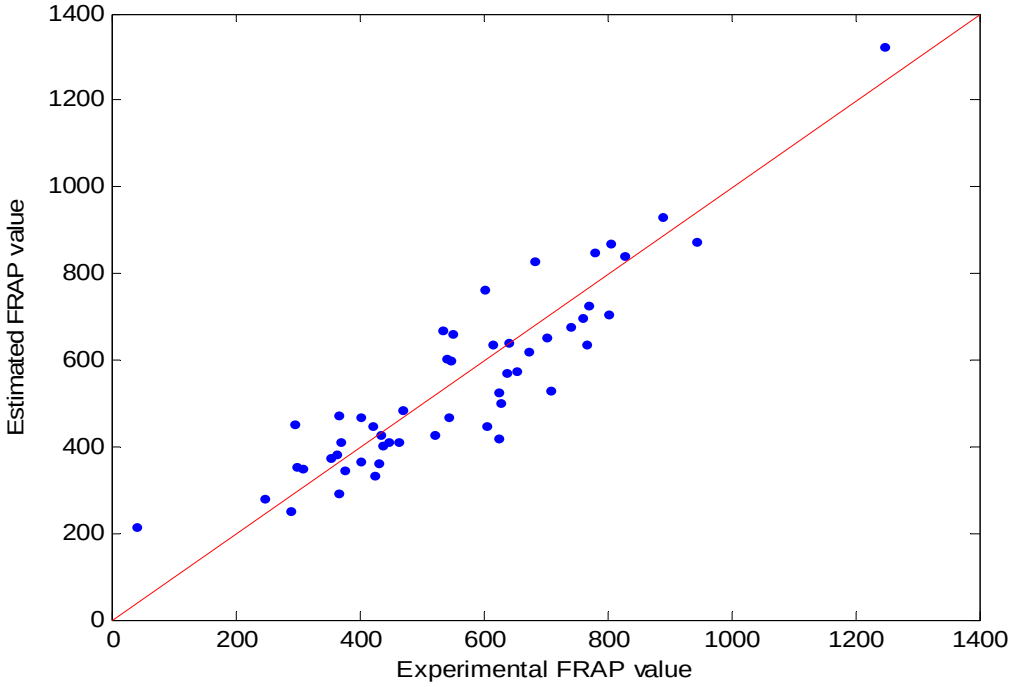


Figure 5.11 Plot of the experimental and estimated FRAP values of the YG samples in the training set utilizing PLS with R=0.9112

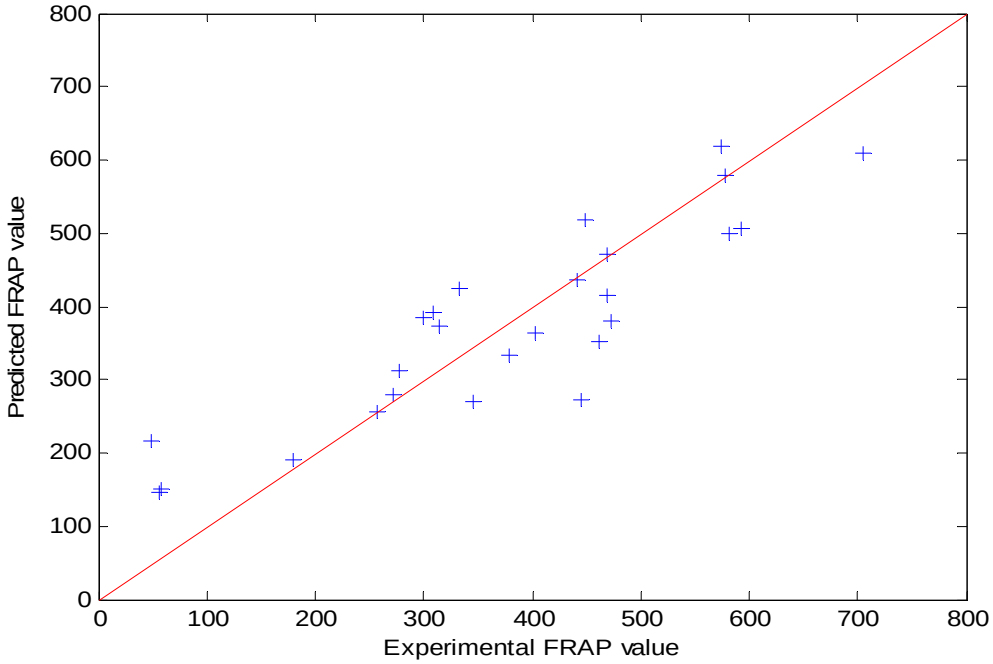


Figure 5.12 Plot of the experimental and predicted FRAP values in the validation set utilizing PLS with R=0.8857

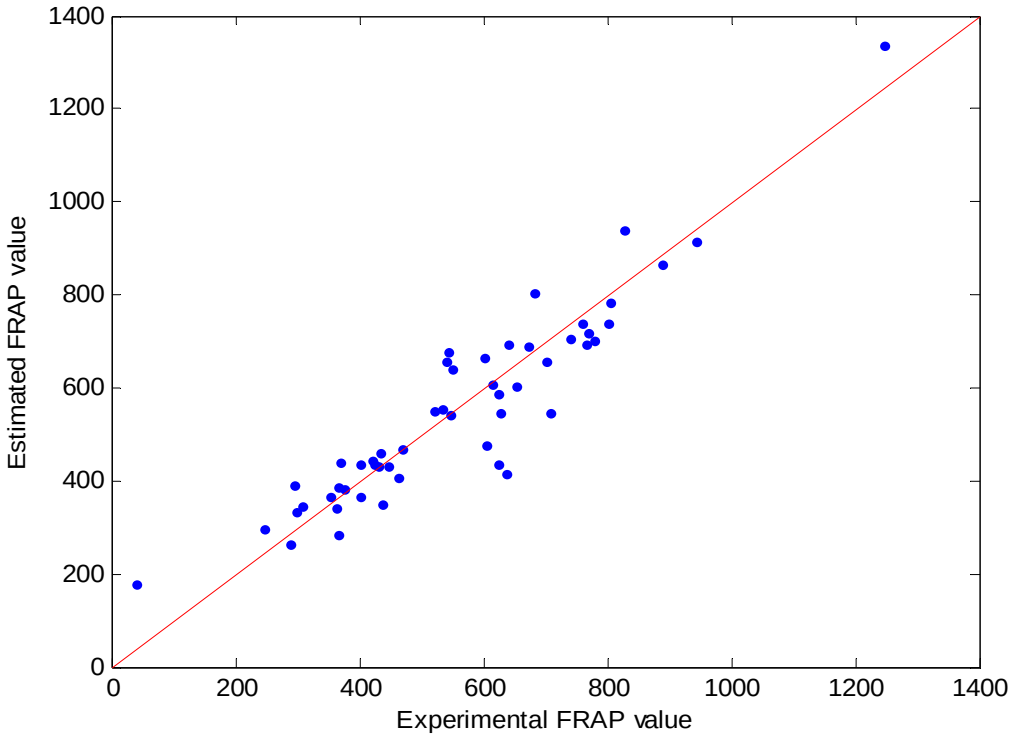


Figure 5.13 Plot of the experimental and estimated FRAP values in the training set utilizing UVE-PLS with R=0.9318

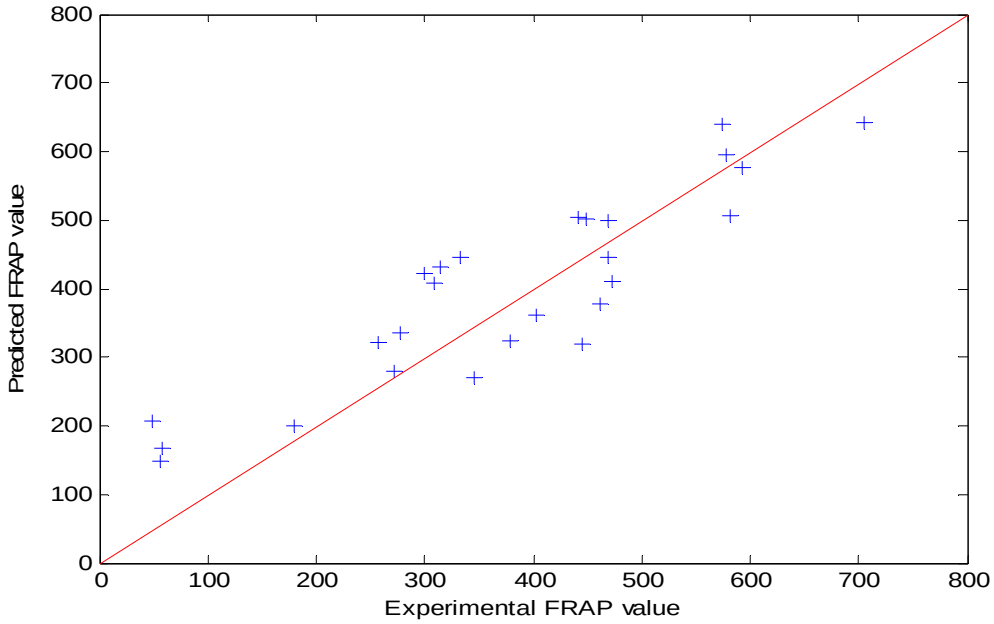


Figure 5.14 Plot of the experimental and predicted FRAP values in the validation set utilizing UVE-PLS with R=0.8926

5.5 Conclusion

Quality evaluation of Herbal Medicine is mainly based on "compound-oriented approach" and "pattern-oriented approach". The former approach focuses on identification and quantification of several selected chemical reference compounds only with no variations of unidentified compounds being considered. Moreover, the therapeutic effect of HM was due to the outcome of multiple ingredients. It is insufficient to authenticate the HM utilizing a few selected reference compounds. Alternatively, the assessment of the quality of HM was carried out through using the whole chromatographic fingerprint representing all the detected components in the latter approach. The change of all these compounds was basically measured qualitatively. Still, the efficacy of HM is not completely confirmed from these current quality control approaches. To cope with this, linkage between the biological activity and chemical composition of HM was constructed in this study with the help of multivariate modeling. In another word, the quality control of HM make possible through our Quantitative Pattern-Activity Relationship (QPAR). Once the relationship is established, the chromatographic fingerprint revealed both chemical constituents and the biological activity of HM.

In this study, authentication of Radix Puerariae Lobatae (YG) was carried out by all these approaches mentioned. In most of the samples, the four isoflavonoids, puerarin (PU), daidzin (DZ), genistin (GE) and daidzein (DE), were identified and quantified. Puerarin was the most abundant isoflavonoid to others in our YG samples. Generally, the contents of the reference compounds varied a lot among these samples. Despite that, the overall patterns of these samples were not deviated prominently from each other. As the unknown multiple components in YG were considered, this indicated that the overall chemical compositions among the YG samples were close to each other.

However, there was a sample, YG-20, that had the similarity indices below 0.8 and the FRAP value deviated from the median value. Among the YG samples, YG-2 exhibited the strongest antioxidant activity while YG-43 was outlier. For those identified reference isoflavonoids, their concentrations in this sample were not the highest. This showed

clearly that it is not necessary to have the highest quantities of the selected components in order to achieve the efficient antioxidant properties. In addition, the antioxidant activity of YG was attributed by the specific combination of the constituents and other unknown components.

Because of this, the relationship of the bioactivity and all the detectable components was established in this study through PLS and UVE-PLS. Both multivariate models were able to predict the antioxidant activity from the chromatographic fingerprint of YG but UVE-PLS could perform better with lower values of RMSE and RMSEP. Consequently, the complete characterization of the chemical compositions of HM in addition to the estimation of the efficacy can be obtained from the chromatographic fingerprints through the QPAR approach.

Chapter 6

Screening the potential antioxidants in Radix Puerariae Lobatae (YG) and the synthetic mixture system (MIX) by Target Projection and Selectivity Ratio (TP/SR) data treatment

6.1 Introduction

Quality assurance of herbal medicine is always important but challenging. It is because the quality of HM would greatly induce the benefits and health risks. The current quality control approaches are mainly based on analyzing the chemical compositions of HM as mentioned before. The quality of a HM is usually measured by the quantities of known reference compounds (markers) or integrity of the chromatographic profiles among the samples. Still, it is insufficient to make certain of the quality. The major problem in "compound-oriented approach" is that the selected compounds may not be pharmacological active. Then, the efficacy of the HM is in question if we focus on monitoring these compounds. It is possible to offer better quality assurance for HM when the bioactivity and chemical composition of HM are linked together [1, 8]. Since HM is a complex system which contains thousand of chemical entities, most of them remained unexplored and very limited knowledge is available. The relationship of the chemical constituents and the bioactivity enables the discovery of the ingredients that are responsible for the biological activity. In this way, it serves the quality control purpose. This could aid in examining the pharmacological mechanism of HM in depth also through the discovered biomarkers and active ingredient. Moreover, the natural product derived drugs have been found to be effective in anticancer, anti-infectives, immunosuppression, and neurological disease therapeutical areas [194]. Because of this, different experimental investigations have been carried out for discovery of the potential drug candidates from HM [9].

Bioassay guided fractionation is a popular technique for finding active ingredients of HM. It is based on repeatable fractionating the herbal extract with bioassay analysis at the same time. Through which, the components that are responsible for the bioactivity could be found [18, 19]. Of course, it is time consuming and labor intensive. Introduction of automated devices helps to certain extent. Apart from that, the use of affinity chromatography such as immobilized DNA or protein could assist in detecting components that bind to biomolecules. It is important to search the components that are capable of binding to the relevant biomolecules to produce the desired therapeutic effect. The compounds that bind to immobilized biomolecules can be determined from the

related biological fingerprint [195]. Despite that, there is a probability of missing the potential candidates due to the selected immobilized biomolecule. Identification of the active components can be done by on-line screening chromatography as well [10, 16, 196]. Measurements of the ability in free radical scavenging and the identification of the components are carried out simultaneously. Up till now, this method can be applied in evaluating the antioxidant properties only. Moreover, comparison of the biological samples like protein, plasma, urine or cell with and without administration of the herbal medicine and the herbal extract itself can be used to determine the possible active components with the help of advanced chromatography [11, 13, 17]. In order to discover the bioactive compounds, the experimental approaches as mentioned above are commonly applied. However, the use of advanced technology in chromatography in addition to special and complicated sample pretreatment process are required.

In this study, we attempt to find out the bioactive candidates in the herbal medicine, a complex mixture system, by making use of the information obtained from chromatographic profiles and relevant bioactivity data without doing any fractionation of the HM extract. As mentioned in Chapter 5, the relationship between the chromatographic fingerprint and bioactivity could be established using our proposed multivariate modeling technique. It is possible to predict antioxidant activity from the chromatographic fingerprint of any samples of Radix Puerariae Lobatae (YG). Once the Quantified Pattern-Activity Relationship (QPAR) is available from this work, we found that the consistency, safety and efficacy of the herbal medicine can be ensured. More importantly, the established relationship or model could help to find out the bioactive candidates that contribute to the bioactivity. This could assist in screening the drug target from thousands of compounds in a HM.

With the application of Target Projection and Selectivity Ratio (TP/SR) here, the possible antioxidant active candidates from YG and the synthetic mixtures (MIX) were explored. YG consists of abundant isoflavonoids. More attention has been paid to isoflavonoid because of their medical importance. Among them, five isoflavonoids were selected as chemical reference compounds in this study for identification and quantification. As the

antioxidant activity was derived from different compounds, other unidentified ingredients may be responsible for the overall antioxidant effect. From the finding of our TP/SR, the bioactive candidates of YG were exposed from all the detectable components. At the same time, fractionation of YG extract was performed in order to support the QPAR prediction. In order to further verify the adequacy of TP/SR, it was also applied to another complex system, MIX, to locate potential antioxidants. The synthetic mixtures were constituted by twelve components from different classes of compounds. In contrast to YG, all the ingredients in this system were known. From chemometrics point of view, it is called white system with sufficient known information for validation and performance evaluation. This could further ascertain and scrutinize the finding of active compounds from the relationship between chromatographic profile and bioactivity.

6.2 Background of synthetic mixtures (MIX)

The synthetic mixtures were prepared by twelve different chemical reference compounds. They referred to phenolic acids, flavonoids, isoflavonoids, triterpenoids and anthraquinone. Each of them had their own specific biological activities and some of them are well known antioxidants. In the following section, the chemical structures and the biological activities of the constituents in MIX will be discussed in details.

6.2.1 Chemical structures of the constituents in MIX

Gallic acid (GA), coumaric acid (CMA) and hydroxybenzoic acid (HB) are phenolic acids. Three hydroxyl group were attached on the benzoic acid to form GA. GA (3, 4, 5-trihydroxybenzoic acid) is polyhydroxyphenolic compound [197], CMA (3-(4-hydroxyphenyl)prop-2-enoic acid) is phenylpropoanoids or hydroxycinnamic acid derivatives and HB is monohydroxy benzoic acid [198].

Flavonoids, quercetin (QT), kaempferol (KF) and rutin (RT), and isoflavonoids, puerarin (PU), daidzin (DZ) and genistein (GS) were also included in composing the synthetic mixtures. Isoflavonoids is a subclass of flavonoids. QT (3, 3', 4', 5-7-pentahydroxyflavone) and KF (3, 4', 5, 7-tetrahydroxyflavone) are aglycone while RT (quercetin-3-rutinoside) is the main glycoside of QT in plant [199, 200]. PU (daidzein-8-

C-glycoside) and DZ (daidzein 7-O-glucoside) are glycoside of daidzein and GS (4', 5, 7-trihydroxy-isoflavone) is aglycone.

The remaining constituents in MIX were from triterpenoids and anthraquinone. Betulinic acid (BA) and ursolic acid (UA) are hydroxyl pentacyclic triterpenoic acid. BA (3β-hydroxy-lup-20(19)lupaen-28-carbonic acid) is lupine-type triterpene [201] while UA (3β-hydroxy-urs-12-en-28oic acid) is oleanan type triterpene [202, 203]. These two are triterpenoids. On the other hand, emodin (ED) (1,3,8-trihydroxy-6-methylanthraquinone) is an anthraquinone derivative [204]. The chemical structures of these chemical constituents are listed in Table 6.1.

6.2.2 Biological activities of constituents

Among the components, phenolic acids, flavonoids and isoflavonoids are referred to the phenolic metabolites which are well known in exhibiting antioxidant activity. These three classes of components constituted the mixtures in majority and they can be found in different natural sources such as tea, coffee, wine, fruit, vegetable and herbal medicine [198-201, 204-217]. The phenolic acids were reported to exhibit anti-allergic, anti-inflammatory, antimutagenic and anticarcinogenic activities [217-219]. Particularly, GA and CMA are potent antioxidants [197, 208].

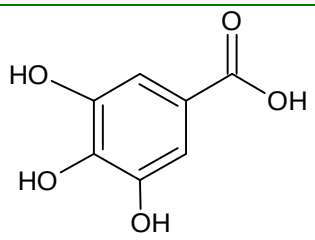
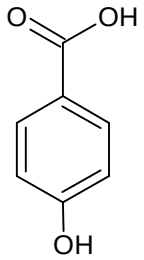
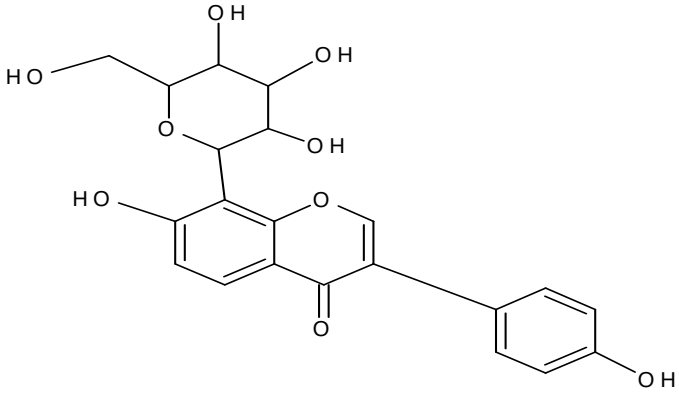
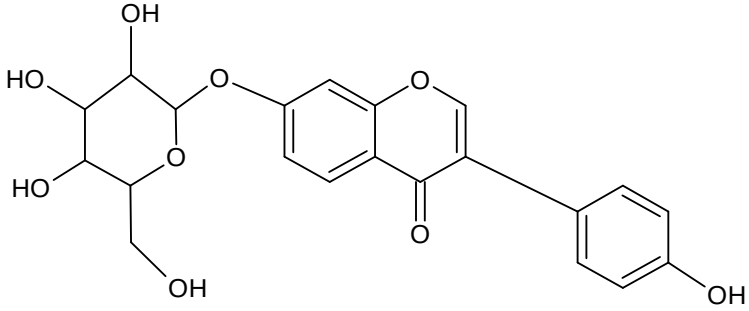
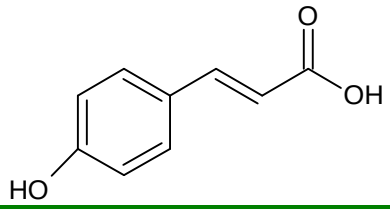
The flavonoids exhibited antioxidant property with inhibitory effects on lipid oxidation, the LDL oxidation and the oxidation of DNA through scavenging the free radicals. Because of these, they are important in treatment of cardiovascular disease and Alzheimer's disease. The risk of ischemia-reperfusion brain injury-related disorders can be greatly reduced [220-225]. Furthermore, they have a variety of biological activities including anticarcinogenic, anti-inflammatory, antibacterial, antiallergic and anti-viral activities [198].

Isoflavonoids have similar bioactivity with the flavonoid compounds such as exhibiting antioxidant activity and anticancer property. The risk of coronary heart disease and Alzheimer's disease are reduced [41, 158, 181]. PU and GA are also able to inhibit the

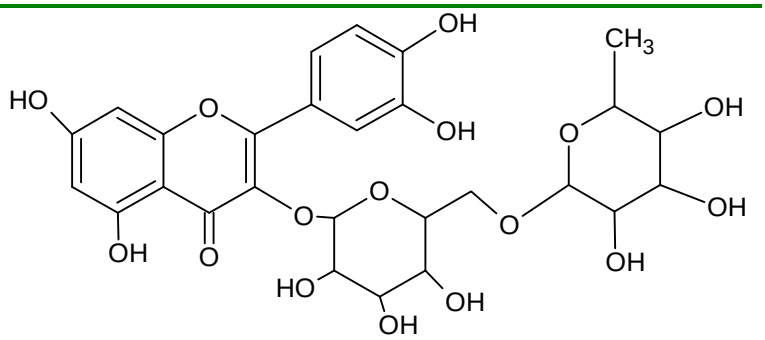
proliferation of the breast cancer cells [172]. Besides, the hazard of osteoporosis and the level of plasma cholesterol can be lowered [40, 42, 182]. Apart from this, DZ has antidipsotropic effect and treatment of the alcoholism [168, 177, 178].

UA, BA and ED exert anti-inflammatory, anti-human immunodeficiency virus (anti-HIV) and anti-cancer activities through inducing apoptosis of cancer cells [201, 203, 204]. Both UA and ED have hepatoprotective effect in addition [203, 204, 206]. Not only this, but also UA exhibits antibacterial activity [202]. Although ED has beneficial effect, it would have toxic effects on the kidney cells [214].

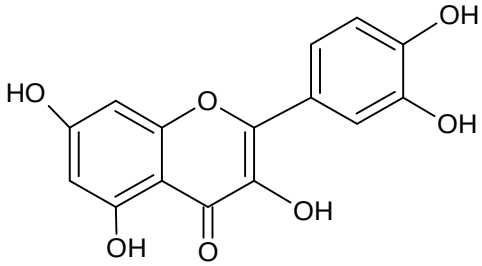
Table 6.1 The chemical structures of the twelve components composing synthetic mixtures (MIX)

Component	Chemical structure
Gallic acid (GA)	
Hydroxybenzoic acid (HB)	
Puerarin (PU)	
Daidzin (DZ)	
Coumaric acid (CMA)	

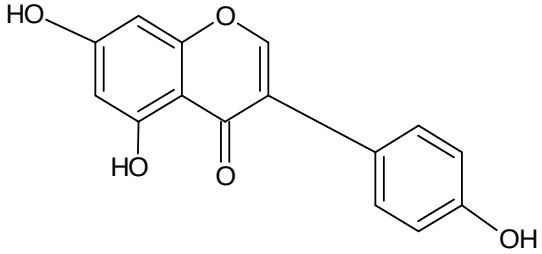
Rutin (RT)



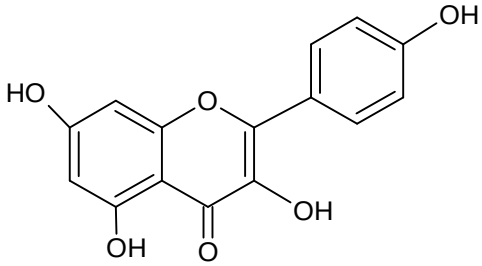
Quercetin (QT)



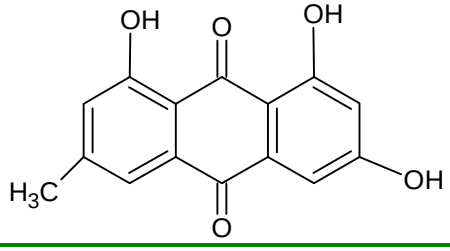
Genistein (GS)

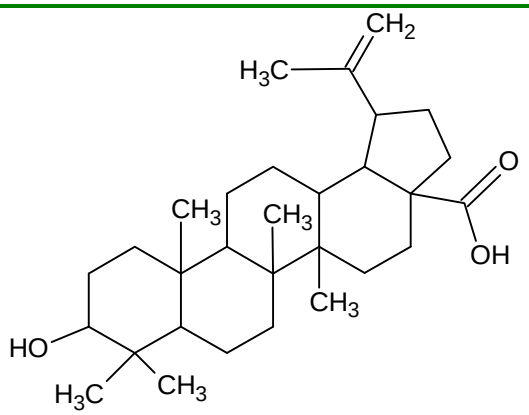
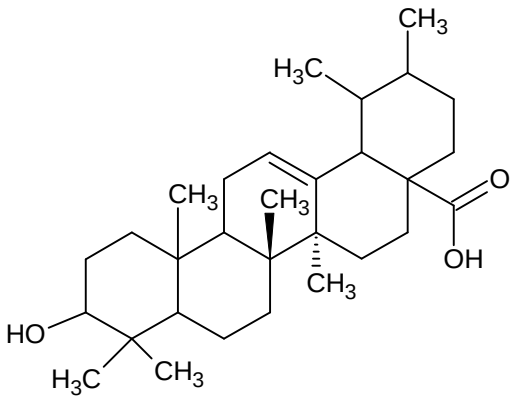


Kaempferol (KF)



Emodin (ED)



Betulinic acid (BA)	
Ursolic acid (UA)	

6.3 Methods of investigation

6.3.1 Target Projection (TP)

Target projection (TP) is one of the multivariate modeling method. It was based on partial least squares (PLS). In PLS, various latent variables represent the combination of the variables and they are related to the response at the same time. One principal component or latent variable is involved in TP in contrast to PLS. Target projection produces a single predictive component by projecting the latent variables decomposition onto the response variable [109]. It can aid in interpreting the model. The target projection model can be expressed in the following.

$$\mathbf{X} = \mathbf{X}_{TP} + \mathbf{E}_{TP} = \mathbf{t}_{TP}\mathbf{p}_{TP}^{T} + \mathbf{E}_{TP} \quad [6.1]$$

The target projected loadings are proportional to the product of vector of regression coefficient and covariance matrix. Both regression coefficient and covariance to variance of the variable must be relatively high in order to obtain the enhanced loading. Thus, the target projected loading combines the prediction ability and the explanatory power. Details about this multivariate modeling were given in Chapter 2.

6.3.2 Selectivity Ratio (SR)

The efficacy of HM is in great concern in these years. Since it is well known that HM is a complex system, the bioactive components that contribute significantly to therapeutic effect are still unexplored in most cases. As mentioned in Chapter 5, contributions of variables on the model can be determined from the regression coefficients and the stabilities that are measured from PLS and UVE-PLS respectively. Other variable selection methods were discussed in Chapter 2. In this investigation, selectivity ratio (SR) coupled with target projection was applied in revealing the potential bioactive components in the complex system [109, 111]. It was found from the explained ($\mathbf{V}_{expl,i}$) and residual variance ($\mathbf{V}_{res,i}$) of the variables.

$$SR_i = \mathbf{V}_{expl,i} / \mathbf{V}_{res,i} \quad [6.2]$$

As described in the above section, the target projected loading of the variables has explanatory power. Making use of this, the ratio of the explained variance and residual variance represents the measure of the variable's ability to discriminate from the rest of

variables. The variable with high value of SR means that it is distinguished from the others. At the same time, the limit was determined by the result of F-test. It aims at selecting the variable without the false selection or losing significant information of the original.

6.4 Results and discussion

6.4.1 Discovery of the potential antioxidants in Radix Puerariae Lobatae (YG)

6.4.1.1 Discovery of the antioxidant active candidates in YG by Selectivity Ratio

As mentioned in Chapter 5, similarities among the chromatographic profiles of seventy-nine YG extracts were evaluated quantitatively by CASE. The results showed that the average similarity indices were above 0.9. That is, the YG samples had similar chemical profiles and overall chemical composition even though the contents of the identified components were different from one another. On the other hand, their antioxidant capacities varied in the range of 39.36 to 2816.73. This represented that the combinations of the components at specific concentration levels were crucial for the antioxidant activity levels of individual YG samples.

Based on the chemical profiles and antioxidant activities of YG samples excluding YG-43, the QPAR relationship was established through the multivariate modeling in this study. YG-43 was considered as outlier. Determination of the antioxidant activities from the chromatographic profiles can be explored. On top of this, it is possible to discover the bioactive candidates using target projection coupled with selectivity ratio. There are multiple methods involved in selecting the variables that are significant to the response [109]. A recent study applied regression coefficient in locating the antioxidant active components in a traditional medicine [226]. The advantage of using TP/SR over the selection based on the regression coefficient and the stability is that it seldom selects the baseline or background absorption. Since PLS model is covariance –based model, the latent variables determined is to maximize the covariance of the variables and response. When the variance of the variables is too large and the correlation to the response is too

small, it would result in a large value of regression coefficient [109]. Also, the cutoff value of the stability in UVE-PLS is based on the random variable which represents the noise. Then, there is a possibility in including the less significant variables from these two parameters.

Here, selectivity ratio was applied to find out the bioactive components of YG. The selectivity ratio is the ratio of total explained variance to the residual variance on the target component. As shown in Figure 6.1, the bioactive candidates of YG were located at (G1) 12.5min, (G2) 13.3min and (G3) 15.6min from the calculation. They were not our selected reference isoflavonoids. Since YG contains abundant isoflavonoids, there is no doubt that other unidentified isoflavonoids compounds were responsible for the antioxidant activity in YG.

6.4.1.2 Verification of the antioxidant active candidates in YG by experimental approach

To support the findings from data analysis, a YG extract was fractionated in this work by utilizing preparative high-performance liquid chromatography into three fractions named as F1, F2 and F3 (Figure 6.2). The first (F1), the second (F2) and the third (F3) fractions were was obtained from 2min to 11min, 11min to 18min, and from 18min to 40min respectively. Among these three fractions, the predicted potential antioxidant active candidates, G1, G2 and G3, were grouped in F2. They were all prepared at the concentrations of 1mg/ml for both chemical analysis and antioxidant assay simultaneously so that comparison can be made on equal mass basis.

6.4.1.2.1 Qualitative analysis of the three YG fractions

It can be seen that the HPLC-UV chromatogram and total ion chromatogram from HPLC-DAD-MS measurement of YG displays multiple detectable components rather than just the selected chemical reference compounds (Figure 6.2). These two kinds of chromatographic profiles were similar to each other which represented the same kinds of components in YG utilizing both diode array detector and mass spectrometer. The same chromatographic analysis was preformed on three fractions as mentioned before. Figures

6.3 and 6.4 depicted the HPLC-UV chromatograms at 254nm of the three fractions and their corresponding total ion chromatograms. It is obvious that the chromatographic profiles of these three fractions resemble closely with the chromatographic fingerprint of whole extract of YG.

In order to confirm the identities of the chemical constituents in the fractions and the original YG sample, matching was done by comparing their retention times, UV spectra and mass spectra obtained from related chromatograms. F1 consisted of unidentified components from 2min to 11min mainly. For F2, three groups of predicted bioactive candidates in addition to puerarin (PU) were detected (Tables 6.2 and 6.3). From the mass to charge ratio reported in the literature, they may be the glycosides of isoflavone. But this required further confirmation by MS/MS study. At the same time, the other reference compounds, daidzin (DZ), genistin (GE) and daidzein (DE) were found in F3 also. These results showed that all these components did not change before and after the fractionation. That is, the chemical compositions of the fractions and the YG sample were the same.

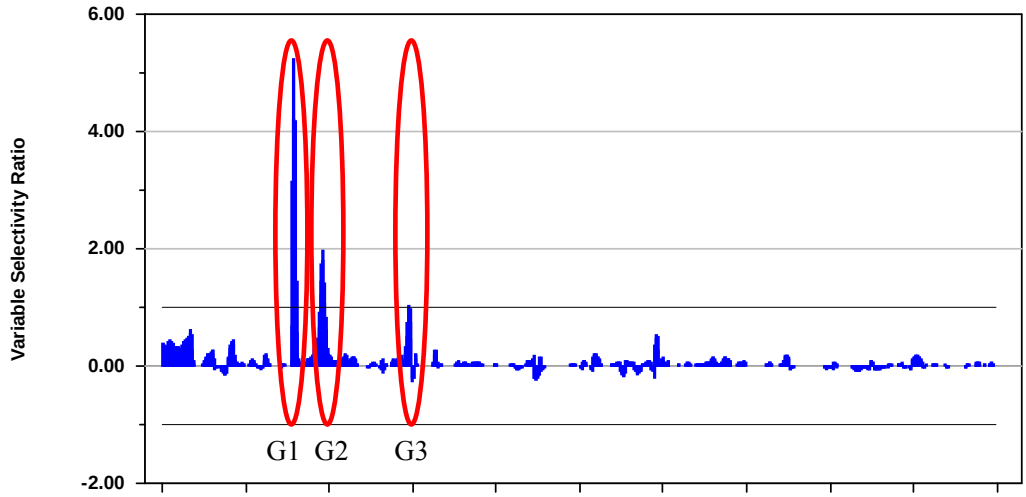


Figure 6.1 The selectivity ratio (SR) of the eluted components of YG at their retention time

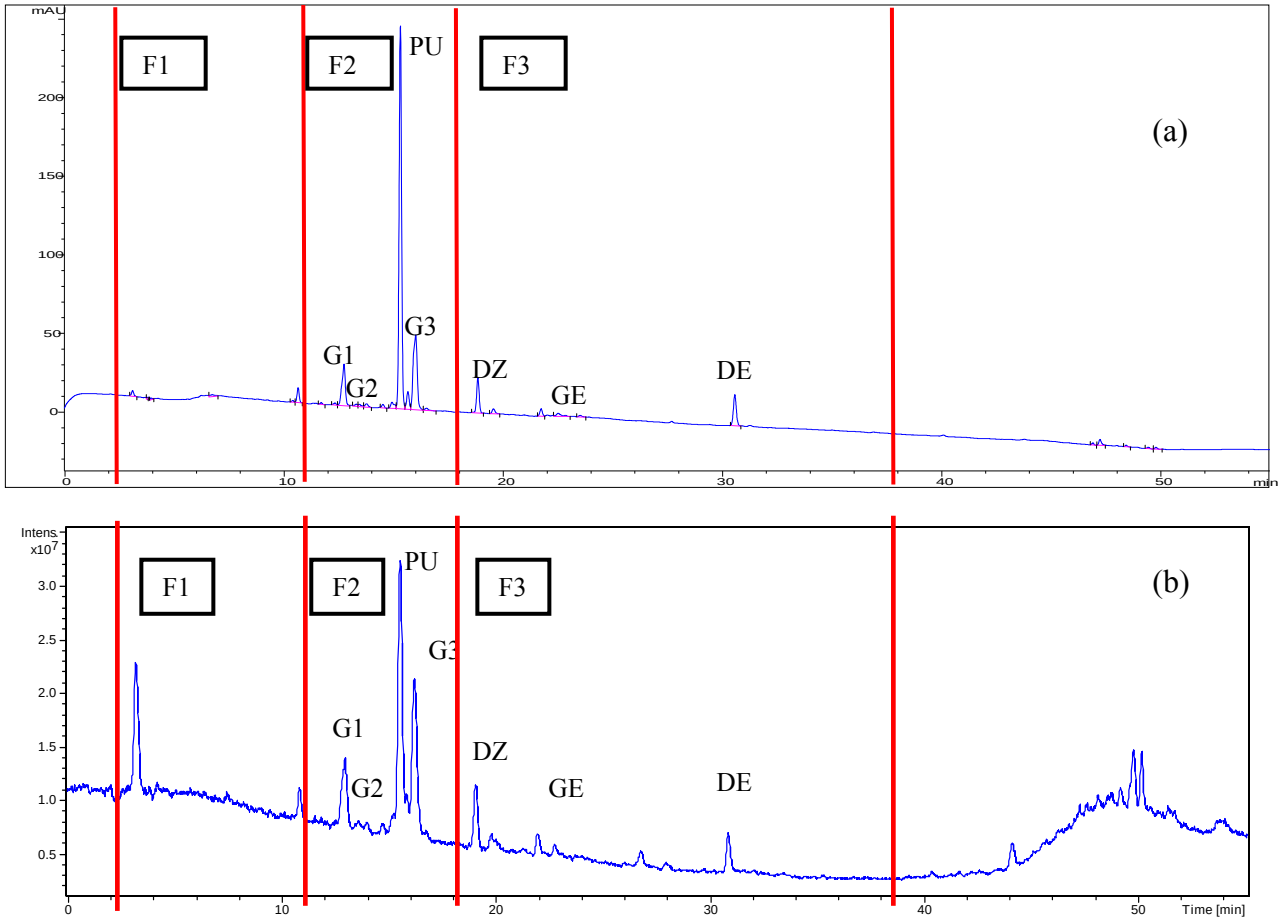


Figure 6.2 (a) The HPLC-UV chromatogram of YG extract at 254nm and (b) the corresponding total ion chromatogram

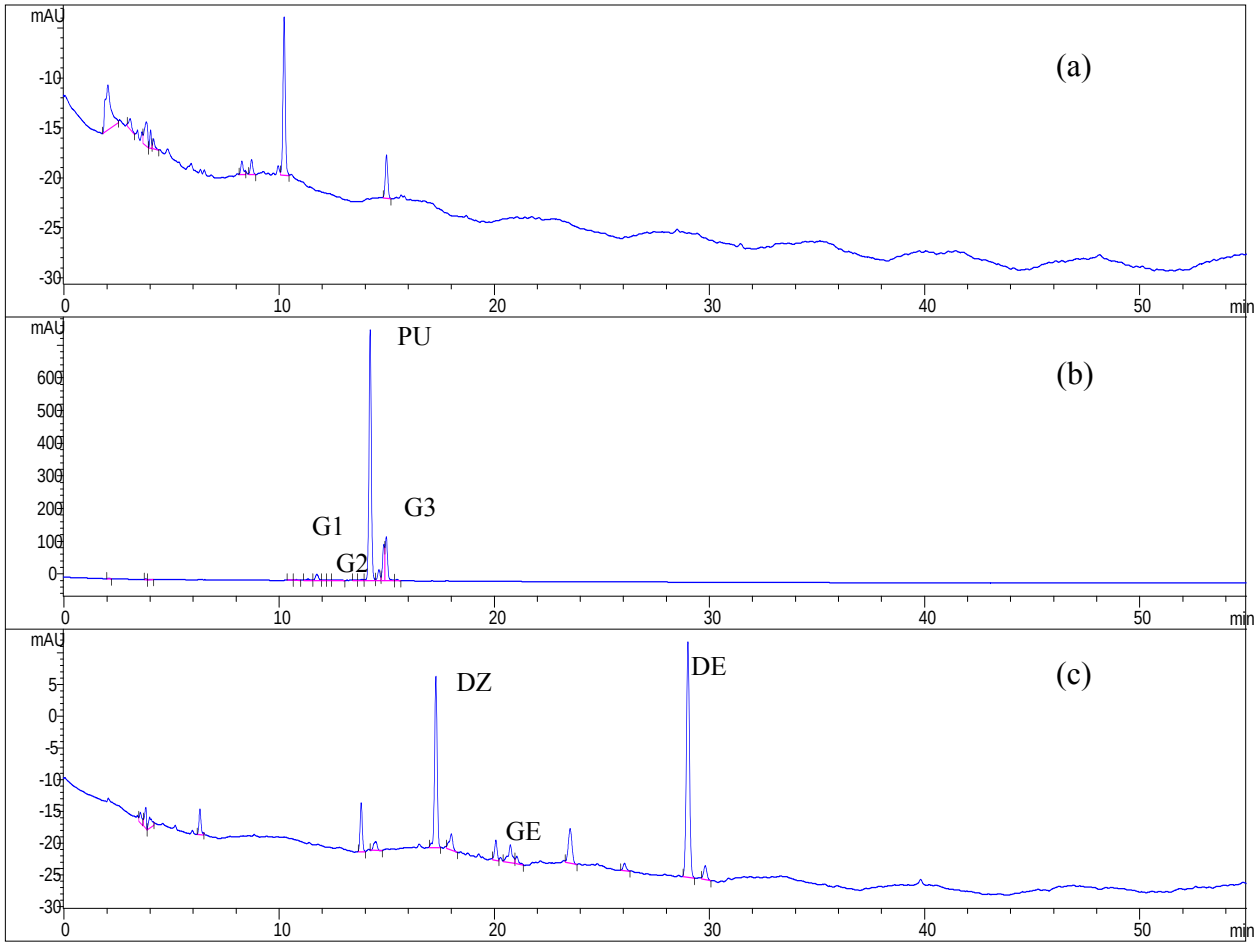


Figure 6.3 HPLC-UV chromatograms of the three fractions (a) F1, (b) F2 and (c) F3 at 254nm

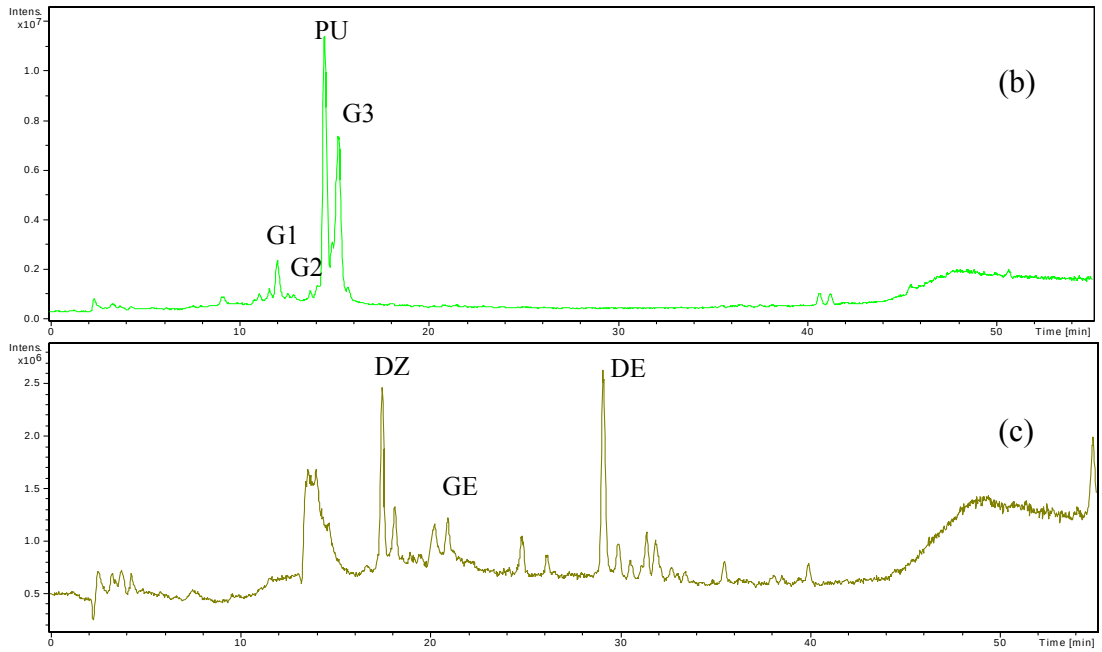


Figure 6.4 The total ion chromatograms of (a) F1, (b) F2 and (c) F3 in positive ion mode

Table 6.2 The UV spectra of the three regions of predicted antioxidants at retention of 12.5min (G1), 13.3min (G2) and 15.6min (G3) in the YG extract and the second fraction (F2)

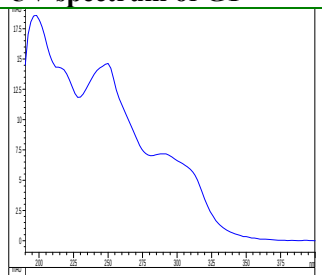
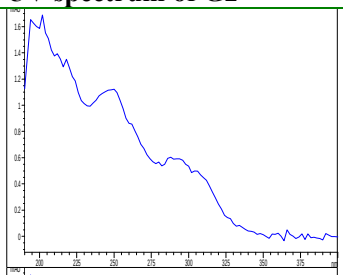
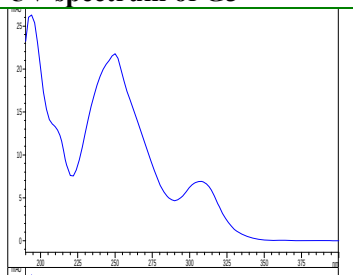
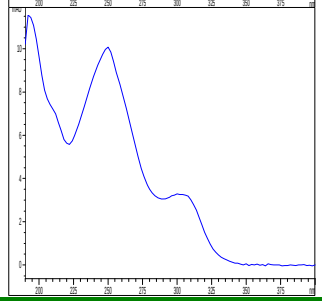
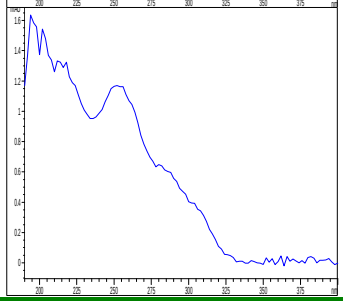
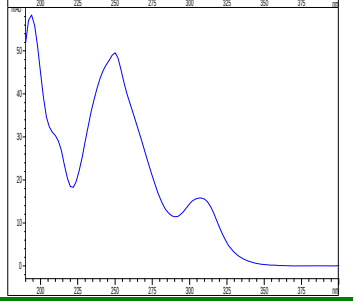
Sample	UV spectrum of G1	UV spectrum of G2	UV spectrum of G3
YG			
F2			

Table 6.3 The mass to charge ratio of the three regions of predicted antioxidants at retention of 12.5min (G1), 13.3min (G2) and 15.6min (G3) in YG extract and the second fraction (F2)

Sample	$[M+H]^+$ of G1 (m/z)	$[M+H]^+$ of G2 (m/z)	$[M+H]^+$ of G3 (m/z)
YG	433, 579	431, 565	447, 549
F2	433, 579	431, 565	447, 549

6.4.1.2.2 Investigation of the antioxidant properties of YG fractions

At the beginning of this work, the antioxidant capacities of the chemical reference isoflavonoids were studied. The quantities of these references, puerarin, daidzin, genistin, daidzein and genistein, differed significantly among the YG samples. The concentration ranges of these compounds were from 9.19ppm to 125.96ppm, 0ppm to 23ppm, 0ppm to 17ppm, 0ppm to 11ppm and 0ppm to 1.1ppm respectively (Tables 5.4). Because of this, the antioxidant activity of each reference isoflavonoid was assessed at its own concentration levels in YG. This could aid in learning the contributions of these compounds to the total antioxidant capacity of YG. In this investigation, all of them were inactive. PU exhibited very low antioxidant activity level with the FRAP value of 26.01 at 125ppm. From the FRAP assay point of view, the reducing power of puerarin would increase with the concentration [186]. Generally, stronger biological activities were exhibited from genistein or daidzein such as anti-proliferation effect or antioxidant activity [42, 188]. As the concentrations of other identified components were low in this test, they were not able to show the bioactivity. Daidzein and genistein were minor metabolites in YG leading to inactive in antioxidant activity. However, a study indicated that the pro-oxidant effect would be exhibited when the concentration of the isoflavonoid is very high [180].

In addition, correlations between the FRAP values and the corresponding concentrations of the reference isoflavonoids in YG were explored in this work. As a whole, the linear relationship between the concentration of individual reference isoflavonoid and its antioxidant activity was very poor with correlation coefficient in the range of 0.0168 to 0.4582. The correlation coefficient of the plots of the FRAP value against the amount of PU, DZ, GE, DE and GS are 0.4582, -0.0541, -0.1212, 0.0168 and 0.2170, respectively. Among the five selected isoflavonoids, it was found that the antioxidant activity of YG is related to the content of the PU component [181]. Based on these, one can conclude that it is not necessary that the extract with the highest amount of these reference isoflavonoids

to have the strongest antioxidant activity. From another point of view, very high content of marker or target compound does not mean that the related extract gives the high bioactivity. This observation is parallel to that reported by Spigno et al. [191]. They found weak correlation between the antioxidant activity and the concentration of polyphenol. Thus, the antioxidant activity of YG did not come from a single component alone.

Here, we also examined the antioxidant property of the mixture of the reference isoflavonoids. It was prepared according to the composition of a YG extract (YG-2). Our result showed that the antioxidant activity of the mixture of our reference isoflavonoids was much weaker than that of the original YG extract. The capacity was only 0.88% to the total antioxidant activity of the YG extract. The reference isoflavonoids were not responsible for the majority of the antioxidant activity of YG. This implied that other unknown isoflavonoids ingredients in YG may be antioxidant active. It was also reported that the isoflavonoids extracted from YG exhibited stronger antioxidant activity than puerarin itself [190, 227]. This may suggest that the synergistic effect of the isoflavonoids leads to high antioxidant activity level of YG. These findings suggest that the three selected bioactive regions with unidentified components as found by our SR calculation might attribute significantly to the YG antioxidant activity.

To further confirm the antioxidant property of the three potential candidates, fractionation was done by preparative high-performance liquid chromatography (PHPLC) as described before. These three fractions possessed different strengths in antioxidant activities. Comparing the antioxidant activities of three fractions, F2 showed the strongest antioxidant activity among the rest of fractions while F1 exhibited the weakest antioxidant activity. This indicated that those unidentified components including G1, G2 and G3 were responsible for the total antioxidant activity. Since these three unknown candidates were found in the most active fraction, this supported to certain extent that our SR analysis is able to reveal the most potential bioactive candidates of YG, a mixture system. Further study is undergoing in this direction.

It is worthwhile to point out again that F2 was the most antioxidant active fractions. In F2,

PU and the potential bioactive candidates, G1, G2 and G3 were found. The quantity of PU was also determined from the calibration curve as mentioned before. PU in F2 was three times more than that in the original YG extract. But the antioxidant activity of F2 was 1.21 times to that of YG whole extract. The antioxidant activity of F2 did not increase in the same proportion with the concentration of PU. Moreover, the poor linear relationship between the concentration of PU and antioxidant activity was shown before. Then, it is less likely that the high antioxidant activity level of F2 related to the concentration of PU in this fraction. Instead, the other compounds such as G1, G2 and G3 were the major constituents in F2 apart from PU. One cannot ignore that the relatively strong antioxidant activity of F2 was due to the presence of these three predicted bioactive candidates.

Usually, aglycone would be more biologically active than glycoside except DE which has low antioxidant activity level in ORAC assay [84]. But F3 which contained DE as well as two other identified glycosides, DZ and GE, showed low antioxidant activity level than F2. These three reference isoflavonoids were unable to display the antioxidant activity at their concentration levels in F3 when they were assessed individually. Moreover, there is lack of correlation between the antioxidant activity and concentrations of these components in the YG samples as discussed before. This said that the antioxidant activity came from multiple ingredients including unidentified components in F3. Furthermore, the contents of DZ and GE were more or less the same in F3 and YG but the concentration of DE was 2 times more than that in YG. Despite that, the resulting antioxidant activity level of F3 was lower than YG extract for four times. Also F1 exhibited much lower antioxidant capacity which was 16.72% to that of YG extract by the unknown compounds. Based on these, the contribution of F2 on antioxidant activity of YG whole extract was quite significant. Additionally, the capacity of the constituents in F1 and F3 on antioxidant activity was less than F2. This further suggested that the predicted bioactive candidates in F2 might contribute significantly to antioxidant activity.

6.4.2 Finding out the antioxidants in the synthetic mixtures (MIX)

6.4.2.1 Development of chromatographic fingerprint of MIX

The finding of bioactive components was also carried out on the synthetic mixture system (MIX) in this work. The mixtures were prepared by different combinations of twelve components that can be found in HMs. As stated in Chapter 3, sixty synthetic mixtures were prepared according to the uniform design table. Then, the antioxidant activity measurements and chemical analyses were done on MIX. The chemical properties of these components in MIX were well known, not the same as that of YG with very little information about its ingredients. This investigation on MIX system can screen in more detail about the applicability and performance of our proposed TP/SR QPAR algorithm. Figure 6.5 shows the chromatographic profile of a MIX at 210nm. It can be seen that all the twelve components were well separated under our chromatographic condition. Their UV spectra and retention times thus obtained are listed in Table 6.4.

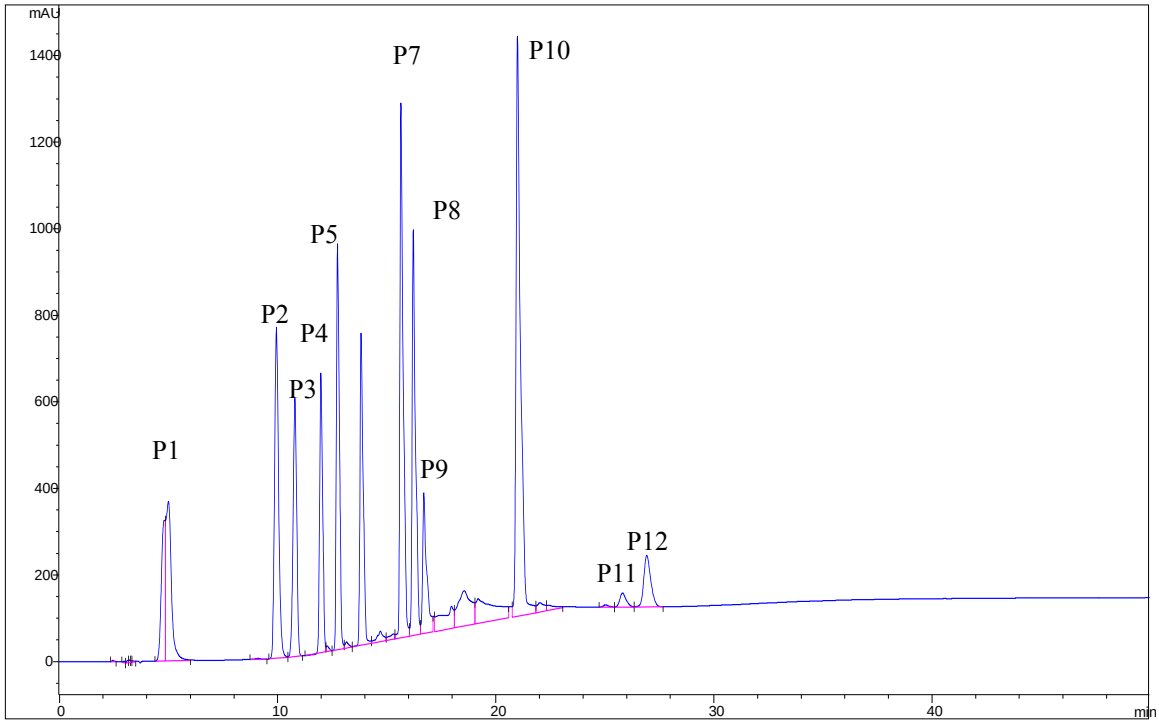
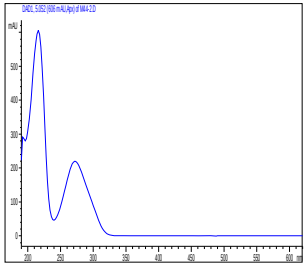
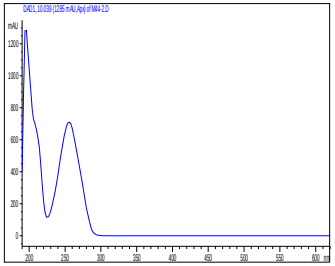
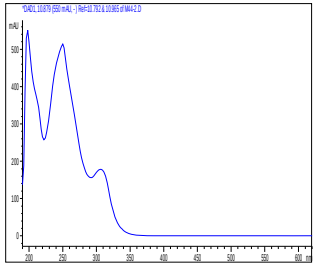
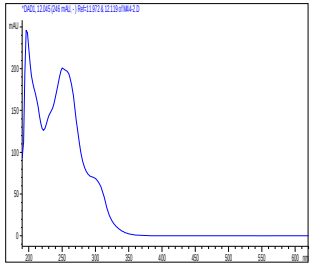
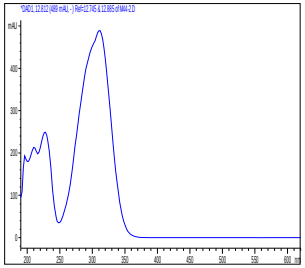
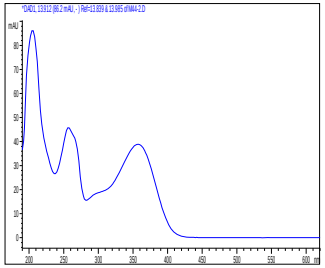
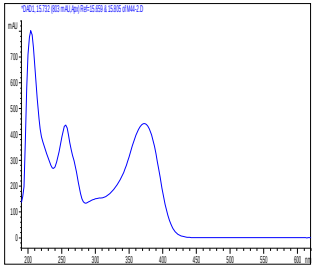
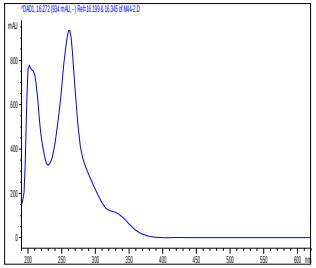
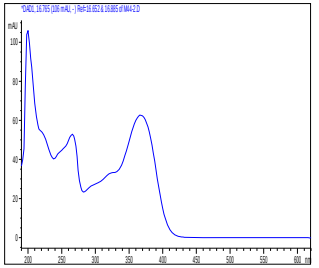
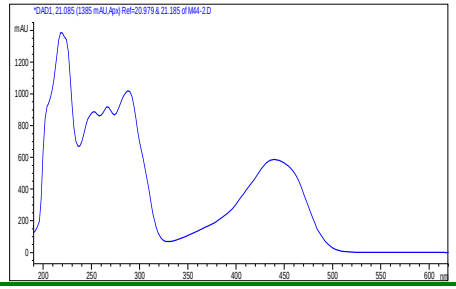
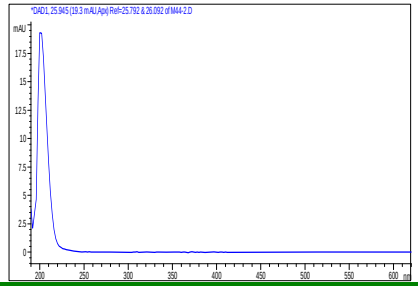
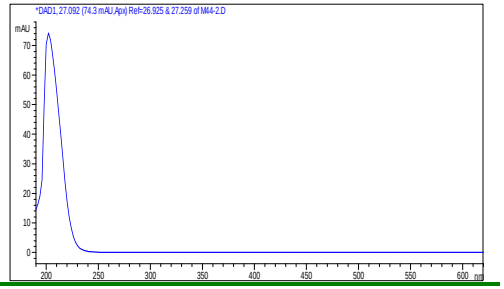


Figure 6.5 A typical HPLC-UV chromatogram of the mixture (MIX 41) with twelve components at 210nm

Table 6.4 The identities of twelve components in the synthetic mixtures (MIX)

Peak	P1	P2	P3	P4
Compound	Gallic acid (GA)	Hydroxybenzoic acid (HB)	Puerarin (PU)	Daidzin (DZ)
Retention time (min)	4.6min	9.9min	10.7min	11.9 min
UV spectrum				

P5	P6	P7	P8	P9
Coumaric acid (CMA)	Rutin (RT)	Quercetin (QT)	Genistein (GS)	Kaempferol (KF)
12.7 min	13.8 min	15.6 min	16.2 min	16.7 min
				

Peak	P10	P11	P12
Compound	Emodin (ED)	Betulinic acid (BA)	Ursolic acid (UA)
Retention time (min)	20.9 min	25.8 min	15.6 min
UV spectrum			

6.4.2.2 Validation of chemical analysis of MIX

Intra-day and inter-day variability were evaluated also in studying the MIX samples. Again, the similar evaluation approach was carried out as that for validating the chemical analyses of DS and YG (see Chapters 4 and 5). The inter-day precision was evaluated by the variations between the three successive injections on the same day while the intra-day variation was performed over five consecutive days. Both intra-day and inter-day variability were determined by the relative standard deviation (RSD) of the peak area and the similarity index among the chromatographic profiles obtained using CASE.

The RSD was found to be less than 1.00% and 10.00% for inter-day and intra-day precision respectively in this investigation (Table 6.5). Apart from this, the similarities of the chromatographic profiles on the same day and on five consecutive days were compared and the similarity indices were computed to be above 0.99 for both intra-day and inter-day variations. Based on these outcomes, our chromatographic analysis of MIX was confirmed to be valid with high precision.

Table 6.5 The RSD values of the chemical constituents in MIX for evaluating both the intra-day and inter-day variability

Chemical component	Intra-day variability (m=3) [a]	Inter-day variability (n=5) [b]
GA	0.51	2.81
HB	0.39	5.11
PU	0.40	5.36
DZ	0.84	6.31
RT	0.41	5.28
QT	0.59	7.15
GS	0.37	5.06
KF	0.72	3.74
ED	0.48	5.31
BA	0.72	5.81
UA	0.58	5.25

[a] m represents the number of injections
[b] n represents the number of days

6.4.2.3 Antioxidant activity of the MIX

Sixty synthetic mixtures were found to have different antioxidant activity levels in the range of 554.58 and 6084.20 with standard deviation within 240.50. This is expected as

their compositions are not identical to one another (see Figure 6.6). Their average FRAP values was 3213.77. Among them, MIX5 exhibited the strongest antioxidant activity while MIX47 was the weakest one. In addition, MIX5, MIX9 and MIX60 were very effective in antioxidant activity which had the FRAP values above 5000. On the other hand, MIX3, MIX25 and MIX47 had the lowest capacity with the FRAP values below 1000 which were much lower than the average values.

Figure 6.7 displays chromatographic profiles of the synthetic mixtures with antioxidant activity levels at the two extremes. There was little retention time shift observed which was caused by the variations in mobile phase composition, column aging and instrumental instability [119, 120]. Also, it should be noted that the chemical compositions were significantly difference between samples with the highest and the lowest antioxidant activity levels. More components were absent in MIX3, MIX25 and MIX47. GA, QT, GS, UA were not found in MIX3. KF, RT, QT and ED were at zero concentration in MIX25. MIX47 had none of GA, QT and ED. On the other hand, those mixtures with high antioxidant activity levels constituted most of the components. Only HB and BA were absent in MIX5. MIX9 and MIX60 contained all the components except PU and HB respectively. At the same time, most of the components in these mixtures were at the high concentration level. The highest concentrations of QT and PU were found in MIX5 and the highest concentrations of GA, QT and BA were found in MIX9. Also, MIX60 had the highest contents of GA and CMA. In contrast, PU, UA and HB were the major constituents in MIX3, MIX25 and MIX47.

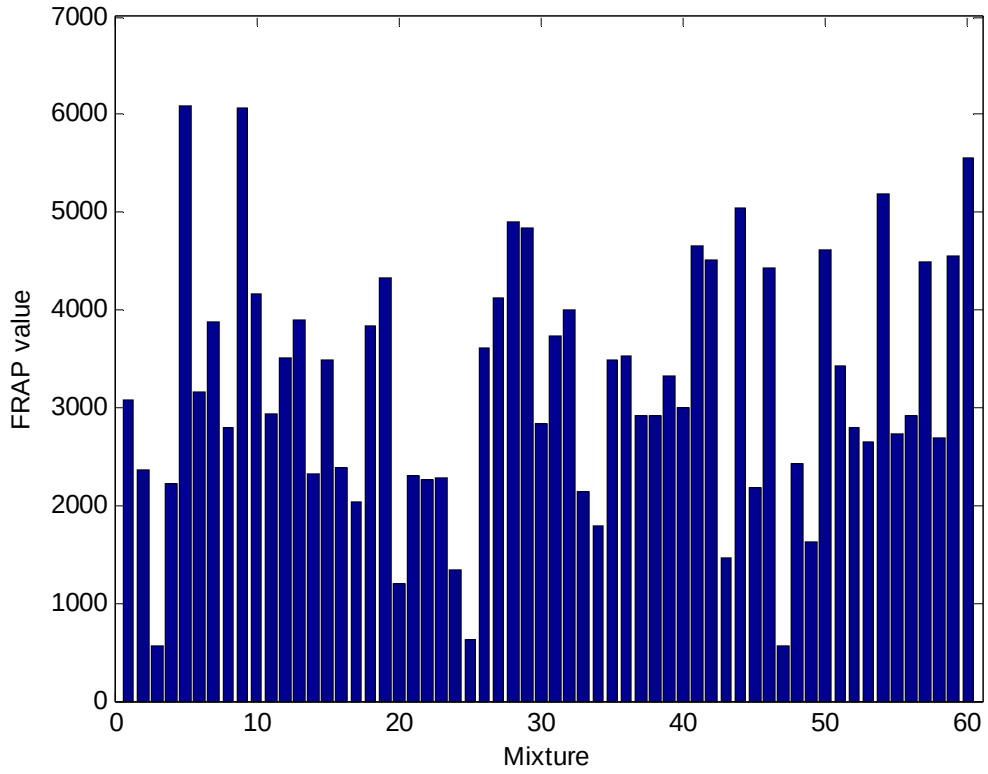


Figure 6.6 A plot of the antioxidant capacities of all synthetic mixtures

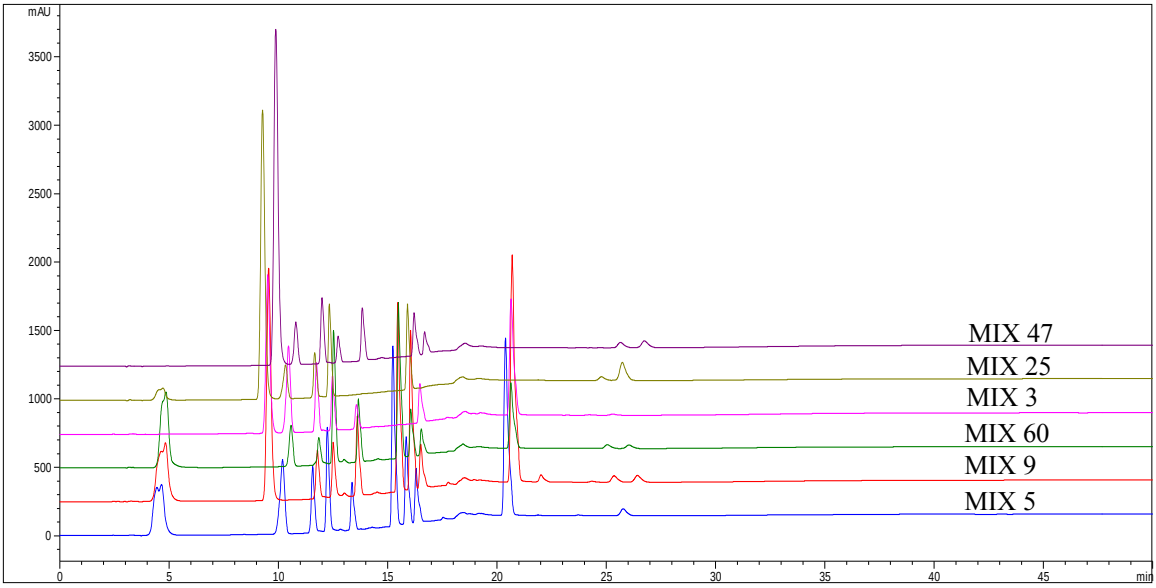


Figure 6.7 The HPLC-UV chromatograms of the synthetic mixtures with the strongest (MIX5, MIX9 and MIX60) and the weakest (MIX3, MIX25 and MIX47) antioxidant activity at 210nm

6.4.2.4 Discovery of the antioxidant active candidates in MIX by Selectivity Ratio

Data preprocessing of the MIX chromatograms was carried out first similar to that on YG. From the established QPAR relationship, the components were revealed from the selectivity ratio plot in the analysis. These potential bioactive candidates were located at retention time of (1) 4.6min and (2) 15.6min (see Figure 6.8). These two regions had the prominent selectivity ratios. That is, GA and QT were discovered as the bioactive components in MIX.

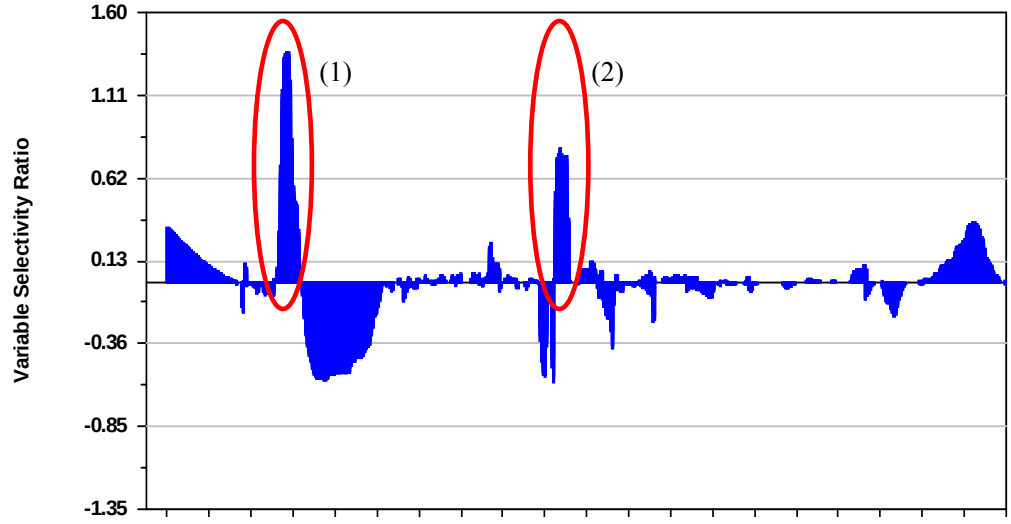


Figure 6.8 The selectivity ratio (SR) of the corresponding variables in the synthetic mixture

6.4.2.5 The antioxidant capacities of individual components in the synthetic mixtures

The components in MIX were the members of phenolic acids, isoflavonoids, flavonoids, triterpenoids and anthraquinone. Here, the antioxidant capacity of each component was measured within their concentration ranges in MIX. Among them, PU, DZ, HB, BA, UA and ED were found to be inactive in their testing concentrations using the FRAP assay although PU, DZ and HB were isoflavonoids and phenolic acids. On the other hand, GA, KF, RT, QT, CMA and GS exhibited the antioxidant activities in different strengths. Still, linear correlation between the antioxidant activity and concentration of the components

were exhibited (Figure 6.9).

In a literature study, QT and KF were reported to possess the strongest antioxidant activity among the twelve components by the FRAP assay. At the same time, the isoflavones exhibited mild antioxidant activity [228]. Similar observation was obtained in our study. Even though the testing concentration range is not the same for each component, the strength in antioxidant activity can be determined from the change of FRAP value with respect to the concentration. That is, the slope of the plot of reducing power against concentration of the component can reflect the strength of antioxidant ability because the linear relationship was confirmed for the active components in MIX. From the slope determined, GA is the most effective one while GS is the least one in this investigation. QT and KF are also antioxidant active. The strength in antioxidant activity of these active compounds can be arranged in the descending order as
GA> QT> KF> RT> CMA> GS

According to the selectivity ratio plot, GA and QT were discovered as the potential bioactive candidates. Considering the experimental observation, the most potent antioxidative components were identified from SR calculation. In other studies, these two components were reported to be strong in free radical scavenging [229, 230], well known antioxidants to have antiproliferation activity on the breast cancer cells [231] and acting as positive control for the antioxidant assays [232].

Phenolic acids, isoflavonoids and flavonoids are well known antioxidants. Phenolic acids including GA, CMA and HB have different antioxidant properties. As mentioned above, GA was the most active one while HB did not have antioxidant activity in our FRAP antioxidant assay. It was not an effective antioxidant in a recent in-vitro assay [233] which may be related to the chemical structures. The monohydroxy group of HB limits its antioxidant activity [198]. The antioxidant activity of CMA was much weaker than GA in this investigation. Another investigation found that the antioxidant activity against the free radical induced low density lipoprotein peroxidation is the lowest among the hydroxycinnamic acid derivatives [233]. Among the flavonoids, RT showed the lower

antioxidant activity level which is caused by the steric hindrance of the sugar moiety. The chemical structure of QT is favorable for the antioxidant activity and demonstrated relatively high activity level [199]. Isoflavonoids are not effective antioxidants in this investigation. GS is the only isoflavonoid in the synthetic mixture system exhibited antioxidant ability at relatively high testing concentration level. PU exhibited antioxidant activity when it was at 125ppm with activity weaker than GS. In addition, other studies also showed that the antioxidant activity of glycosides was reduced when compared with the aglycone [28, 198]. Also, concentration is one of the factors in affecting the antioxidant activity. Otherwise, GS became an inactive component when the concentration is as low as in the YG samples.

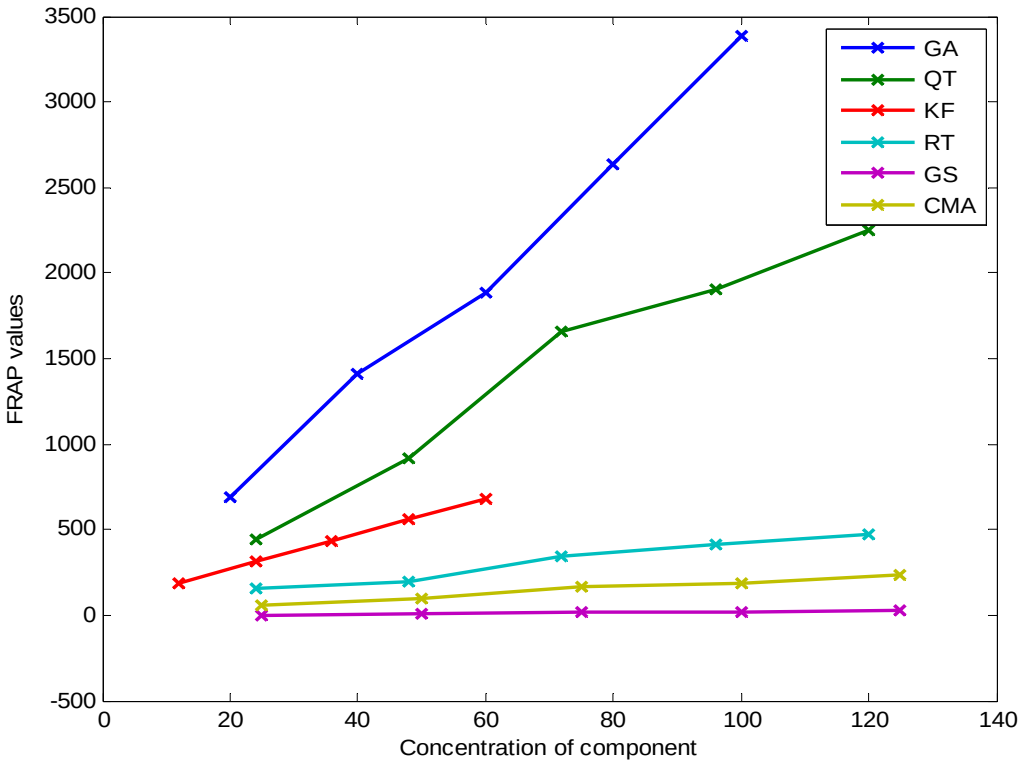


Figure 6.9 Plots of the concentrations of individual active components alone with their FRAP values

6.4.2.6 Relationship of the components on the antioxidant activity of MIX

It is interested in examining how the antioxidant activity of the synthetic mixtures related to the concentrations of the constituents. As mentioned previously, MIX5, MIX9 and

MIX60 were the most active ones with the FRAP values above 5000. These were very active mixtures can be explained by the concentration of the active compounds. For instance, GA, QT and CMA were found at the highest concentrations. Particularly, MIX5 consisted of more GA, KF and QT than MIX 9 and MIX 60 and it had the potent antioxidant activity. On the contrary, MIX3, MIX25 and MIX47 showed the weakest antioxidant activity with the FRAP values below 1000 owing to the lack of active components. The major compounds in these mixtures were PU, UA and HB which are inactive. The most active components, GA and QT were absent in MIX3 and MIX47 and the flavonoids were not found in MIX25. The results showed that MIX3 and MIX47 were less antioxidant active than MIX25. This indicated that the presence of GA and QT were important in the antioxidant activity of the mixture.

Correlations between the contents of the constituents and the antioxidant activities of all the mixtures were studied. It could reflect the contribution of the components to the total antioxidant activity. The activity of the mixtures did not increase with the total concentrations of all the constituents with very low correlation coefficient being 0.2791 only. Then, the antioxidant activities of synthetic mixtures did not relate to the amount of all components. This should be applicable to other mixture system like HM. At the same time, the effects of active and inactive components on the antioxidant property were considered respectively. From further study, relative linear relationship was found between the total concentration of all the active components, GA, QT, KF, RT, CMA and GS, and the antioxidant activities with higher correlation coefficient of 0.6447. On the contrary, there is no linear correlation among the antioxidant activities and the total amounts of all the inactive components. The correlation coefficient has a very low value of 0.0097. This indicated that the antioxidant activities of the mixtures depended very much on the amounts of the active components instead of the inactive ones.

Among the active components, genistein (GS) is the least effective one. Thus, the correlation between all the active components and antioxidant activities except GS was investigated. The result indicated that the antioxidant activities of the mixtures increased with the concentrations of the active components even excluding GS with the correlation

coefficient being 0.7175. This said that the effect of GS on the activity is not significant. In fact, the linear relationship improved when all four most active components were considered. The correlation coefficient obtained was 0.8103. In individual evaluation of antioxidant activity, GA, QT, KF and RT were much stronger than CMA and GS. This implied that the more active components had contributed more to the antioxidant activity of the mixture.

Linear relationship was found between the more active components included in the above analysis. The higher the correlation coefficient value was obtained. The correlation between the three most active components, GA, QT and KF, and the antioxidant activities was 0.9103. With the most active components of GA and QT only, the correlation coefficient of their total concentrations and the total antioxidant activities had value of 0.8988. Here, the relationship of the antioxidant activity of the synthetic mixtures with the concentration of single active components was investigated as well. The results indicated that the total antioxidant activity of the mixture did not increase markedly with the concentration of GA, KF or QT. Their corresponding correlation coefficients were 0.7353, 0.1993 and 0.5928. All these indicated that the antioxidant activity of the mixture is related to the total concentrations of the most active components. More importantly, this implied that the antioxidant activity of mixture is the result of the contribution by the relatively more active constituents.

According to our experimental findings, GA and QT were the most active compounds individually. Moreover, the total antioxidant activities of the mixtures are strongly related to the concentrations of these two most active ingredients. They played important role in the antioxidant activity aspect. At the same time, the most antioxidant active compounds were revealed in the SR plot. Consequently, our study support that TP/SR is capable of finding the possible bioactive components in a multi-component mixture system.

6.5 Conclusion

The quality of herbal medicine (HM) is always in great concern. Recently, there is increasing application of HM on the treatment of disease. It is crucial to guarantee the

consistency, safety and efficacy of HM. But there are certain limitations of the current quality control measures. For example, the efficacy of HM cannot be evaluated with respect to its chemical composition and the reference compounds may not be the bioactive components in the HM concerned. Apart from this, discovery of bioactive components in HM with known efficacy could assist in drug development, examining its pharmacological activity and upgrading the level of quality control. Currently, different experimental approaches have been developed to find out the bioactive candidates from complex systems like HM. However, they are time consuming and require the specific instruments and experimental set ups. In this study, another strategy in discovery of the bioactive components in HM was proposed. The robust method in revealing the potential bioactive components rapidly and accurately was utilized through building up the linkage of chromatographic profile and biological activity. The target projection coupled with selectivity ratio (TP/SR), a chemometric method, was applied to the chemical and bioactivity data of mixtures systems for uncovering the antioxidant active candidates of herbal medicine and the synthetic mixtures.

According to the selectivity ratio plot, three regions of Radix Puerariae Lobatae (YG) were found to be potential antioxidant active. They were not identified as any reference isoflavonoids in our study beforehand. At the same time, fractionation of YG was performed in order to confirm the findings. From our experimental results, the fraction consisted of predicted active components by TP/SR algorithm exhibited the strongest antioxidant activity. In addition, the potential active components in our known mixture system MIX, GA and QT, were revealed by the QPAR method developed here. They were the most active components when they were evaluated individually. Furthermore, the antioxidant activity of the mixture increased linearly with the concentrations of these components as well. Because of these, the detection of the bioactive candidates using target projection coupled with selectivity ratio is applicable to both herbal medicine and a complex mixture system under study. It can be seen that our QPAR approach is very helpful in both drug development and upgrading the quality control level of HM when both the pharmacological property and full set of detectable chemical ingredients are considered.

Chapter 7

Investigation of the relationship of the herbal formula, Danshen-Gegen Tang (DGT) with different combination ratios of Radix et Rhizoma Salviae Miltiorrhizae (DS) and Radix Puerariae Lobatae (YG) through their chemical compositions and antioxidant activities

7.1 Introduction

Herbal formulae which composed of more than one component herb have been used for long long time in healing disease for centuries in China, Japan and Korea. According to the theory of TCM, these herbs are classified according to four elements: Jun (king or master), Chen (minister), Zuo (assistant) and Shi (servant or convey) and they work in harmony together to achieve the purpose [56]. Jun or the master is responsible for the essential therapeutic effect. It is accelerated by Chen or minster and directed by convey to the proper channel or site of action. At the same time, assistant could help towards the accomplishment of Jun [234]. As a result, the beneficial effect of HM can be enhanced while its undesired side effect can be greatly reduced [1]. It was found that patients with infectious diseases and cancer as well have been benefited from the combination chemotherapy with the application of several drugs as the standard of care [235]. Similar concept has been used long time ago for treatment using herbal formula which is commonly applied to handle flu, liver disease, menstrual disorder, cardiovascular disease or relieve pain. For example, Jakyak-Gamcho Decoction has been utilized for relieving abdominal pain [236]. The well known and thorough studied herbal formula, Danggui Buxue Tang, could improve menopausal symptoms [56, 237]. The prescription of Xiao-chia-hu Tang was good for the liver diseases [160].

Both Radix Salviae Miltiorrhizae (DS) and Radix Puerariae Lobatae (YG) could reduce the blood pressure, show lipid lowering effects, exhibit antioxidant activity and improve the microvascular circulation which are important in preventing or healing cardiovascular disease [238]. DS when combined with other herbs like Panax notoginseng, Rhizoma Chuanxiong, Flos Carthami etc. in the Danshen dripping pill and Guan-Xin-Er-Hao served for this purpose [133, 140, 239]. Besides, DS and YG were part of component herb in Xin-Ke-Shu and Jingtongping tablets for protection of cardiovascular system [55, 238]. Another herbal formula composed of Danshen and Gegen mainly and named as Danshen –Gegen Tang (DGT) for healing cardiovascular disease. It was originated in "Zhong Yao Fang Ji Xian Dai Yan Jiu Da Dian" (中藥方劑現代研究大典). They used together in the treatment of atherosclerosis disease (heart attack and stroke) [3, 45].

It was reported that the mixture of Danshen and Gegen in the ratio of 7:3 in water had the ability to modulate key early events in atherosclerosis [44]. The mixture of the characteristic ingredients, puerarin in YG and danshensu in DS in the ratio of 1:1 exhibited antioxidant activity and anti-lipid peroxidation properties. These effects are significant in preventing the cardiovascular disease [45]. Furthermore, a clinical study demonstrated that it was able to have secondary prevention on the atherosclerosis through improving the vascular function and structure without any side effect on the patients. Hence, it is effective as an alternative and complementary medicine [3]. These investigations indicate that the efficacy of DGT is related to its antioxidant property. In fact, the therapeutic effect of herbal formula is affected by the combination ratios of its component herbs. The optimal combination ratio of DS and YG in Danshen-Gegen Tang (DGT) has not been reported so far. In addition, identification of bioactive compounds has not been conducted yet even though the DGT biological activity has been shown. Thus, it is desirable to find them out for further studies.

In this investigation, the chromatographic analysis of DGT was performed by rapid resolution liquid chromatography (RRLC) coupled with a diode array detector (DAD). It is because the characterization of multiple constituents in HM through RRLC-DAD can be carried out rapidly with good resolution and sensitivity. At the same time, the antioxidant activities of different combinations of YG and DS were measured by the FRAP antioxidant assay. The aim of this study is to maximize the antioxidant activities of DGT through optimizing the combination ratios of DS and YG. Furthermore, the correlation between the identified components and antioxidant activity of the DGT formula was investigated. This would provide certain information on the contribution of the components in the DGT.

7.2 Results and Discussion

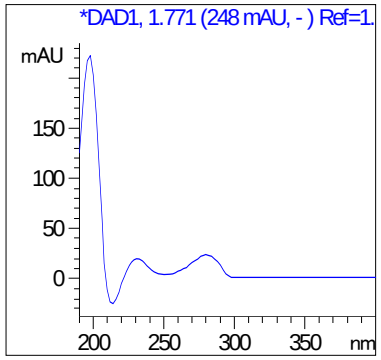
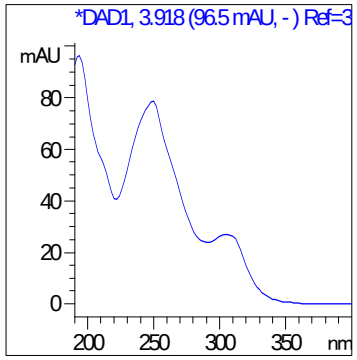
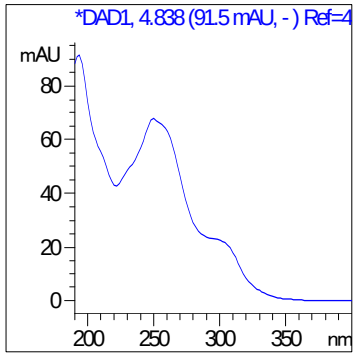
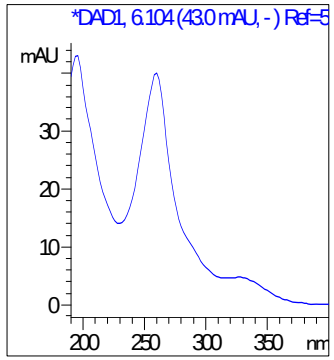
7.2.1 Chemical analyses of the DGT samples with different ratios of the component herbs, DS and YG

Two extraction methods were applied in preparing the DGT which were ultrasound

assisted extraction and boiling as mentioned in Chapter 3. The same extraction solvent, duration of extraction and solid to liquid ratio were set to both extraction methods. DS and YG were mixed in different combinations with mass ratio of 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8 and 1:9 such that the total fresh mass of DS and YG in the formula was the same and equaled to 1g. Throughout this study, the ratios were labeled according to the ratio of DS to YG. Then, DS and YG were extracted together utilizing ultrasound assisted extraction and boiling respectively and the DGT samples thus obtained were labeled as FA and FB accordingly. In parallel, DS and YG were prepared under the same extraction conditions. For example, the ultrasonic extract of DS was named as DSA while the extract prepared by boiling was labeled as DSB. The same labeling applied to YG extracts as well. Instead of having extraction of two component herbs together, another set of samples was prepared by mixing the dried extracts of DS and YG over the same range of ratios as stated above. The mixture of DSA and YGA was known as FA2 and the mixture of DSB and YGB was known as FB2.

RRLC-DAD was applied in analyzing DS, YG and DGT. The analytical time of these three aqueous extracts were greatly reduced to 10minutes compared with the HPLC-DAD analysis which required more than 55minutes for each one. Here, eight target compounds were selected for identification and quantification. They were puerarin (PU), daidzin (DZ), genistin (GE), daidzein (DE), genistein (GS), danshensu (DA), rosmarinic acid (RA) and salvianolic acid B (SAB) which referred to isoflavonoids and phenolic acids. Since the aqueous extracts were examined, the phenolic acids from DS were focused only in our work. Identification was done by matching their retention times and UV spectra with the references and were listed in Table 7.1. At the same time, the quantities of these reference compounds in the extracts were determined by their individual calibration curves (Table 7.2). The quantification of isoflavonoids and phenolic acids were carried out using data acquired at 254nm and 280nm respectively as they have maximum absorption at these two wavelengths.

Table 7.1 The retention times and UV spectra of the reference isoflavonoids and phenolic acids in DGT

Compound	Danshensu (DA)	Puerarin (PU)	Daidzin (DZ)	Genistin (GE)
Retention time (min)	1.7min	3.9min	4.8min	6.1min
UV spectrum				

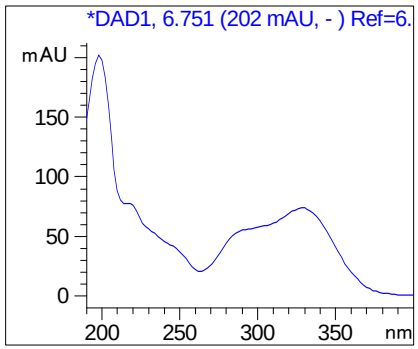
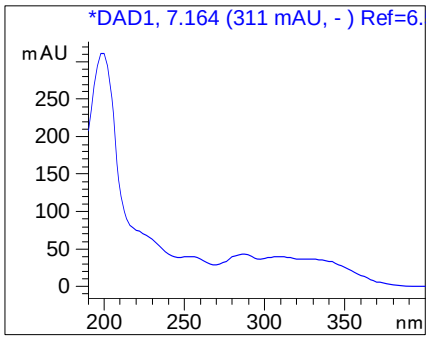
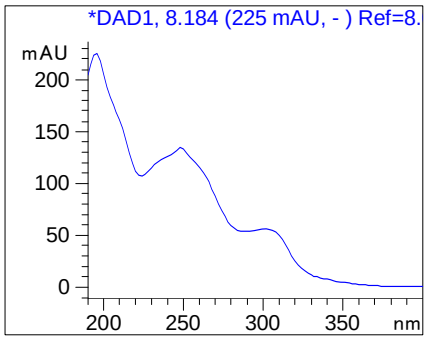
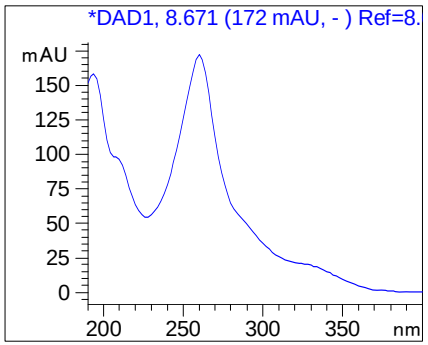
Rosmarinic acid (RA)	Salvianolic acid B (SAB)	Daidzein (DE)	Genistein (GS)
6.7min	7.1min	8.2min	8.6min
			

Table 7.2 The regression equations, correlation coefficients and linear ranges of the selected chemical reference compounds of DGT obtained in this study

Reference compound	Regression Equation	Correlation coefficient	Linear range (ppm)
DA	y = 0.71x-1.41	0.9998	1.50-160
PU	y = 4.28x-17.69	0.9998	0.23-300
DZ	y = 4.88x-6.08	0.9997	0.23-100
GE	y = 5.16x-7.78	0.9994	0.23-100
RA	y = 2.09x-5.52	0.9994	1.50-160
SAB	y = 1.11x-1.10	0.9989	3.75-320
DE	y = 6.90x-8.24	0.9999	0.23-200
GS	y = 7.86x-10.68	0.9998	0.23-300

7.2.1.1 Chemical analyses of DS and YG

The chromatographic fingerprints of DS and YG that were extracted by boiling are displayed in Figure 7.1. All target compounds except genistein (GS) could be extracted by two extraction methods but differed in contents. DA, RA and SAB were in higher concentration level in DSB than DSA. The amounts of PU, DZ and DE were much higher in YGA than in YGB (Figure 7.2). But similar concentrations of genistin (GE) can be obtained in both YGA and YGB. Based on these, we found that ultrasound assisted extraction and boiling were favorable extraction methods for YG and DS respectively. The extraction method is one of the parameter in acquiring the target compounds.

7.2.1.2 Chemical analyses of the DGT prepared by combined extractions of DS and YG at different combination ratios

Figure 7.1 shows the chromatographic fingerprint of FB (6:4) in addition to those of DSB and YGB. Two detection wavelengths, 254nm and 280nm, were selected for the DGT as the maximum absorption of DS and YG were different. It can be seen that the components of the DGT is made up of the components from DS and YG. All the identified components can be found in FB (6:4). The components eluted at retention time from 1.6min to 2min, 6.5min to 7.5min that could be detected in both DSB and FB at 280nm. The peaks from 2min to 6.5min and from 7.5 min to 10min referred to YGB and FB at 254nm. Similar results were obtained for other combination ratios of DS and YG as well as FA. This explained that the FA and FB were the consequence of the combined extracts of DS and YG without the detection of the new components. The major difference among the DGT samples was due to the quantities of the target compounds to be extracted.

The combination ratio of two component herbs is another factor in the extraction of the target compounds with different quantities. PU, DZ and SAB are most commonly found in all the studied DGT (FA and FB). Yet, GE, DE, DA and RA were identified in certain extracts only. Moreover, the average concentrations of PU, DZ, GE, DE, DA, RA and SAB were 42.50ppm, 4.26ppm, 2.05ppm, 2.76ppm, 6.05ppm, 6.93ppm and 22.12ppm respectively. PU and SAB were the major components in the DGT while the rest of them

were the minor ones. Figure 7.2 depicts the contents of each chemical reference compounds in the DGT.

In general, the concentrations of PU and DZ increased with the mass ratio of YG in the DGT samples (see Figure 7.2). Both FA and FB with the ratio of 1:9 has the highest quantities of PU comparing with other combination ratios. In addition, its concentration in FA is much higher than in FB (Figure 7.2). This shows that both the extraction method and mass ratio of YG in DGT affected most on the contents of PU. Similar observation applies to DZ. The highest concentration of DZ can be found in FA (1:9) and FB (1:9) as well. But its content in these two samples were comparable to each other. It seems that the content of PU was affected by the mass ratio of YG in the DGT mostly. Still, the relative amount of DS and YG in the DGT could not be neglected. For example, the DGT with the ratio of 6:4 had the relative high concentrations of PU and DZ. The content of DZ in FA (8:2) was also higher than that in FA (1:1) with higher mass in YG. This might be caused by the extraction efficiency of the two component herbs together. In turn, both the concentrations of PU and DZ could be increased at certain ratios.

The change in composition of the other reference isoflavonoids with the mass ratio of YG in DGT was not clear. GE could be found in the DGT with more YG in mass under boiling at the ratio of 6:4, 4:6, 3:7, 2:8 and 1:9. These extracts contained trace amount of it. It could also be found in FA (7:3), FA (6:4) and FA (1:9) only but not in extracts with other ratios using ultrasound assisted extraction. Despite the extraction efficiency of GE in YG under the two extraction methods being similar, the extraction of GE in the DGT using boiling was more favorable. That is, the combination ratio of DS and YG affected the extraction of GE most. In contrast, DE could be extracted under ultrasound assisted extraction instead of boiling method. It could be identified in most of the DGT with different ratios of DS and YG under ultrasound assisted extraction but it could be found in the two extracts with the ratio of 6:4 and 4:6 under boiling. This indicated that the extraction method and combination ratio affected the amounts of DE obtained.

The contents of phenolic acids did not increase with the mass ratio of DS in the DGT

(Figure 7.2). It seems that the extraction of these two chemical components depended on the combination ratio of DS and YG but not the mass ratio of DS only. As mentioned previously, boiling is the preferred extraction method of DS to yield the optimal amounts of the reference phenolic acids. Hence, the concentrations of DA and RA were relatively higher in the FB samples at certain combination of DS and YG (Figure 7.2). Unlike DA and RA, higher amount of SAB was found in FA instead of FB. All of the reference phenolic acids could be found in the three DGT samples, FA(6:4), FB(6:4) and FB(9:1) (Figure 7.2). Among them, FA(6:4) consisted of the highest amounts of the target phenolic acids instead of FB(9:1). At the ratio of 1:9, FA and FB contained none of DA, RA and SAB. This also suggested that the quantities of the phenolic acids were related to the ratio of DS in the herbal formula. However, the effect was not so dramatic. The influence of the extraction efficiency of DS and YG together was more dominant on the extraction of the phenolic acids.

In summary, extraction method was one of the factors in obtaining the target compounds in the FA and FB samples. Apart from that, the amounts of PU and DZ found in most of the FA and FB samples were related to the mass ratio of YG in DGT. For the other target compounds, the combination ratio of YG and DS influence their quantities to be extracted most.

7.2.1.3 Chemical analyses of the DGT samples prepared by mixing the dried extracts of DS and YG at different mass ratios

In this study, the dried extracts of DS and YG were mixed together in a series of studied ratios. In this way, there was no possible chemical reaction occurred between the constituents of the two herbs during the extraction process. It can be seen in Figure 7.3 that the chemical compositions of FA2 and FB2 were related to the constituents of their DS and YG extracts directly. FA2 and FB2 were the mixtures of DS and YG dried extracts which were prepared under two extraction methods respectively. In addition, there were no additional chromatographic peaks at the detection wavelengths other than 254nm and 280nm. It is impossible that interactions induced among DS and YG occurred during mixing.

In this kind of DGT samples, all the identified components except DZ and GE were in lower concentrations than their contents in DS and YG (see Figure 7.2). Considering those samples with these selected chemical reference compounds, the mean contents of PU, DZ, GE, DE, DA, RA and SAB were 47.17ppm, 5.45ppm, 2.45ppm, 1.87ppm, 3.78ppm, 3.87ppm and 10.01ppm respectively. PU and DZ could be identified over the studied combination ratios and their amounts increased with the ratio of YG in FA2 and FB2. GE and DE can be obtained in most of the DGT especially those composed of more YG in mass (Figure 7.2). At the ratio of 9:1, they were absent in both FA2 and FB2. Additionally, these two compounds were not contained in FB2 with the ratios of 8:2 and 7:3. The compositions of all target isoflavonoids in FA2 and FB2 at the ratio of 1:9 were close to those of YGA and YGB respectively. Also, their contents in FA2 were generally higher than FB2 over the investigated mixing ratios.

Similar result was obtained for phenolic acids as that of isoflavonoids as described above. More DA and SAB were found in FA2 and FB2 with larger proportion of DS. Figure 7.2 shows that DA was absent in DGT samples at the ratio of 2:8 and 1:9. SAB could be quantified in all the DGT samples except FA2 (1:9). Unlikely, RA was found in FB2 samples but not FA2 over the combination ratios studied. In addition, the amounts of phenolic acids were relatively higher in the FB2 samples. DS and YG were extracted by boiling and then dried for mixing in different ratios for this kind of DGT samples. As mentioned previously, the quantities of phenolic acids in DSB were generally more than those of DSA. This means boiling was a more favorable extraction method for DS. Because of this, less DA and SAB and none of RA can be obtained in FA2 samples. Moreover, the content of DA in the dried extract of DS was quite low and leads it to be not detected in the DGT with low mass ratio of DS. Similar result was got for the rest of phenolic acids.

In general, the contents of the targeted compounds in this kind of sample depended on the mass ratio of YG or DS. At the same time, it is strongly related to the individual extraction efficiency of two component herbs. That is, the compositions of FA2 and FB2

samples were totally related to the constituents in the dried extract of DS and YG.

7.2.1.4 Comparison of the compositions between the two different kinds of DGT samples

At the two selected detection wavelengths of 254nm and 280nm, the chromatographic patterns (Figure 7.4) of FA2 and FB2 were similar to those of FA and FB. The constituents of these kinds of samples are the results of the combined constituents of DS and YG together. That is, no new detected chemical components were formed during the extraction or mixing process. Furthermore, the yields of the reference components in the DGT samples with both kinds of sample preparation were usually not as high as that of DS or YG. But the amounts of the identified components in these two kinds of DGT varied from each other.

By comparing each reference isoflavonoids, the concentrations of all glycosides, puerarin (PU), daidzin (DZ), and genistin (GE) in FA or FB were lower than FA2 or FB2 mostly. Opposite result was got for that of DE, the aglycone of PU and DZ. The contents of PU and DZ are closely depended on the mass ratio of YG. But the combination effect of DS and YG dominated the extractions of genistin (GE) and daidzein (DE) in FA and FB samples. Instead, the contents of the isoflavonoids depended on the mass ratio of YG in FA2 or FB2 samples. Referring to Figure 7.2, the elevated quantities of GE and DE in both FA2 and FB2 with more YG in mass ratio were shown.

More phenolic acids were found in FA or FB samples when compared with FA2 and FB2 samples. Also, the linear relationship between the concentrations of the phenolic acids in FA2 or FB2 with the mass ratio of DS was illustrated. However, no such kind of relationship was found in FA or FB. Therefore, this suggested that the extractions of the target phenolic acids in FA or FB depended on the extraction efficiency of DS and YG simultaneously while preparing DGT. On the other hand, the quantities of these components in FA2 and FB2 were related to the mixing ratio of DS and YG. As a result, extraction of DS and YG together would enhance the extracted amount of phenolic acids while those of isoflavonoids dropped.

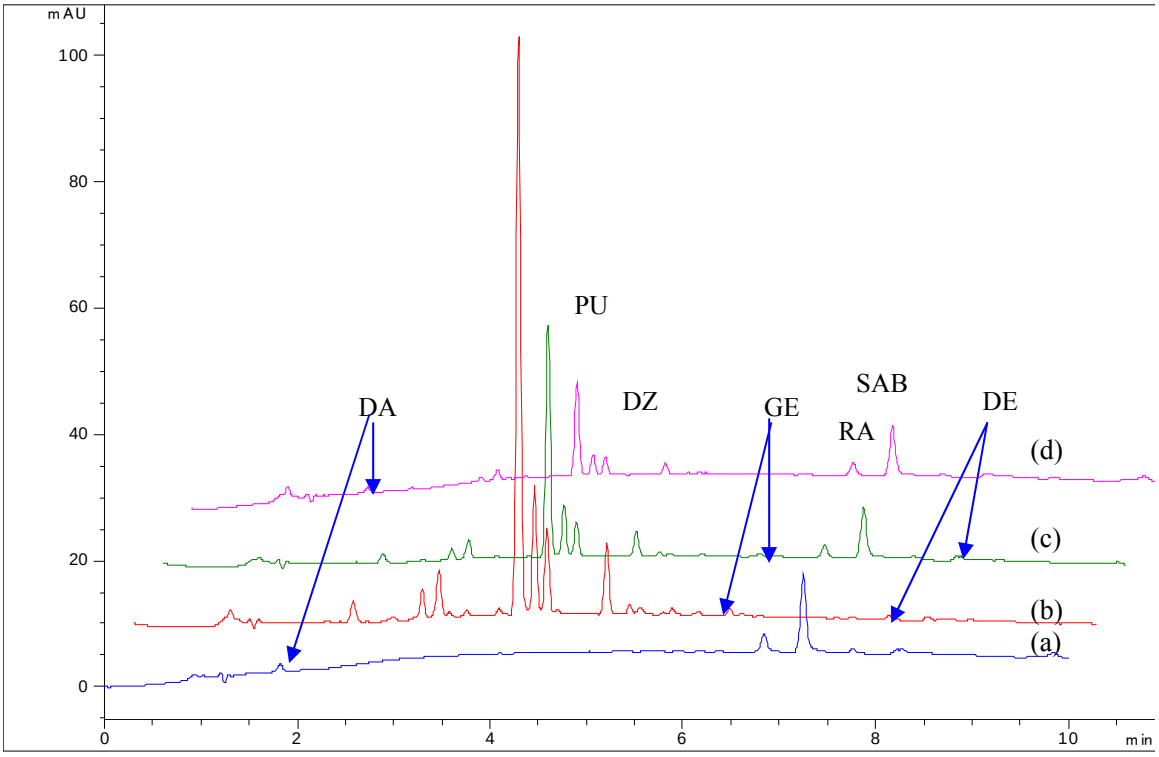
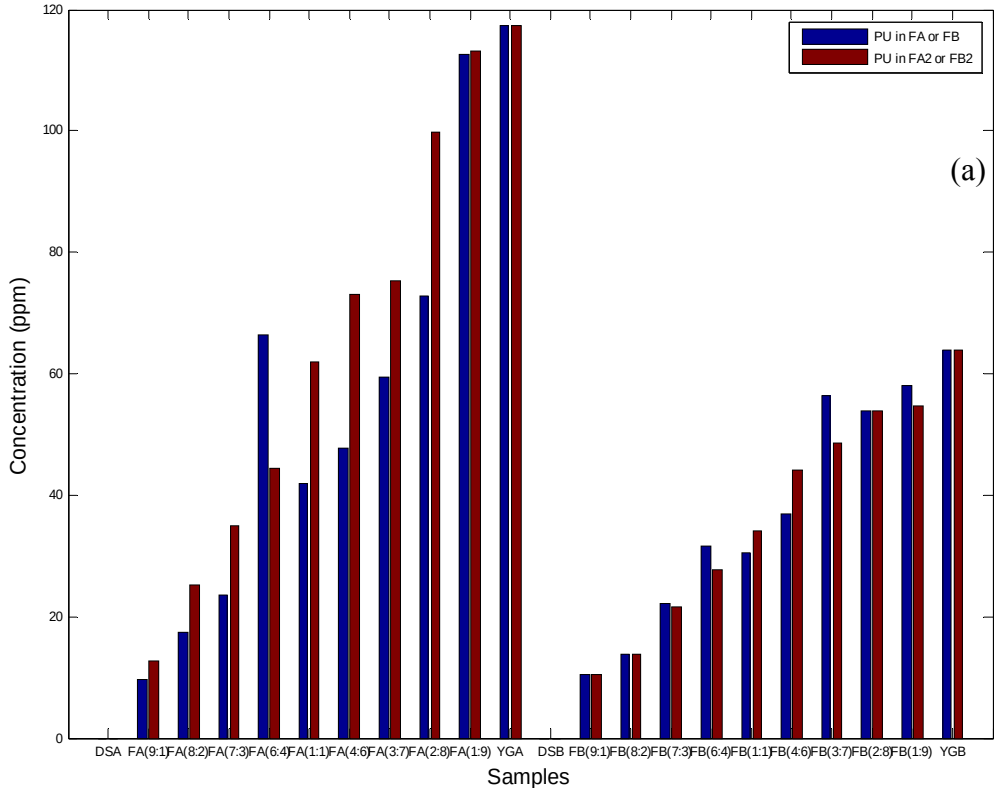
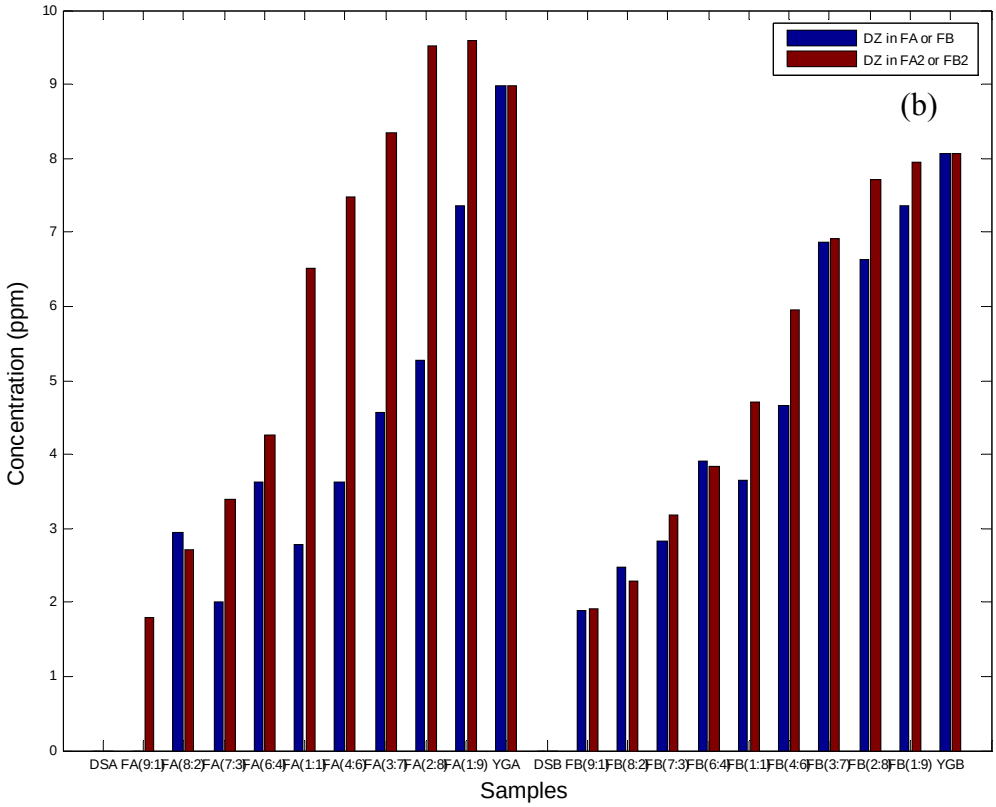
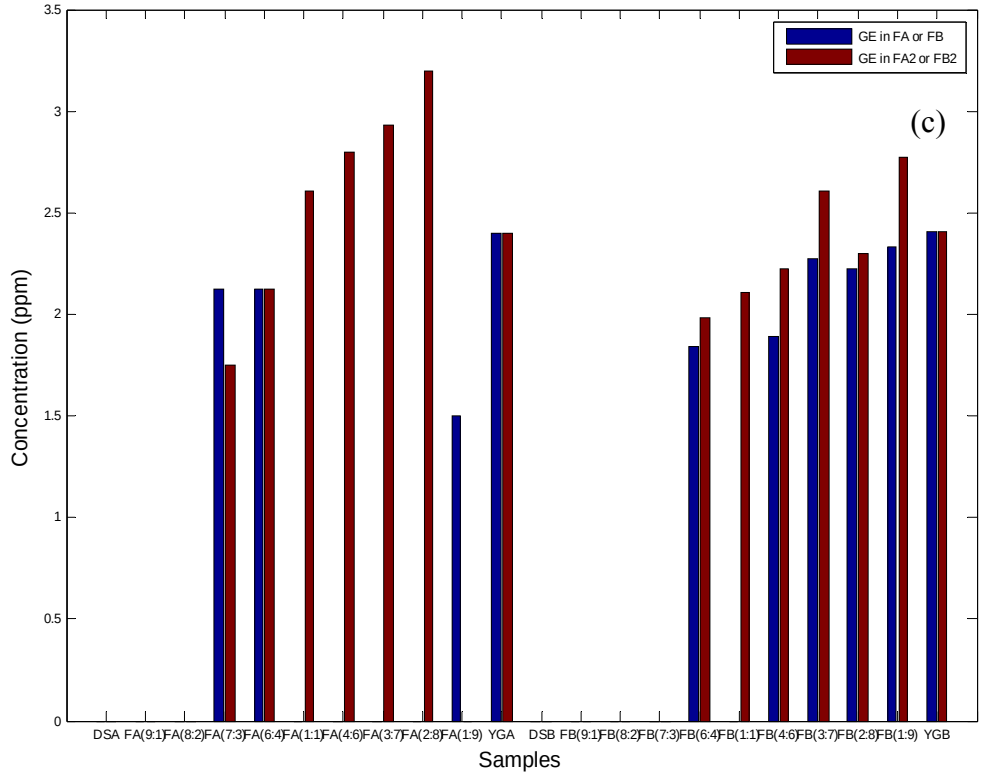
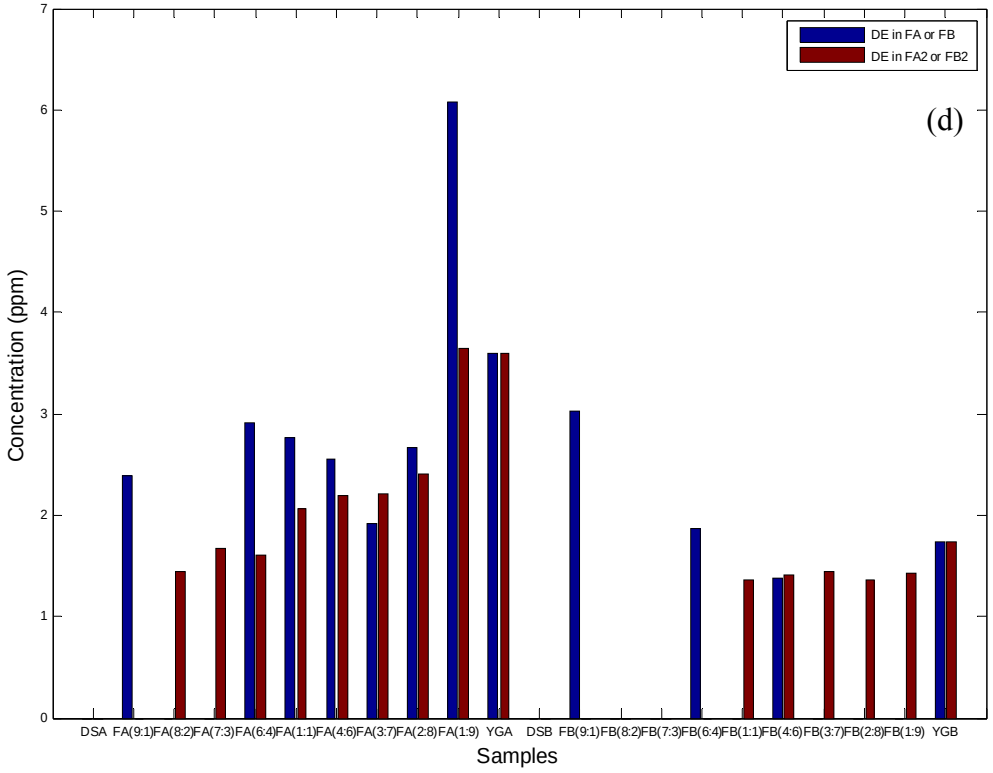


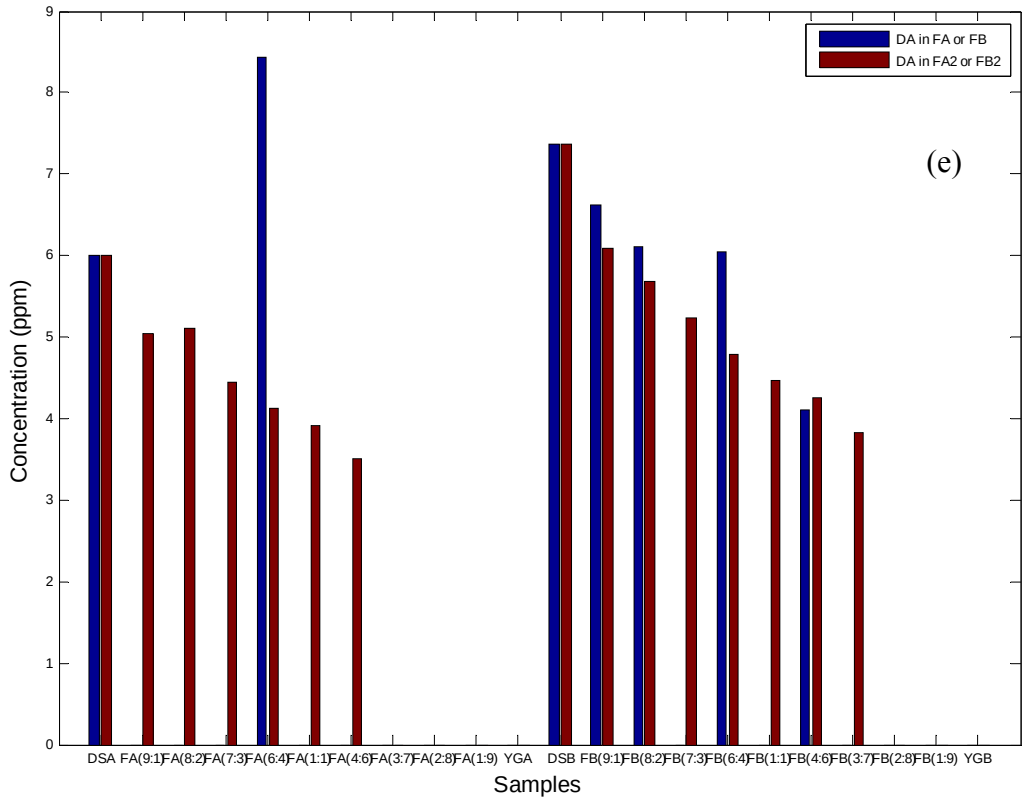
Figure 7.1 The RRLC-DAD chromatograms of (a) DSB at 280nm, (b) YGB at 254nm, (c) FB (6:4) at 254nm, (d) FB (6:4) at 280nm with the reference compounds marked


PU in FA or FB
PU in FA2 or FB2
(a)
Concentration (ppm)
Samples
DSA FA(9:1) FA(8:2) FA(7:3) FA(6:4) FA(1:1) FA(4:6) FA(3:7) FA(2:8) FA(1:9) YGA DSB FB(9:1) FB(8:2) FB(7:3) FB(6:4) FB(1:1) FB(4:6) FB(3:7) FB(2:8) FB(1:9) YGB


DZ in FA or FB
DZ in FA2 or FB2
(b)
Concentration (ppm)
Samples
DSA FA(9:1) FA(8:2) FA(7:3) FA(6:4) FA(1:1) FA(4:6) FA(3:7) FA(2:8) FA(1:9) YGA DSB FB(9:1) FB(8:2) FB(7:3) FB(6:4) FB(1:1) FB(4:6) FB(3:7) FB(2:8) FB(1:9) YGB


GE in FA or FB
GE in FA2 or FB2
(c)
Concentration (ppm)
Samples
DSA FA(9:1) FA(8:2) FA(7:3) FA(6:4) FA(1:1) FA(4:6) FA(3:7) FA(2:8) FA(1:9) YGA DSB FB(9:1) FB(8:2) FB(7:3) FB(6:4) FB(1:1) FB(4:6) FB(3:7) FB(2:8) FB(1:9) YGB


DE in FA or FB
DE in FA2 or FB2
(d)
Concentration (ppm)
Samples
DSA FA(9:1) FA(8:2) FA(7:3) FA(6:4) FA(1:1) FA(4:6) FA(3:7) FA(2:8) FA(1:9) YGA DSB FB(9:1) FB(8:2) FB(7:3) FB(6:4) FB(1:1) FB(4:6) FB(3:7) FB(2:8) FB(1:9) YGB


DA in FA or FB
DA in FA2 or FB2
(e)
Concentration (ppm)
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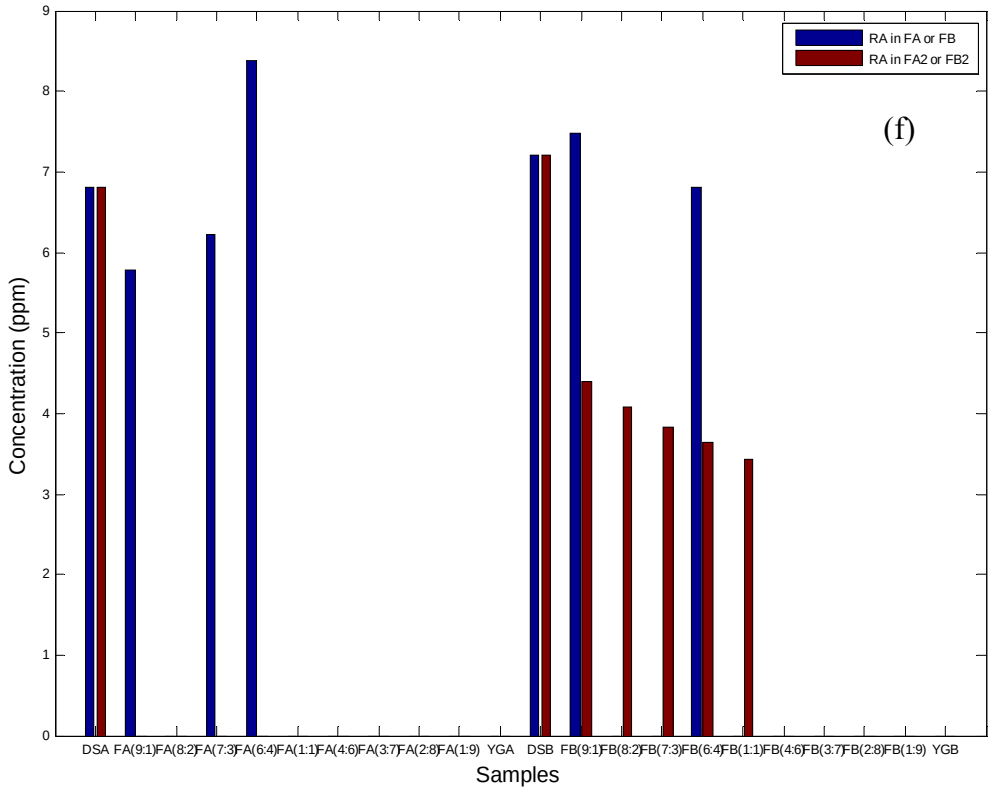
DSA FA(9:1) FA(8:2) FA(7:3) FA(6:4) FA(1:1) FA(4:6) FA(3:7) FA(2:8) FA(1:9) YGA DSB FB(9:1) FB(8:2) FB(7:3) FB(6:4) FB(1:1) FB(4:6) FB(3:7) FB(2:8) FB(1:9) YGB
Samples


RA in FA or FB
RA in FA2 or FB2
(f)
Concentration (ppm)
0
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9
DSA FA(9:1) FA(8:2) FA(7:3) FA(6:4) FA(1:1) FA(4:6) FA(3:7) FA(2:8) FA(1:9) YGA DSB FB(9:1) FB(8:2) FB(7:3) FB(6:4) FB(1:1) FB(4:6) FB(3:7) FB(2:8) FB(1:9) YGB
Samples

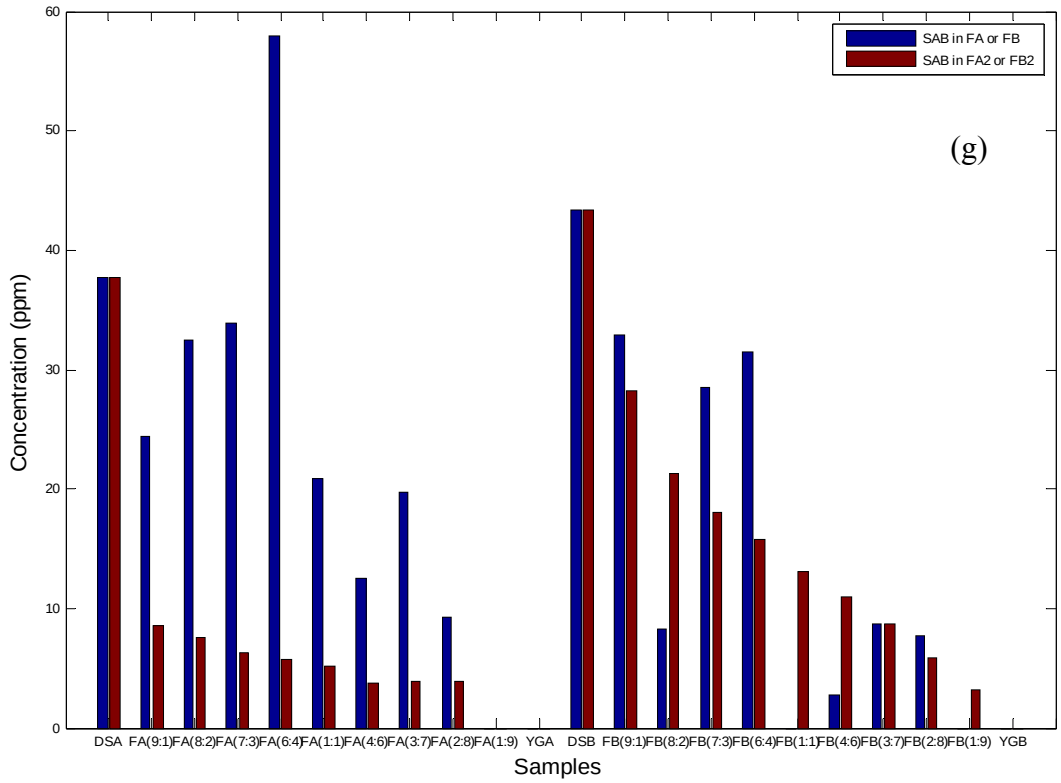

Figure 7.2 The concentrations of (a) puerarin, (b) daidzin, (c) genistin, (d) daidzein, (e) danshensu, (f) rosmarinic acid and (g) salvianolic acid B in DGT with different ratios of DS and YG

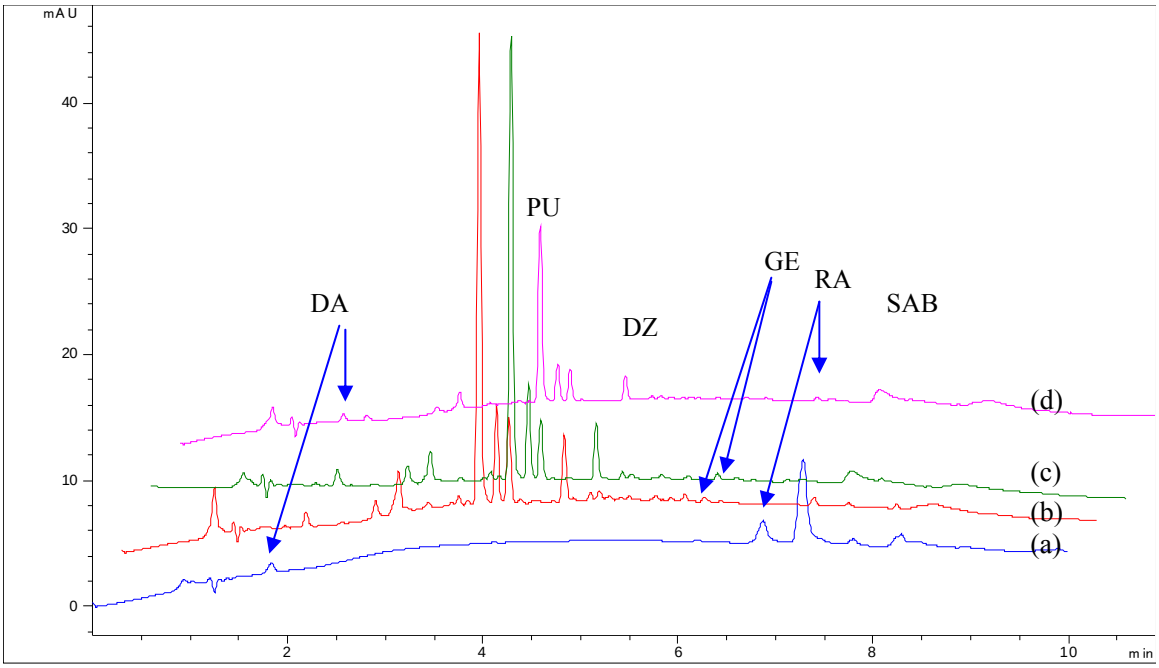

Figure 7.3 The RRLC-DAD chromatograms of (a) DSB at 280nm, (b) YGB at 254nm, (c) FB2 (6:4) at 254nm, (d) FB2 (6:4) at 280nm

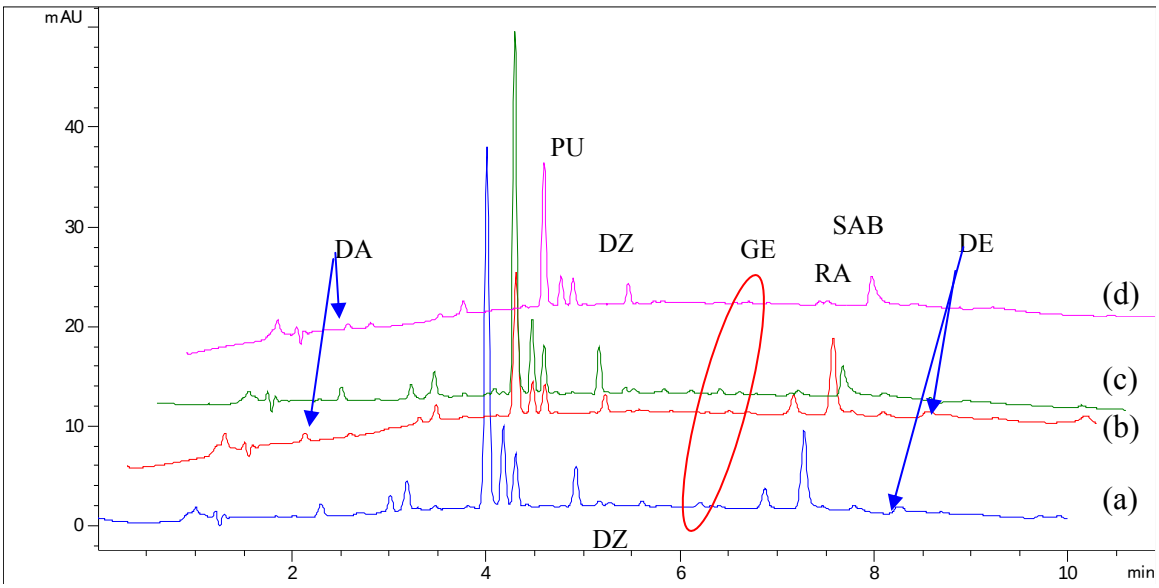

Figure 7.4 The RRLC-DAD chromatograms of (a) FB (6:4) at 254nm and (b) FB (6:4) at 280nm, (c) FB2 (6:4) at 254nm and (d) FB2 (6:4) at 280nm

7.2.1.5 Validation of the chromatographic analysis

Our analytical procedures were validated by linearity, intra-day and inter-day variability.

The linear relationship between the peak area and the concentration of the solution of the chemical reference compounds was indicated in the correlation coefficient. They all had values greater than 0.99 (Table 7.2). Apart from that, the precision of the chromatographic method used were measured in terms of the intra-day variability and inter-day variability. The difference among the three consecutive injections on the same day was used to assess for intra-day variability. The variations among the analyses on five consecutive days were determined for inter-day variability. The relative standard deviations (RSD) of the peak areas of the chemical reference compounds present in the RRLC-DAD chromatograms concerned were computed and the results are summarized in Table 7.3.

Table 7.3 The RSD values of the chemical reference compounds in DS, YG and DGT obtained from evaluation of both the intra-day and inter-day variability

Reference compound	Intra-day variability (m=3) [a]			Inter-day variability (n=5) [b]		
	DS	YG	FB	DS	YG	FB
DA	1.30	-[c]	0.19	1.07	-	0.27
PU	-	0.07	0.42	-	0.33	0
DZ	-	3.17	0	-	1.51	5.66
GE	-	1.54	2.59	-	2.02	1.06
RA	2.94	-	1.67	3.26	-	2.05
SAB	4.02	-	2.73	2.97	-	1.11
DE	-	2.84	2.00	-	1.17	0.36
GS	-	-	-	-	-	-

[a] m represents the number of injections
[b] n represents the number of days
[c] "-"denotes that the compound was not detected

7.2.2 Comparison of the antioxidant activities of the chemical reference compounds and the DGT samples

7.2.2.1 Evaluation of the antioxidant activity levels of the chemical reference compounds in DGT

Two classes of compounds, isoflavonoids and phenolic acids, were focused in this investigation and they have their own specific biological activities as mentioned before. In our work, the antioxidant activities of PU, DZ, GE, DE, DA, RA and SAB were evaluated individually over a series of concentrations. The testing concentration ranged

from 31.25ppm to 500ppm. Figures 7.5 and 7.6 depict the linear relationships between the concentrations of these reference isoflavonoids and phenolic acids and their antioxidant capacities respectively. That is, the contents of all the reference compounds increased with their concentrations with different extent.

The antioxidant activities of phenolic acids were found to be much stronger than those of isoflavonoid as observed (Figures 7.5 and 7.6). At the same concentration level, the antioxidant activities of phenolic acids were hundred times more than those of isoflavonoids. Danshensu (DA) was the most active one while daidzin (DZ) was the weakest one in the testing concentration range. DA is the characteristic component in DS. As mentioned in Chapter 4, the selected phenolic acids in Danshen were principal components in protecting cardiovascular system through scavenging the free radicals. SAB and RA were reported to exhibit antioxidant properties and protective effect against peroxidative damage [10]. Although it was found in Zhao et al. study [82] that salvianolic acid B was stronger in antioxidant activity than danshensu, both results indicated that these two components were essential in antioxidant activities.

As stated in Chapter 5, isoflavonoids are phytoestrogen which could reduce the risk of cardiovascular disease and osteoporosis and prevent cancer. Among the isoflavonoids, daidzein (DE) gave the strongest antioxidant activity. Usually, glycosylation decreases the antioxidant activity in comparison with the corresponding aglycones [14]. Furthermore, pharmacological studies on antiproliferation effect and estrogenic activity reported that the aglycones were more effective than their corresponding glycosides [42, 188]. Although PU and DZ are glycoside of daidzein, they possessed a variety of antioxidant activities in FRAP assay. Also, similar chemical structure of DZ and GE did not result in similar antioxidant activity between them. GE is a more effective antioxidant than DZ. In Russo et al. study, DZ was unable to counteract the human tumor cells while GE exhibited the anticarcinogenic property [43]. Besides, it was reported by Lee et al. study that GE had higher activity level in scavenging free radical and reducing power than DZ [182]. Here, puerarin and daidzein exhibited the antioxidant activities more efficiently and this finding was parallel to that of Cherdshewasart et al. study [181].

However, they were not as effective as the phenolic acids in this aspect.

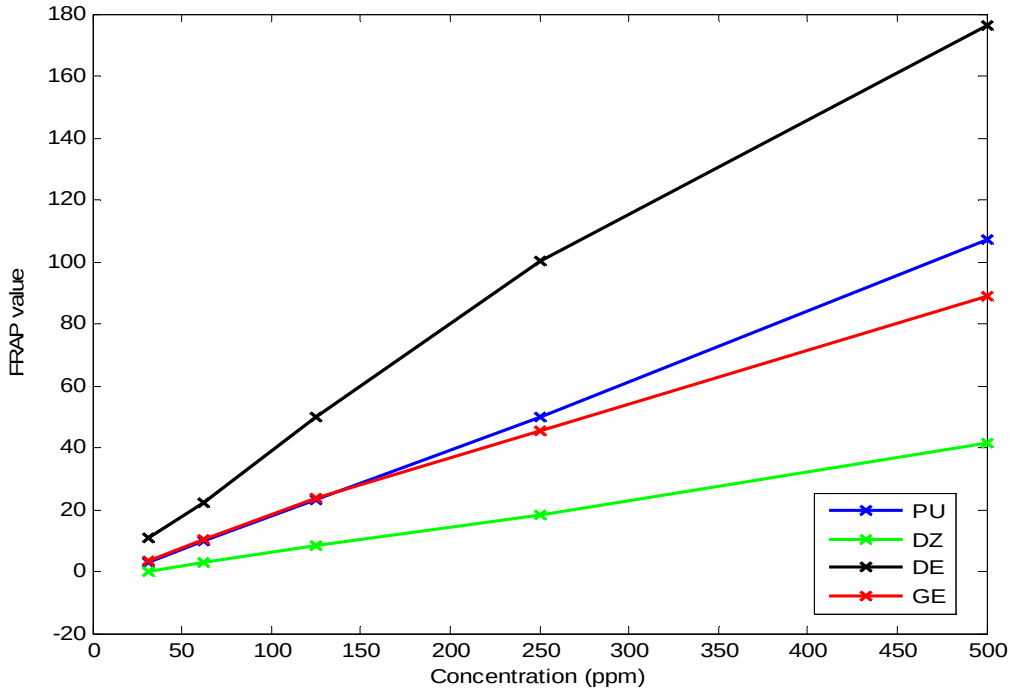

Figure 7.5 Plots of the antioxidant capacities of the four reference isoflavonoids at different concentrations

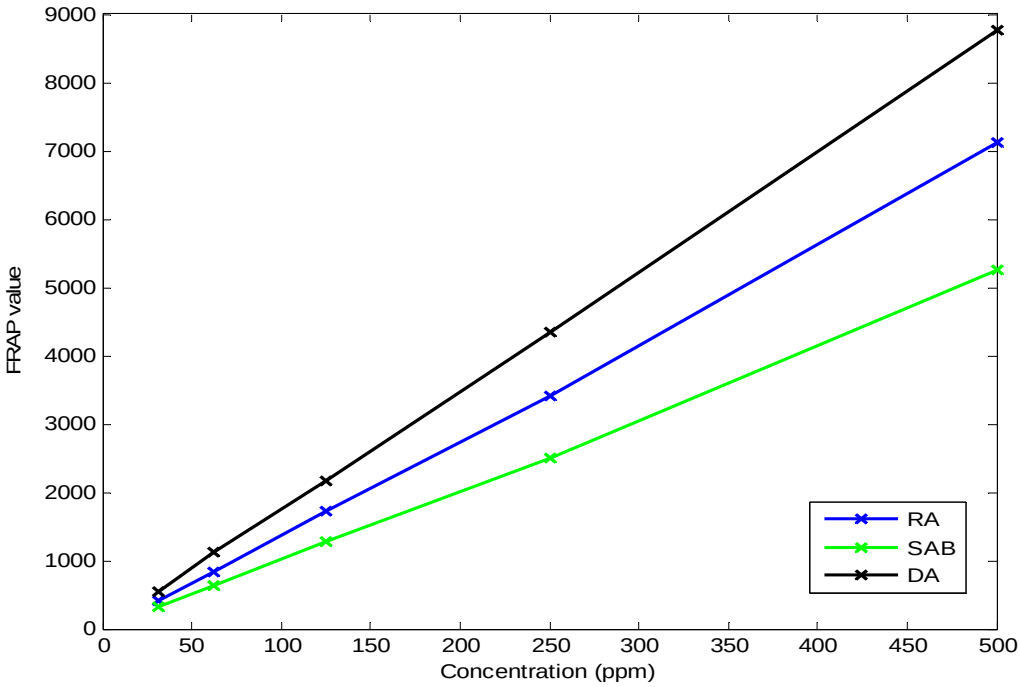

Figure 7.6 Plots of the antioxidant activities of the three reference phenolic acids at

different concentrations

7.2.2.2 Evaluation of the antioxidant activity of the DGT extracts prepared by two different extraction methods of ultrasound assisted extraction and boiling

Better therapeutic effect may be achieved when two or more herbal extracts utilized together. For example, the combined usage of Radix Salviae Miltiorrhizae and Radix Ginseng in two-dragon –broth could increase blood flow and improve the myocardial ischemia [240]. The pharmacological activities of Danggui Buxue Tang improved markedly when compared to those of single herb [56, 241, 242]. It is very common to use the compound prescription to treat disease so as to give better pharmacological activities than a single HM. Both DS and YG were important in the treatment of cardiovascular disease. So their joint usage might aid in protecting the cardiovascular system. As the protection effect is due to partly the antioxidant activity of DGT, the efficiency of different combination ratios of YG and DS on antioxidant activity was examined.

As mentioned before, DS is a well known HM with antioxidant activity through its own reducing ability and capability in scavenging the free radical [140]. YG contained abundant isoflavonoids which could scavenge the free radical and prevent the lipid peroxidation. They contribute to the pharmacological activities of YG [36, 41]. In this investigation, YG played a less important role in antioxidant activity than DS while screening by our two proposed extraction methods (Figure 7.7). The activity of DS extract was 1.5 times and 3 times to YG extract under ultrasound assisted extraction and boiling respectively. As mentioned previously, the antioxidant activity of phenolic acids were found to be hundred folds stronger than that of isoflavonoids when they were evaluated individually in our testing concentrations. Considering the contents of the identified reference compounds and the antioxidant activity together, we recommend preparing DS and YG by boiling and ultrasound assisted extraction respectively. Although there was significant difference in the contents of PU and DE in YGA and YGB, the variation in the antioxidant activities of these two samples was not as large as that. Thus, the antioxidant activity of YG is the consequent of the combined effort of different

components. Similar message was received from DS.

DGT samples with different combination ratios contributed distinct strength in antioxidant capacities. The average antioxidant activity level of the DGT was 776.77 and the range covered 437.10 to 968.46. The activity reduced when the ratio of YG in the DGT increased. As expected, the antioxidant activity of DGT with more YG is close to that of YG while DGT with high ratio of DS had activity close to that of DS here. FA (9:1) and FB (9:1) did not exhibit the highest antioxidant activity level. Instead, the sample at the ratio of 6:4 prepared by boiling showed the strongest antioxidant activity with a FRAP value of 968.46 among FA and FB samples. The DGT at the ratio of 8:2 using ultrasound assisted extraction (FA(8:2)) exhibited the second strongest antioxidant capacity with the FRAP value of 939.77. They have relatively high concentrations of phenolic acids. Usually, the antioxidant activity of FA or FB increased with the mass ratio of DS. The DGT with more than 60% DS in mass gave antioxidant activities above the average value. For the DGT with strong antioxidant activity, they were composed of more than 50% of total amount of target phenolic acids in reference to total concentration of all identified compounds. Both isoflavonoid and phenolic acids were required to enhance the antioxidant activity of DGT as a whole. Thus, it can be seen that the combination ratio of DS and YG is an important factor to this activity.

On the contrary, FB (1:9) had the lowest antioxidant activity level among FA and FB samples. It constituted isoflavonoid majority. The relatively lower activity level was obtained when the DGT had higher YG mass ratio. They gave antioxidant activity level lower than the average value when the mass ratio of YG increased from 50% to 90%. In these extracts, the total content of all identified phenolic acids was less than 20%. It may be due to the phenolic acids in DS were significant in antioxidant activity in this DGT formulation. At the same time, the antioxidant activities of FA and FB at the ratio of 1:9 were higher than that of YG for more than 10%. This implies that the addition of DS to YG in the DGT would increase the antioxidant activity.

Since the extraction method did also affect the contribution of antioxidant activity by the

component herbs, the DGT with higher mass ratio of YG resulted in stronger antioxidant activity when preparation was done under ultrasound assisted extraction. For those DGT composed with more DS in ratio, they were more effective in antioxidant activity when they were prepared by boiling. In general, the antioxidant activities of FB were higher than the activity of FA especially at the ratio with more DS except 7:3. But the difference at these ratios with more DS is not remarkable. Since the antioxidant activity of YGB is much lower than that of YGA and the variation in activity of DS under the two extraction methods was not so noteworthy, the total antioxidant activity of YGA and DSA in FA would be at higher level than FB if there is no interaction among YG and DS. The experimental results showed that the difference among FA and FB became larger than the variation between the antioxidant capacities as estimated from the summation of DS and YG under our two extraction methods. Moreover, FB with more DS gave stronger antioxidant activity than FA. Therefore, boiling YG and DS together would enhance the resulting antioxidant activity with more mass ratio of DS.

The antioxidant activities of those FA samples with ratios of 1:1, 4:6, 3:7, 2:8 and 1:9 were stronger than the FB samples with the percentage difference up to 40%. This can be explained by that the contents of the SAB in these FA samples were much higher than its contents in FB samples. The antioxidant activity of SAB is linearly correlated to the concentration when it is analyzed individually as mentioned above (Figure 7.2). In addition, the amounts of PU and DE in FA were higher than in FB at these examined ratios. These two components were also potent antioxidants among the isoflavonoids. The other reference isoflavonoids, DZ and GE were not as effective as PU and DE even though their amounts in FB were much higher than in those in FA. The antioxidant capacities of FA and FB were related to their chemical compositions which were affected by extraction methods and combination ratios of two components herbs.

7.2.2.3 Evaluation of the antioxidant activity of DGT prepared by mixing the dried extracts

The dried extracts of DS and YG were also mixed in different ratios in this study and they were labeled as FA2 and FB2. The range of their antioxidant activities was from 382.39

to 982.35 and the average value is 736.25 (Figure 7.7). There is a decline in antioxidant activity when the mass ratio of YG in FA2 or FB2 increased and approached to the antioxidant activity of YG and vice versa. Still, the activity of FA2 at the ratio of 1:9 was stronger than that of YGA with more than 10%. This showed the importance of the addition of DS in improving the antioxidant activity. Among all FA2 and FB2 samples, FA2 (8:2) and FB2 (9:1) were the strongest ones while FA2 (2:8) and FB2 (1:9) were the weakest two using ultrasound assisted extraction and boiling respectively (Figure 7.7). Again, this showed that the antioxidant activity is strongly related to the mass ratio of DS and YG in the DGT.

In general, the antioxidant activity of FA2 was stronger than FB2 except at the ratio of 9:1. The difference in antioxidant activity was greater than 10%. At the ratio of 9:1, the higher antioxidant activity level of FB may be related to the relatively higher concentrations of phenolic acids. Usually, FA2 samples were composed of more target isoflavonoids in total than FB2. All the target isoflavonoids in FA2 constituted more than 50% to total contents of all target compounds. Yet, the contents of reference phenolic acids in FA2 were lower than FB2. As the total target compounds were focused, the other unidentified compounds were not quantified. The unknown active ingredients might have joint effort with the two classes of compounds on the total antioxidant activity. Of course, the combination of different components in the samples would have assorted activities. Otherwise, the relatively stronger antioxidant activity of FB2 would show as the antioxidant activities of phenolic acids are much stronger than the reference isoflavonoids.

7.2.2.4 Comparison of the antioxidant activity of two kinds of DGT samples

Both chemical compositions and the antioxidant activities of two kinds of DGT samples in this investigation were not identical. This may relate to the difference in the sample preparations. The variation between the antioxidant activities of these is not significant except at certain ratios (Figure 7.7). These two kinds of samples had similar capacities with only 5% difference in their average values.

From the antioxidant capacities of two kinds of samples, the addition of DS to the YG

extract would improve the activity of the whole sample. At higher mass ratio of DS, the antioxidant activity of the DGT was getting close to the antioxidant activity of DS. This may be referred to the relatively higher contents of phenolic acids. Despite that, most of the FA or FB samples were slightly stronger than FA2 or FB2. That is, the component herbs extracted together would enhance the antioxidant activity. This implied that extraction of the component herbs would have better effect on antioxidant activity than simply mixing together the extracts of component herbs which were prepared separately. Furthermore, the optimal combination ratio of DS and YG among these samples was not the same. FB (6:4) and FA2 (8:2) were the most active. Not only they were differed in the mass ratio, their compositions of target phenolic acids and the isoflavonoids were not the same also. But they had the highest activity level among the two kinds of samples studied. It may be related to the presence of other unidentified compounds which are significant in this aspect and the specific composition of the individual components in the DGT.

In addition, the compositional difference between these two kinds of samples may be referred to the sample preparation methods. Then, FA and FA2 samples at the ratios of 9:1 and 6:4 exhibited quite a large difference in the activities (Figure 7.7). At the ratios of 8:2, 7:3, 6:4, 2:8 and 1:9, the samples prepared by boiling were generally more active than those prepared by mixing dried extracts at these ratios. The compositional variation in the two classes of compound is the major reason of the variation in antioxidant activities.

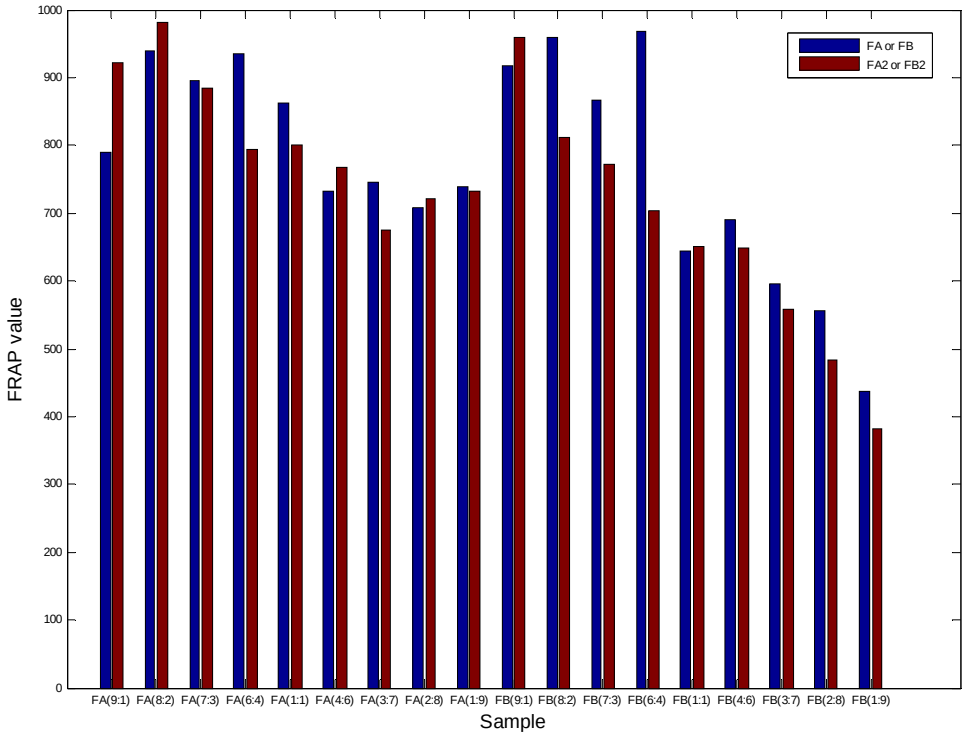

Figure 7.7 The antioxidant activities of the DGT samples with different combination ratios of DS and YG in addition to different sample preparation methods

7.2.3 Relationship between the composition of reference compounds in the DGT and the antioxidant activity

The contents of reference compounds in the two types of DGT samples were less that those in DS and YG respectively. It is because the composition of the DGT is related to the extraction efficiency of DS and YG. The relationship between the contents of the reference compounds and mass ratio of component herbs is not clearly known. Therefore, it is indivisible to consider the extraction efficiency of DS and YG simultaneously in FA and FB samples. On the other hand, the concentrations of isoflavonoids or phenolic acids are linearly related to the mass ratio of YG or DS in the DGT in FA2 and FB2 samples. Thus, the chemical composition of this kind of DGT samples relied on the extraction efficiency of DS and YG separately.

As stated previously, the antioxidant activities of the reference isoflavonoids were shown at high concentration level when they were analyzed individually. But the phenolic acids with low concentrations showed antioxidant activity already. Based on the amounts of identified compounds in DS and YG extracts, they were at the concentration levels lower than the testing concentration of the components when they were analyzed individually. Despite that, both DS and YG were still very effective antioxidant. This indicated that the presence of other unidentified active ingredients and the contribution of all components played an important role in the activity.

Similarly, the concentrations of the identified components included both isoflavonoids and phenolic acids in the DGT were lower than their testing concentration ranges during the measurement of the individual capacity. Their average quantities except PU were less than 31.25ppm which was the lowest testing concentration level. Here, it was suggested that the DGT was not effective in antioxidant activity because the corresponding activity of the target compounds would be low at their low concentration level. But it is not so in this case. It seems that all the constituents in the DGT were responsible for the antioxidant activity.

At the same time, the change of the composition with the antioxidant activity of the DGT was explored. This would provide certain information on the possible components that contributes to the antioxidant activity. Negative correlation was found for puerarin, daidzin, and genistin with values of correlation coefficient being -0.4095, -0.7021 and -0.5078 respectively. That is, the DGT with high concentrations of PU, DZ and GE did not exhibit stronger antioxidant activity. Thus, the activity of FA is stronger than FB even though FA had lower concentrations of GE and DZ. Besides, the concentration of daidzein (DE) increased with the antioxidant activity with a low value of correlation coefficient 0.3476. This suggested that daidzein is more important than the rest of isoflavonoids on the antioxidant activity of the DGT. On the contrary, positive correlation between the amounts of the three phenolic acids with antioxidant activity was found. From the correlation coefficients, the antioxidant activity of the DGT depended on DA and RA with the respective values of 0.6508 and 0.7259. Those samples with either DA

or RA also demonstrated the relatively high antioxidant activity level. The relatively weak linear relationship was shown for SAB with the value being 0.5434. All the correlations between the identified phenolic acids and antioxidant activity were statistically significant.

Up to now, the antioxidant activity of the DGT was found to relate to the high concentrations of phenolic components. But the presence of the aglycone, DE, is needed which had slightly linear relationship with the antioxidant activity. According to the correlation between the concentration of the individual component and antioxidant activity of the DGT, they did not exhibit very linear relationship. That is, the activity of DGT was under the influence of the presence of other components but it is not affected by one constituent alone. Moreover, the optimal antioxidant activity was exhibited in the DGT contained both phenolic acids and isoflavonoids. This suggested that the presence of isoflavonoid is required to have better antioxidant activity level. Thus, the presence of DS and YG is crucial in getting high antioxidant activity level. Besides, other unidentified isoflavonoids and phenolic components would be this activity of DGT as well. Further study on this through studying the relationship of all the detectable components and the antioxidant activity is required.

7.3 Conclusion

The efficacies of various herbal formulae have been demonstrated to certain extent through different scientific studies. It is believed that the application of two or more HM could improve the pharmacological activity of single herbs. Danshen and Gegen have been utilized for cardiovascular disease treatment because of their antioxidant activities. Then, the protection of the cardiovascular system from the oxidative damage would be enhanced. In this study, this antioxidant activity and the chemical compositions of different Danshen-Gegen Tang (DGT) samples were analyzed. They were composed of different ratios of Danshen and Gegen. The investigated ratios of DS to YG were 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8 and 1:9. Two kinds of samples were prepared under different extraction methods. DS and YG that were mixed in different ratios and extracted together were labeled as FA or FB samples here. Alternatively, the DGT that were prepared by

mixing different ratios of dried extracts of DS and YG were labeled as FA2 or FB2 samples. Our experimental findings showed that the antioxidant activities and chemical compositions of FA or FB were affected by the combination ratio of DS and YG as well as the extraction method. For FA2 or FB2, the mixing ratio is the key factor that affected the antioxidant activity and composition of two reference components.

Among different ratios of DS to YG, the highest antioxidant activity was exhibited at the ratio of 6:4 under boiling (FB(6:4)). The comparatively higher quantities of phenolic acids were found. But they were not at the highest concentrations among different combination ratios studied. In fact, there was no linear relationship found between the composition and the mass ratio of DGT. Despite that, the relatively strong antioxidant property was exhibited when the total concentration of the reference phenolic acids was 50% towards the total concentrations of all identified constituents. Thus, the combined effort of DS and YG should not be ignored.

Apart from that, another kind of DGT samples was prepared by mixing the dried extracts of DS and YG which were extracted independently at different ratios. Different antioxidant capacities and the chemical compositions were obtained when compared with the DGT prepared by simultaneous extraction of two component herbs as mentioned previously. FA2(8:2) and FB2(9:1) gave the highest antioxidant activity levels under two examined extraction methods. Also, the chemical compositions on both isoflavonoids and phenolic acids were associated with the mass ratio of DS and YG in the DGT. Still, the antioxidant capacity was the result of the joint efforts among the constituents.

In order to study the effect of the composition and the antioxidant activity of the DGT, the relationships between the two kinds of information obtained were investigated. The negative correlations between puerarin, daidzin and genistin on the antioxidant activities were found. On the contrary, the relatively linear relationships between daidzein and the phenolic acids in the DGT were obtained. This suggested that DS plays a more important role than YG on the antioxidant activity in the DGT. Yet, inclusion of YG should not be neglected.

To conclude, the present result showed that the DGT with the ratio of DS to YG being 6:4 exhibited the strongest antioxidant activity under boiling compared to other extraction method. But the contents of the reference constituents were not the highest at this ratio. This implies that both DS and YG have effects in the DGT to contribute to the antioxidant activity. Future work relating to the optimization of the extraction method will be carried out to get DGT with better antioxidant effect.

Chapter 8

Conclusion

Application of Herbal Medicine (HM) to treat diseases has been widely accepted by people from around the world. Yet, lots of them cast doubts on its safety and efficacy. One of the main reasons is that the contents of the bioactive components within are greatly affected by the environment and other factors. Hence, its consistency is one way to assure its quality. It is a crucial issue to ascertain the effectiveness of HM by using appropriate quality control measures. Through characterizing the chemical components in HM by hyphenated instruments, the consistency and safety of HM with focus on the chemical composition can be evaluated to certain extent. In addition, the biological activity should be assessed to assure the HM efficacy. With the consideration of both chemical and biological information of HM, better quality control measures can be provided. In this research work, two herbs Radix et Rhizoma Salviae Miltiorrhizae (Danshen, DS) and Radix Puerariae Lobatae (Gegen, YG) were investigated by both chemical and biological screening method. In addition, the herbal formula, Danshen-Gegen Tang (DGT), with these two herbal medicines was examined as well. A brief description of these studies is given in the following paragraphs.

In Chapter 2, different quality control approaches and chromatographic technologies were discussed. At present, most of the quality control approaches are inadequate to secure the effectiveness of HM. Then, the chromatographic fingerprint and biological activity of HM were correlated to give higher level of quality control. In this review, different in-vitro antioxidant assays were discussed because antioxidant activity was the target in our bioactivity study. Two kinds of data obtained can be analyzed and linked through the chemometric techniques. Here, the multivariate modeling methods and the variable selection methods were introduced. The variable selection could improve the prediction performance and indicate the significance of the variables involved. Besides, other kinds of chemometric techniques such as MSCC, PCA and similarity evaluation could aid in data interpretation of the complicated systems. The commonly used ones were mentioned in this chapter.

Detailed experimental procedures for chemical analyses and biological assay of two herbal medicines and the herbal formula in addition to a synthetic mixture system were

reported in Chapter 3. It included the experimental procedures of sample preparation, chromatographic studies, antioxidant assay, and fractionation using preparative high-performance liquid chromatography.

Quality of the HM product relates closely to the manufacturing procedures. Sample extraction of HM would affect the amounts of biological active compounds to be obtained. As interaction among the HM components is possible, the efficacy or toxicity would be altered and reduced accordingly. It is one of the issues to be taken care of during quality assurance of HM. Also, the therapeutic effect of HM is due to multiple constituents. Hence, in Chapter 4, the response surface methodology was applied in optimizing the antioxidant activity of Radix et Rhizoma Salviae Miltiorrhizae (DS) with respect to several extraction parameters. Bioactivity instead of the content of the selected chemical reference compounds was the focus. In order to have optimal antioxidant activity, DS was extracted under 90ml of 80% methanol for 45minutes utilizing ultrasound assisted extraction. Under this condition, the antioxidant activities and chemical compositions of twenty DS samples were studied and compared. The samples from different origins of P. R. China exhibited variations in these two properties. It was found that the phenolic acids plays more important role in antioxidant activity when compared with another class of compound, diterpenoids. For those extracts having the highest level of antioxidant activity, they did not have the large amounts of the identified reference compounds. Thus, it is better to optimize the sample extraction with the consideration of bioactivity as found in our investigation.

The ideal authentication method of HM aims at considering consistency, safety and efficacy of HM instead of just a few markers like most do currently. Hence, linkage of the chemical composition and biological activity of HM together for HM quality assessment is the key issue in Chapter 5. The quality of Radix Puerariae Lobatae (YG) was first evaluated through “compound-oriented approach” and “pattern-oriented approach”. Five target isoflavonoids, puerarin, daidzin, genistin, daidzein and genistein were selected for identification and quantification of seventy nine YG samples collected from different regions of P.R. China. They contained a wide range of concentrations of these five

reference compounds. Among them, YG-41, YG-38, YG-29, YG-9 and YG-18 have the highest content of these isoflavonoids. Furthermore, comparison of the chemical compositions among the YG samples was carried out via their chromatographic fingerprints. The variations among all the detectable components observed in these fingerprints were assessed by their similarity indices (SI). Most of the samples were similar to each other considering their chemical compositions and their SI were above 0.9. But this approach measured only the difference in chemical compositions among samples. In addition to the chemical analysis, the antioxidant activities of the YG samples were measured. YG-2 was found to be the most antioxidant active. For those samples with the significant amounts of reference isoflavonoids, they did not exhibit the strongest antioxidant activity. Both the chemical composition and antioxidant activity of YG were also correlated with the help of multivariate modeling in this work. The model thus established was applied to predict the antioxidant activity from the related chromatographic fingerprint using uninformative variable elimination by partial least squares method (UVE-PLS). The performance of the multivariate modeling was evaluated by RMSE, RMSEP and correlation coefficient. In the training set, the square error of estimation was 76.88 (6.37%) and the correlation coefficient between the predicted and experimental value was 0.9318. Also, the root mean square error of prediction in validation set was 79.59 (6.60%) and the correlation coefficient was 0.8926. Through the result obtained, one can easily find that the chemical profiles of HM revealed both efficacy and the chemical compositions of HM through the quantified relationship obtained.

Complexity of HM hinders the study of therapeutic effect of HM in detail with more active ingredients being considered. In Chapter 6, application of the target projection coupled with selectivity ratio (TP/SR) in screening the beneficial components was done on YG and our synthetic mixture system (MIX). It would be the starting point to investigate the mechanism of the disease treatment of this HM. Moreover, isolation of the YG constituents is not needed in this stage. All the YG components that appeared in the fingerprint were related to the YG antioxidant activity in the TP/SR data analysis. Through which, three groups of potential bioactive candidates were found out by SR.

Their retention time are (G1) 12.5min, (G2) 13.3min and (G3) 15.6min. In order to confirm the predicted result, fractionation of the YG extract was preformed by preparative HPLC and the fraction with these three potential antioxidative components showed the strongest antioxidant activity. Moreover, a series of synthetic mixtures were prepared to verify the adequacy and performance of TP/SR. The results confirmed that this data processing method was able to uncover the most active components, GA and QT. This further substantiated the findings from our established relationship between the chemical profiles and antioxidant activity. These strongly supported that TP/SR is a reliable method which can reveal the bioactive components in a mixture system including HM accurately and rapidly.

Additionally, the antioxidant activities of the combination of two herbs, DS and YG in the Danshen-Gegen Tang (DGT) were investigated. In this study, DS and YG were mixed systemically with ratios of 9:1, 8:2, 7:3, 6:4, 1:1, 4:6, 3:7, 2:8 and 1:9 before extraction. Apart from this, the antioxidant activities of the mixtures of DS and YG dried extracts at these ratios were assessed. Here, the effect of boiling and ultrasound assisted extraction on the contents of reference compounds selected and the antioxidant activity were inspected as well. In order to improve the resolution and speed up the analysis of the complicated mixtures system, rapid resolution liquid chromatography coupled with diode array detector (RRLC-DAD) was utilized in analyzing the DGT. The boiling extract with the combination of DS to YG in the ratio of 6:4 gave the strongest antioxidant activity. Yet, it did not contain the highest amounts of the reference isoflavonoids or phenolic acids. This indicated that the antioxidant activity is related to the combination of the constituents including those unidentified active ingredients in the DGT. Moreover, extraction of the component herbs together could possess the antioxidant activity more effectively than the mixture of individual dried extracts in this case. This also suggested that the extraction of DS and YG together would enhance the antioxidant property of DGT.

To conclude, the relationships of the chemical compositions and antioxidant activities of Radix et Rhizoma Salviae Miltiorrhizae (DS), Radix Puerariae Lobatae (YG) and their

combination of Danshen-Gegen Tang (DGT) were explored. We tried to investigate whether this can lead to better understanding of the pharmacological property of HM and quality control measures at higher level or not. In this work, the chromatographic fingerprint and the antioxidant activity of YG were correlated and our statistic model was built up with the help of multivariate modeling. This proposed methodology successfully determined the antioxidant activity and discovered the bioactive components in YG. Therefore, this approach provides a reliable quality control way to YG. From the correlation of the antioxidant activity and the contents of selected components obtained, we found that optimization of DS based on antioxidant activity can help in setting up standardization of the DS extract. In addition, the optimal combination ratio of the components herbs in the herbal formula, DGT, can be determined with the consideration of both chemical compositions and antioxidant activities.

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