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Applications of Chromatographic, Chemometric Techniques and *in-vitro* Bioassay to Investigate the Chinese Medicines, Radix Ginseng, Radix Panacis Quinquefolii, Ganoderma Amboinense, Danggui Buxue Tang and RDLP

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A thesis submitted in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

September, 2007

CERTIFICATE OF ORIGINALITY

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ABSTRACT

Abstract of the thesis entitled

"Applications of chromatographic, chemometric techniques and *in-vitro* bioassay to investigate the Chinese medicines, Radix Ginseng, Radix Panacis Quinquefolii, Ganoderma Amboinense, Danggui Buxue Tang and RDLP"

submitted by LAU Tsui-yan

for the degree of Doctor of Philosophy

at The Hong Kong Polytechnic University in September, 2007.

Chinese medicine (CM) is now regarded as potential supplements and alterative medicine throughout the world in the past decades. However, quality, safety and efficacy of CMs are of great concern owing to limited scientific evidence available about its chemical composition, pharmacology and related properties. In this study, hyphenated chromatographic, chemometric data processing and biological screening techniques were applied to investigate the CMs, Radix Ginseng, Radix Panacis Quinquefolii and Ganoderma amboinense, and two CM formulations of RDLP and Danggui Buxue Tang (DBT) for both qualitative and quantitative analyses.

Radix Ginseng and Radix Panacis Quinquefolii are two well-known and popular CMs and their dietary supplements can be found easily in health food stores. In this study, a fast extraction and a chromatographic procedure for high performance liquid chromatography-diode array detector-evaporative light scattering detector (HPLC-DAD-ELSD) were developed for introducing the basic concept of chemical analysis of natural

products to tertiary students. The experiment designed was flexible for tertiary institute with different instruments.

HPLC-DAD together with a chemometric method, evolving window orthogonal projections (EWOP) method was used to analyze the six nucleosides components, adenine, adenosine, inosine, guanosine, uracil and uridine, in the cap, the stipe and fruit body of Ganoderma amobinense (DH Lingzhi). Adenosine, guanosine, uridine and uracil, were identified in all the DH Lingzhi samples by using the experimental method. However, their accurate contents were difficult to determine by their peak areas as these their chromatograms were affected by the instrumental noise and other interferences. Hence, EWOP method was employed to eliminate the interferences in these data sets. As a result, both qualitative and quantitative analyses of the nucleosides components in DH Lingzhi samples were achieved.

RDLP is a CM formulation developed by a Hong Kong CM practitioner and it is good for liver cancer. In this work, preparative HPLC-DAD and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay were applied respectively to analyze and fractionate the chemical constituents, and the safety and the anti-cancer activity of RDLP on liver cancer cell lines. The MTT cell proliferation assay was used as guidance for the fractionation of the chemical components of RDLP by PHPLC, so-called bioassay-guided fractionation approach. Four fractions of the RDLP extract were obtained, and their anti-cancer activities were studied. One of four fractions was found to contain potential bioactive components by us in RDLP. The RDLP extract was also less harmful to normal liver cell lines.

DBT has been used for the improvement of the menopausal symptoms to woman since 1247 A. D. The formulation is composed by 10 qian Radix Astragali (Heungqi) and 2 qian Radix Angelicae Sinensis (Danggui). In this investigation, HPLC-DAD combined with Multicomponent spectral correlative chromatographic (MSCC) method Similarity index (SI) were utilized for investigating the chemical constitutions of DBT and its two herbs. Their extracts were prepared by the boiled and soaked methods. Then they were measured by HPLC-DAD. Moreover, the chromatographic data acquired were further treated with MSCC and SI to find out the chemical compositions of the samples. The variation of the chemical components found in the two DBT extracts was helpful to evaluate the bioactivities of DBT.

PUBLICATIONS AND PRESENTATIONS

List of Publications and Conference / Symposium Presentations

Journal Articles

Lau, T.Y., Ng, K.M. and Chau, F.T. "Quantitative HPLC analysis of Ginseng dietary supplement: Simultaneous Determination of Six Ginsenosides". Manuscript under preparation.

Wei, S.Y., Xu, C.J., Mok, K.W., Cao, H., Lau, T.Y and Chau, F. T. "Analytical Comparison of Different Parts of Radix Angelicae Sinensis by Gas Chromatography Coupled with Mass Spectrometry". Paper submitted.

Conference / symposium Presentations

Lau, T.Y., To, M.L. and Chau, F.T. "Isolation Active Extracts or Ingredients by Preparative Liquid Chromatography (PHPLC) from a Traditional Chinese Medicine (TCM) formulation". Poster presentation at the 7th Asian Conference on Analytical Sciences, , Hong Kong, 28 -31 July 2004.

Lau, T.Y., To, M.L. and Chau, F.T. "Identification of Active Ingredients of RDLP a Traditional Chinese Medicine (TCM) formulation to treat cancer". Poster Presentation at

the 25th International Symposium on Chromatography (ISC), Paris, France, 3-8 October 2004.

Lau, T.Y. and Chau, F.T. "Study of active components of Shan Zha on cardiovascular disease". Poster Presentation at the 12th Symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 23 April 2005.

Lau, T.Y., Ng, K.M. and Chau, F.T. "Quick Procedure for qualitative and quantitive analyses of six ginsenosides in the commercial products of Koran white ginseng (*Panax ginseng*) and American white ginseng (*Panax quinquefolius*) using HPLC-DAD-ELSD". Poster Presentation at 2005 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 12 August 2005.

Lau, T.Y. and Chau, F.T. "Development of an HPLC procedure for simultaneous analysis of the anti-tumor drugs". Poster Presentation at the 13th Symposium on Chemistry Postgraduate Research in Hong Kong, Hong Kong, 22 April 2006.

Lau, T. Y. and Chau, F. T. "HPLC analysis of ten commercial standards found from different traditional Chinese medicines". Poster Presentation at 2006 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 17 August 2006.

Lau, T. Y and Chau, F. T. "Correlation of the Antoxidative activity of a Chinese Medicine herb to its Chemical composition". Poster Presentation at the 14th Symposium on Chemisty Postgraduate Research in Hong Kong, Hong Kong, 28 April 2007.

Xu, C. J., Lau, T. Y., Chan, H. Y., Cheung, C. Y. and Chau, F. T. "Pre-preocessing of Liquid Chromatography / Mass Spectrometry Data in Herbal Medicine System – a Comparison Study". Poster Presentations at the 14th EuroAnalysis, Antwerp, Belgium, 9-14 September 2007.

Course attendance

'Method Validation Key Issues and Tools', lectured by Dr. Jerome Vial and Dr. Jean-Michel Menet. A course offered by three different societies (AFSep - Association Francophone des Sciences Séparatives (France), The Chromatographic Society (UK), and Gesellschaft Deutscher Chemiker, Fachgruppe Analytische Chemie, Arbeitskreiss Separation Science (Germany).) at the 25th International Symposium on Chromatography, Paris, France, 4 October, 2004.

'TCM Fingerprint Techniques and Chemometrics', lectured by Prof. Foo-tim Chau, Prof. Olav M. Kvalhim, Prof. Peishan Xie, Ms. Yuzhen Yan and Prof. Yizeng Liang. A course offered by the Chemometrics and Herbal Medicines Laboratory Research Group at the Department of Applied Biology and Chemical Technology of The Hong Kong Polytechnic University, Hong Kong, 22 February 2006.

'Recent Development of TCM Fingerprint Analysis Techniques and the New SFDA TCM Stabliity Guidelines' lectured by Prof. Foo-tim Chau, Prof. Peishan Xie, Prof. Yizeng Liang, Dr. Daniel Kam-wah Mok and Dr. Chi-on Chan. A course offered by the Chemometrics and Herbal Medicines Laboratory Research Group at the Department of

Applied Biology and Chemical Technology of The Hong Kong Polytechnic University, Hong Kong, 7 June 2007.

Seminars attended

'Development in HPLC and LCMS Column Technologies from Thermo Electron Corporation', offered by GAT Asia Limited, 2003, Hong Kong, 21 October.

'ICMCM2004 Seminar and Exhibition', offered by Modernized Chinese Medicine International Association, Hong Kong, 12 -13 August 2004

'Advancing Life Science Mass Spectrometry', offered by Applied Biosystems, Hong Kong, 1 April 2005.

'Innovations of Separation Science', Waters China Limited, Hong Kong, 29 June 2005.

'Hong Kong Chinese Materia Medica Standards Volumn 1', offered by the Chinese Medicine Division of the Department of Health, Hong Kong, 6 September 2005.

'Analytical Techniques for Quality Products Symposium' offered by Modernized Chinese Medicine International Association, Hong Kong, 15 September 2005.

Committee for Symposium

2005 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 12 August 2005.

ACKNOWLEDGEMENTS

I would like to thank the Research Committee of The Hong Kong Polytechnic University for providing a research fund, and the University Grant Council (UGC) of Hong Kong Special Administrative Region via the Area of Excellence (AoE) of "Chinese Medicine Research and Further Development" (AoE/B-10/01) for the kind support in granting this project including support for my attendance of the course "Method Validation Key Issues and Tools" offered in the 25th International Symposium on Chromatography (ISC) in 2004 in Paris and different symposia held in Hong Kong. I also wish to express my gratitude to all those have helped in various ways during the course of this work for support of this project.

I would like to thank Professor Foo-tim Chau, my supervisor, for his critical advice and supervision of this project as well as his comments on the final draft of this thesis and the publications. I am greatly indebted to Dr. Daniel Kam-lun Lee, my co-supervisor, for the helpful advice and support of biological studies and instruments in this project. Special thanks are due to Dr. Daniel Kam-wah Mok, Dr. King-wah Ma, Mr. Kin-man Ng, Dr. Venus Man-ling To, Dr. Sally Shui-yin Wei, Dr. Chi-on Chan and Miss Tweety Chui-yee Cheung for their technical advice and assistance.

Finally, I am thankful to all the members of my family, piano teacher, Ms. Jessica Yuen-han Lok, and best friend, Miss Joey Wai-chung Leung for their constant encouragement during the course of this work especially in the hard times.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

2D	Two dimension
3D	Three dimension
ACN	Acetonitrile
AGS	Radix Panacis Quinquefolii / American ginseng
ALC	Analytical high-performance liquid chromatography
CAM	Complementary and alternative medicine
CM	Chinese Medicine
C.V.	Coefficient of variation
DAD	Diode array detector
DBT	Danggui Buxue Tang
DG	Radix Angelicae Sinense / Danggui
DH Lingzhi	Ganoderma ambinense / Deer Horn Lingzhi
DMEM	Dulbecco medified Eagle's medium
DMSO	Dimethyl sulfoxide
EFS	Evolving factor analysis
ELSD	Evaporative light scattering detector
EtOH	Ethanol
EWOP	Evolving window orthogonal projections method
FBS	Fetal bovin serum
FDA	US Food and Drug Administration
GC	Gas chromatography
GC/MS	Gas chromatography-mass spectroscopy
HepG2	Human hepatoma cell line at early stage
Hep3B	Human hepatoma cell lines at last stage
HELP	Heuristic evolving latent projections
HP	Hewlett Packard

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
HQ	Radix Astragali / Huangqi
IC50	50% inhibition percentage
LC	Liquid chormatography
LC-MS	Liquid chromatography-mass spectroscopy
LLS	Local least squares
MEM	Minimum essential medium
MeOH	Methanol
MRC-5	Human lung normal cell line
MS	Mass spectormety
MSCC	Multicomponent spectral correlative chromatographic
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
$(NH_4)H_2PO_4$	Ammonium phosphate
O.D.	Optical density
OGS	Radix Ginseng / Oriental ginseng
PBS	Phosphate-buffered saline
PC	Principal component
PCA	Principal component analysis
PHPLC	Preparative high-performance liquid chromatography
RDLP	One of the CM formulations studied
RPMI	RPMI medium 1640
S.D.	Standard derivation
SFC	Supercritical-fluid chromatography
SI	Similarity Index
TLC	Thin layer chromatography
UV	Ultra-violet
WHO	World Health Organization

WRL-68	Human liver normal cell line
ZCR	Zero-concentration region
ZCG	Zero-concentration graph

Symbols

A_{mxn}	Matrix A with dimension m x n	Eq. [2.1]
A_i^T	Different submatrices	Eq. [2.2]
C	Concentration profile	Eq. [2.1]
$\tilde{c}_a$	Concentration profile of component a	Eq. [2.4]
d_P and d_A	Diameter of column	Eq. [5.1]
E	Array of the measurement noise and experimental error	Eq. [2.1]
F_P and F_A	Volumetric flow rate of column	Eq. [5.1]
I	Identity matrix	Eq. [2.2]
L_P and L_A	Length of column	Eq. [5.1]
$\lambda_{\max}$	Maximum absorption wavelength	
n	Number of measured chromatographic point	Eq. [2.3]
P_i	Orthogonal projection matrix	Eq. [2.2]
$\left\|P_i v_a\right\|$	Euvlidean norm of the vector	Eq. [2.3]
R_{N_2}	Nitrogen gas flow rate	
R_s	Peak resolution	Eq. [3.1]
r^2	Correlation coefficient	
r_a	Residue vector of component	Eq. [2.4]
re_i	Length of residue vector a	Eq. [2.3]
S	Spectra	Eq. [2.1]
S_p	Diagonal matrix	Eq. [2.5]
T_d	Draft tube temperature	
t_R	Retention time	Eq. [3.1]

U_p	Score matrix	Eq. [2.5]
V_p	Loading matrix	Eq. [2.5]
v_a	Abstract spectrum of component a	Eq. [2.4]
W	Peak width	Eq. [3.1]
w	Size of window	Eq. [2.3]
X_P and X_A	Injection amount of column	Eq. [5.1]
X_a^0	Zero-concentration region of component a	Eq. [2.4]
$\tilde{X}$	Matrix with resolution information of component a	Eq. [2.4]
X_{target}	Target chromatographic peak cluster of X	Eq. [2.5]
Y_{test}	Test chromatographic peak cluster of Y	
y_j	Original spectrum at the chromatographic point j	Eq. [2.7]
y_j^{op}	Series of corresponding residuals	Eq. [2.7]

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Chapter 1

Introduction

1.1 Background

Chinese medicine (CM) has been used for more than thousands years in Asian countries of China, Japan and Korea (Emboden, 1979). In recent years, it has made the headlines many times worldwide as it is recognized as alternative treatment modalities for treating chronic and age-related diseases, and it is particularly attractive for the prevention of disease and health maintenance. It is also found that the world trade of such natural products is more than US $15 billion per year with 10% annual growth rate (Lee, 2004). Thus, CM plays an important role in the international nutraceutical industries. CM is, however, not regarded as a mainstream medicine in Western medical community because of such complicated composition and lack of sufficient scientific support, which is recalcitrant to current Western medicine. In order to increasingly escalate the role of CM in the mainstream pharmaceutical market, several fundamental issues should be considered, in particular, quality control, safety and proven efficacy.

Nowadays, several techniques can be applied to identify and quantify the complex chemical constituents of CM including Thin layer chromatography (TLC), High performance liquid chromatography with diode array detector (HPLC-DAD), Liquid Chromatography with mass spectrometry (LC-MS), and Gas chromatography with mass spectrometry (GC/MS). In some case, the huge amount of complex data is acquired and difficult to interpret during the analysis of CM with the above instruments. Chemometrics is then a useful data processing tool to analyze and interpret such complex data. It makes use of mathematical, statistical and other logic-based methods for interpreting, predicting chemical data and extracting information, especially in analytical

chemistry. For evaluating of safety and efficacy of CM, appropriate preclinical studies are highly desirable. Details of the chromatographic techniques, chemometric methods and bioassay applied in this study can be found in Chapter 2.

In this research project, three CMs, Radix Ginseng, Radix Panacis Quinquefolii. and Ganoderma amboinense, and two traditional CM formulations, RDLP and Danggui Buxue Tang (DBT) were chosen to investigate their chemical constituents by using chromatographic techniques combined with chemometric methods and / or bioassay. A fast procedure was developed to analyze the commercial products of Radix Ginseng and Radix Panacis Quinquefolii by using HPLC coupled with DAD and evaporative light scattering detector (ELSD) in series. Moreover, the chemical constituents of Ganoderma amboinense and DBT were studied with HPLC-DAD and chemometric methods. Preparative HPLC (PHPLC) technique was applied to isolate the fractions of the water extract of a CM formulation RDLP and to find out its potential active fraction which benefit to anti-cancer activity with *in-vitro* bioassay.

As mentioned, the rapid annual growth rate of the commercial product of Chinese medicine (CM) trades in the world resulting in the quality control, safety, and efficacy of CM have been paid attention in the world, and various approaches have been reported in mainstream journals. Apart from research, education of the quality control of CM is important for tertiary students in Hong Kong and other regions to connect science with their daily life. In order to introduce the concept of its quality control, the commercial products of Radix Ginseng (Oriental ginseng, OGS) and Radix Panacis Quinquefolii (American ginseng, AGS) were selected in this work because these are one of the most

popular CM in the world, and their herbal markers, ginsenosides, are available commercially.

To provide laboratory lesson of the quality control of the Ginseng products, the procedure found in most literatures are quite time consuming and complicated. Thus, the extraction method and the chromatographic condition of HPLC-DAD-ELSD for this analysis were optimized in this work. Here, we succeeded to develop a fast procedure to distinguish OGS and AGS, as well as to identify and quantity the ginsenosides in the samples. Also, this procedure was suitable and flexible enough to conduct the laboratory lesson in tertiary institutes. Details of the development of the fast procedure are discussed in Chapter 3.

Apart from ginseng, *Ganoderma lucidum* is another ancient Chinese medicine which is highly revered in Asian countries and the West, and regarded as an elixir of life for thousand of years. Modern research has revealed that *Ganoderma lucidum* contains a variety of chemical ingredients, including simple and complex carbohydrates, amino acids, organic germanium, triterpenes, nucleosides, alkaloids and numerous mineral elements. In addition, triterpenes and polysaccharides have found to possess potent anti-tumor.

A rare species of an antler form *Ganoderma lucidum*, Ganoderma amboinense (Deer Horn Lingzhi, DH Lingzhi), is found to have most of its chemical constituents at level higher than *Ganoderma lucidum*. However, there is no report found in the literature about the content of nucleosides in DH Lingzhi. In Chapter 4, nucleosides in DH Lingzhi and its cap and stipe were analyzed with HPLC-DAD. It was difficult to determine the

nucleosides content of the complex multi-component system of the DH Lingzhi samples simply using chromatographic techniques. Then, a chemometric method, Evolving window orthogonal projections (EWOP) method, was employed to extract the useful information from the DH Lingzhi samples for quantitative analysis of nucleosides with success.

Based on traditional CM theory, balancing Yin and Yang in body is important to be restored by directing multiple targets to treat a totality of symptoms. In general, a signal herb used for remedy may be insufficient to tackle multiple targets. This is why mixture of herbal medicines, traditional CM formulation, is a major approach for the treatment of illnesses. Hundreds to thousands of different combinations of traditional CM formulations have also been documented and prescribed, from 110 in *Chin Kuei Yao Leuh* to 100,000 in *I Fang Chi Chieh* (Lee, 2004). In this way, chemical constituents in CM formulations become more complicated, and their quality control is a significant challenge. Hence, bioassay-guided fractionation or isolation is introduced to identify the bioactive fractions or compounds from such formulations.

To conduct this approach, preparative chromatographic technique and appropriate bioassay are necessary for fractionation or isolation, and bioactivity assessment respectively. In Chapter 5, the CM formulation RDLP, which has been used for more than 20 years to treat liver cancer, was selected to demonstrate the approach with preparative HPLC (PHPLC) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay for finding out potential bioactive fractions

possessing anti-cancer activity. MTT assay was also utilized to estimate the safety and efficacy of RDLP.

Authentication of traditional CM formulations is commonly taken place by identifying a few chemical markers of component herbs, i.e. "marker approach". However, in some cases, unique markers of component herbs are not available in the commercial market, and the selected markers can not be identified in the formulation. Two-way data acquired by hyphenated chromatographic instruments including HPLC-DAD, LC-MS and GC/MS, can be facilitated by "multi-component" and "pattern" approaches using chemometrics to get more information and achieve the target to larger extent..

DBT, documented for thousands years, is one of the simplest traditional CM formulation from the thousands. It is composed by Radix Angelicae Sinense (Danggui, DG) and Radix Astragali (Huangqi, HQ), and prescribes for women's ailments. Here, three markers of DG and two markers of HQ were selected for authenticating DBT by using HPLC-DAD. Adversely, "multi-component" and "pattern" approaches were carried out by chemometric methods of Multicomponent spectral correlative chromatographic (MSCC) and Similarity index (SI) respectively for analyzing DBT. Details of the investigation are given in Chapter 6.

In this research work, analytical and preparative HPLC were applied to study the selected Chinese medicines and CM formulations. With the helps of chemometric methods and bioassay, additional information of the chromatographic analysis was extract to enhance quality control, safety and efficacy of CM.

Chapter 2

Review on techniques for chromatographic separation, chemometric data treatment and *in-vitro* bioassay

2.1 Introduction

Chinese medicine (CM) has been widely used for thousands of years in many oriental countries, like China, Japan and Korea. During the last decade, use of CM has expanded rapidly in western countries as well. However, the multi-component system of CM is so complicated and its chemical composition depends on the natural habitat including season, humidity, geographical features and others. This may be the major reason why quality control of CM is still much more difficult than that of western drug. As mentioned in "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines", "Despite its existence and continued used over many centuries, and its popularity and extensive use during the last decade, traditional medicine has not been officially recognized in most countries. Consequently, education, training and research in this area have not been accorded due attention and support. The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use world-wide. The reasons for the lack of research data are due to not only to health care policies, but also to a lack of adequate or accepted research methodology for evaluating traditional medicine" (World Health Organization, 2000). This is implied that the quality control of CM has been paid attention to in the world and is significant to the internationalization of CM.

In fact, the quality control of Chinese medicine is a great challenge to scientists as CM contains hundreds of unknown phytochemical components and many of them are unknown. Fortunately, several chromatographic techniques, including Thin layer chromatography (TLC), Gas chromatographic (GC), High performance liquid

chromatography (HPLC), Capillary electrophoresis (CE) and Supercritical-fluid chromatography (SFC), are available and can separate such complex chemical components in CM into individual components to certain extent and can even fractionate the CM extract. Recently, chromatographic instruments coupled with different kinds of spectroscopic detector systems like High performance liquid chromatography-diode array detector (HPLC-DAD), Gas chromatography-mass spectroscopy (GC-MS) and Liquid chromatography-mass spectroscopy (LC-MS) are well developed and advanced and they are widely utilized in the separation science, especially for the separation of CM, such complex system like in CM, because both separation and the spectral information of the system concerned are acquired simultaneously (Leung *et. al.*, 2000; Gong *et. al.*, 1999; Bogusz and Erkens, 1994; Keller *et. al.*, 1992). The chromatographic hyphenated techniques is not only very useful for the qualitative and quantitative analysis, but can also combine with chemometric approach to improve chemical analysis of complex mixtures by minimizing the undesired instrumental interferences, correcting retention time shift and resolving overlapping peaks (Gong *et. al.*, 2001; Manne *et. al.*, 1999; Shen *et. al.*, 1999; Liang and Kvalheim, 1994). Combination of the hyphenated techniques and the chemometric approaches is powerful enough to reveal clearer pictures behind. Thus, they have been applied extensively in recent years to the quality control of CM. Besides, pharmacologists are interested in active ingredients of CM. In order to fulfill such interest, preparative liquid chromatography (PLC) is increasingly utilized to isolate and purify quantity amount of individual components or fractions from CM for further investigation.

Apart from the phytochemical analysis, safety and pharmacological efficacy of Chinese medicine (CM) are of great concern in the field of quality control of CM. Therefore, international health organizations and many governments have developed regulatory requirements for assessing these aspects of CM (Ho *et. al.*, 2003; World Health Organization, 2000). As suggested in the guideline of World Health Organization, toxicity tests including immmunotoxicity, genotoxicity, carcinogenicity and reproductive toxicity are encouraged to be done on CM and CM formulations, with long history, but no documentation available on their side-effects, about the safety assessment, and the requirements for proof of their efficacy (World Health Organization, 2000). In order to assess the safety and efficacy of CM, in general, *in-vitro* bioassays have been regarded as preliminary tests as such results provide indication for designing *in-vivo* tests based on animal test and clinical test. These bioassay are often relatively quick and cheap (Genshow*et. al.,* 2002; Dean, 1997; Lebrec *et. al.,* 1995; Frazier, 1992). In addition, *in-vitro* bioassays can serve as an aid for the discovery of potential active ingredients from CM through different isolation techniques such as preparative HPLC (PHPLC) (Demirezer *et. al.*, 2006; Mandal *et. al.,* 2006; Cottiglia *et. al*., 2005; Ismaili *et. al.*, 2004; Lo *et. al*., 2004; Fall *et. al.,* 2003; Penna *et. al.,* 2001). Hence, this kind of bioassays plays an important role to the assessment of safety and efficacy of CM.

In this research programme, hyphenated chromatographic instruments were the core devices applied to chemical analyses of the selected CMs, Radix Ginseng, Radix Panacis Quinquefolii and Ganoderma amboinense, and the two CM formulations, RDLP and Danggui Buxue Tang (DBT). Chemometric techniques were also included for processing chromatographic data of Ganoderma amboinense (Chapter 4) and DBT (Chapter 6).

Moreover, an *in-vitro* bioassay was utilized to assess the safety and efficacy of the CM formulation RDLP and find its potential active fractions (Chapter 5). In this chapter, all these techniques are briefly described.

2.2 Chromatographic techniques

The first chromatographic technique was invented by Russian botanist Mikhail Semyonovich Tsvet in 1900 during his research on plant pigments, and the colour bend appearance of the separation results was taken as an advantage for the naming of this technique, called chromatography (Skoog *et. al.*, 1996). In the past century, various techniques of this kind, for example, TLC, HPLC, GC etc., have been developed for separating substances with different physical and chemical properties. No matter what differences in these techniques are, their working principles are more or less the same of exploiting the differences in partitioning behavior of analytes between a mobile phase and stationary phase to separate components in a mixture. For example, in HPLC, a liquid mobile phase carries a mixture of analytes passing through a silica stationary phase to separate the analytes from the mixture. Nowadays, chromatography in analytical chemistry is widely applied for the separation, identification and determination of the chemical components in mixtures.

Among these chromatographic techniques, TLC has a long history for the analysis of CM, and very often been recommended by pharmacopoeias including American Herbal Pharmacopoeia, Chinese drug monographs and analysis, and Pharmacopoeia of the People's Republic of China. In recent years, other instrumental chromatographic techniques like HPLC and GC have been introduced and become popular. Apart from

qualitative and quantitative analyses, chromatographic techniques are also applied for isolation and purification of the CM extracts. Preparative liquid chromatographic is a typical example for the application. In this work, both analytical and preparative HPLC were applied and brief descriptions are given below.

2.2.1 Analytical HPLC

HPLC has received the most extensive application in the analysis of CM, and over 1000 articles have been reported. They cover a vast range of applications especially in the area of pharmaceutical and drug analysis and other biologically important compounds (Li *et. al.,* 2005; Robards *et. al.,* 1994). Moreover, HPLC is another major technique for the authentication of CM in the Latest 2005 edition of Pharmacopoeia of the People's Republic of China. Its popularity is owing to its versatility, automation, good reproducibility, and unlimited by the stability of the sample compound (Liang *et. al.,* 2004).

To HPLC separation, column is the spiritual component. Based on the polarity and / or molecular weight of analytes, reversed-phase, normal phase, adsorption, ion exchange and gel exclusion columns can be chosen for HPLC. Among these, reversed-phase (RP) is widely utilized for separating chemical components of CM as they most likely are nonionic polar and non-polar. Examples of this kind are triterpene, flavonoids, nucleosides, tannins, lignans and quinines (Cai *et. al.*, 2006). Also, factors like the composition of mobile phases, their pH value and flow rate are needed to be optimized with the column used to achieve the best performance. On the other hand, detector is important to provide the signals good for characterization of the analysts as well. The

most common detectors coupled with hyphenated HPLC instruments are diode-array detector (DAD), mass spectroscopy (MS) and evaporative light scattering detector (ELSD). DAD and ELSD were employed in this work, and are described in Section 2.2.1.1 and 2.2.1.2.

2.2.1.1 Diode-array detector (DAD)

As mentioned in Section 2.1, DAD is a one of the widely used detectors for liquid chromatography. The detection of DAD is based on absorption of ultraviolet or visible radiation by the samples. Using our DAD, absorption in the range of wavelengths (190-400 nm) can be measured simultaneously (Skoog *et. al.*, 1996). Since the spectra acquired related to the structural features of the organic functional groups of the analyte concerned, this information is good for identification and/or confirmation of the target and the unknown chemical compounds. This is especially helpful to the analysis of Chinese medicine (CM), on top of the retention times of the CM constituents that are used solely in this kind of study by most analytical chemists.

The data set obtained from HPLC-DAD is called two-way data sets in chemometrics with one way for chromatogram and another way for spectrum. These information-rich data sets can give more chance to solve the difficult problems in the CM analysis with the aids of chemometrics. This is another reason why DAD is a powerful tool for the analysis of CM and was employed in our work.

2.2.1.2 Evaporative light scattering detection (ELSD)

ELSD is the mainstream of detection choices of liquid chromatography separation for the application of carbohydrate (Bento and Sa, 1998; Herbreteau *et. al.,* 1992; Macrae and Dick, 1981), lipid (Sugawara and Miyazawa, 1999; Elfmanborjesson and Harrod, 1997; Pons *et. al.,* 1997; Vaghela and Kilara, 1995) and amino acid (Chaimbault *et. al.,* 2000). It is also an attractive complement to spectroscopic detectors and HPLC-DAD-ELSD for other applications in order to compensate the limitations of spectroscopic detections (Young and Dolan, 2003). It is not only capable to detect non-chromophoric compounds, but also can eliminate the solvent front peak and produce a flat and stable baseline during gradient elution. Because of these advantages, a serial coupling of DAD and ELSD were applied also in this study as mentioned in Chapter 3.

The working principle of ELSD can be broken down into three stages of nebulization, evaporation and detection (Alltech, 2000). In the first stage, the chromatographic effluent is nebulized by a stream of pressurized nitrogen into droplets, from which the mobile phase can be easily evaporated at the second stage. Next, the droplets are carried by the nebulizing gas toward the evaporator, where evaporation occurs and more volatile mobile phase is converted to gas, and the non-volatile analytes remain as particle. Finally, the solute particles emerging from the evaporator enter the light cell where they are directed toward a laser beam. The light scattered by the analyte particles is then measured by a photomultiplier. The signal peak area is related to the concentration of the analyte in the effluent.

2.2.2 Preparative HPLC (PHPLC)

PHPLC was firstly introduced in 1980s for the production of specialty chemicals (Miller, 2003). In comparison to analytical HPLC (ALC), the basic principle of PHPLC is almost the same, but their applications are quite different. ALC is to separate a mixture of compounds for identification and quantitative analyses; while PHPLC is to isolate in quantity the separated compound or fraction from mixtures for further study like structural determination, bioassays, production of pharmaceuticals etc.

In practice, optimization of the chromatographic condition of PHPLC is quite complicated because many factors including column properties, packing quality, particle size and shape of column and sample solubility should be taken into account, and these are rarely considered at ALC. Therefore, in the past twenty years, many reports have suggested different guidelines for dealing with this aspect (Miller, 2003; Wennberg *et. al.*, 2001; Heuer *et. al.*, 1996; Porsch, 1994; Hairsine, 1986). Fundamentally, ALC should carry out preliminary runs first to allow scale up and provide an initial predicted value for the optimization in the preparative scale with the same type of stationary phase material (Verrall, 1998).

PHPLC is regarded as a costly method in its initial capital investment and running cost. However, in the past decades, the uses of PHPLC keep increasing especially in pharmaceutical industry. It is not only due to its flexible of scale for sample recovery from micrograms to kilograms, but also its speed and efficiency making the fast turnaround to get profits (Miler, 2003).

2.3 Chemometric data processing techniques

Chemometrics began in the mid 1970s, and its most popular definition is that the chemical discipline that use mathematical, statistical and other methods employing formal logic to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information by analyzing chemical data (Massart, 1988). Currently, chemometrics are widely applied in analytical chemistry for experimental design, pattern recognition, and data processing.

As mentioned, combination of the hyphenated chromatographic techniques, HPLC-DAD, LC-MS and GC-MS, and chemometric techniques is a very powerful approach for quality control of Chinese medicine. Many literatures have also reported this approach. For instance, HPLC-DAD with principal component analysis (PCA) was applied in the quality control and discrimination of *Pericarpium Citri Reticulatae* and *Pericarpium Citri Reticulatae Viride* (Yuan *et. al.,* 2007). HPLC-DAD with local least squares (LLS) and PCA was adopted to evaluate the quality of 33 different *Erigeron breviscapus* herbal samples (Li *et. al*. 2004). GC-MS with evolving chemometric approach was used to resolve the essential constituents of Ramulus cinnamomi (Xu, 2001). In this study, Evolving window orthogonal projections (EWOP) method, Multicomponent spectral correlative chromatographic (MSCC) method and Similarity index (SI) were applied for resolving the overlapping chromatographic peaks and facilitate the comparison of relevant chemical components from different herbal samples respectively. Their methodologies are described in the following sections.

2.3.1 Evolving window orthogonal projections (EWOP) method

The chemometric method EWOP was designed by Xu *et. al*. and published in 1999 (Xu *et. al.,* 1999). Its theory is briefly described here and more detail can be found in the reference. When a sample is measured by HPLC-DAD, the two-way data set can be expressed as a matrix A (mxn). Here $A_{m\times n}$ with dimension mxn represents an absorbance matrix expressing the chromatographic profiles of m retention time points measured at n different wavelengths. Based on the Lamber-Beer Law, the matrix can be represented by the product of the concentration profile (C) and the spectra (S) as follow:

$$A_{m\times n} = CS^{T} + E \qquad [2.1]$$

Where the superscript T denotes matrix or vector transposition and E is the array of the measurement noise.

For the resolution of a chromatographic overlapping peak profile, the zero-concentration region (ZCR) and the selective region are two essential parts. The former one is the region with zero concentration profile of one certain component; while the latter one is the region of pure concentration profile of that component. In order to obtain the ZCR of a certain component accurately, its extracted pure spectrum is projected upon a series of evolving windows. By using the orthogonal projection approach, the orthogonal projection matrix P_i on the complementary subspace A_i^T is defined as

$$P_i = I - A_i^T \left(A_i^T\right)^{+} \qquad [2.2]$$

Where the superscript + denotes the Moore-Penrose pseudoinverse, I is the identity matrix, and A_i^T represents different submatrices, which are a series of fixed size window matrices moving along the chromatographic direction. Then the length of residue vector (re_i) is given by

$$re_i = \left\| P_i v_a \right\|^2 (i = 1,2,..., n - w + 1) \qquad [2.3]$$

Here, v_a denotes the abstract spectrum of component a and $\left\| P_i v_a \right\|$ designates the Euclidean norm of the vector, n is the number of measured chromatographic point and w is the size of window.

Plotting the value of re_i against the index i, the zero-concentration graph (ZCG) is obtained and the ZCR (X_a^0) and the selective region of say, component a can be identified immediately. In the ZCG, the ZCR is discerned by the region where re_i has a value significantly greater than zero; otherwise selective region is found. Afterwards, the concentration profile of components ($\tilde{c}_a$) can be calculated by the following equation:

$$\tilde{c}_a = \frac{\tilde{X} r_a}{v_a \left\{ I - (X_a^0)^T \left[(X_a^0)^T \right]^+ \right\} v_a} \qquad [2.4]$$

Where $\tilde{X}$ is a matrix that holds resolution information of component a, and r_a is the residue vector of component a. Finally, the concentration of component a can be determined.

2.3.2 Multicomponent spectral correlative chromatographic (MSCC) method

The MSCC method was developed by the research group of Prof. Liang in Central South University in 2004 (Hu *et. al.*, 2004). It is very useful for quality control of CM by comparing the relevant chemical components from different herbal samples. This method is briefly described here and the details can be found in the publication of Hu.

Two-way data matrices X and Y generated by hyphenated instrument represent two chromatograms of different samples. Their submatrix $X_{t\arg et}$ is a target chromatographic peak cluster of X, and Y_{text} is a tested chromatographic peak cluster of Y, which is spectral correlative to $X_{t\arg et}$. In order to extract the spectral features from $X_{t\arg et}$, the target cluster of $X_{t\arg et}$ can be decomposed by singular value as below,

$$X_{t\arg et} = U_p S_p V_p^T + E \qquad [2.5]$$

where U_p and V_p are the score and loading matrices, respectively, and S_p denotes a diagonal matrix. The subscript p represents the number of principal components (PCs) contained in $X_{t\arg et}$. The superscript T indicates the transpose of matrix. E is the instrumental noise, experimental error, and the part of the data of $X_{t\arg et}$.

Now, an orthogonal projection matrix P can be constructed as

$$P = I - V_p V_p^T \qquad [2.6]$$

P depicts a complementary space of V_p, which spanned by the p principal spectral features, and I denotes an identity matrix.

Subsquently, every original spectrum y_j is projected and recorded at the chromatographic point j of the two-way spectochromatogram Y onto this operator P, and a series of corresponding residuals y_j^{op} are acquired, by the following way.

$$y_j^{op} = Py_j (j = 1,...,m) \qquad [2.7]$$

Where m means the chromatographic length of y measured at regular time intervals. If the spectrum y_j embraces the spectral information correlating to p feature spectra of V_p, the correlative information would be removed from the original y_j, and the Euclidean norm (re_j) of its projected residual should be zero theoretically,

$$re_j = \left\| y_j^{op} \right\| = 0 \qquad [2.8]$$

Actually, because of the influence of instrumental noise and experiment error, the projected residual norm can only be closed to zero. Therefore, the inner-product coefficient between the original y_j and its projected residual y_j^{op} was proposed in the method, that is,

$$r_j = \frac{y_j^T y_j^{op}}{\left\| y_j \right\| \left\| y_j^{op} \right\|} (j = 1,...,m) \qquad [2.9]$$

The values r_j (j = 1,…,m) are in the range of $0 \le r_j \le 1$. A projection curve about r_j (j = 1,…,m) can be obtained accordingly in the chromatographic direction of spectrochromatogram Y. This curve exhibits the spectral information correlating to the peak cluster X_{target}. From the definition, one can easily find that if correlation between y_j and the feature spectra within V_p is very high, the values of r_j (j = 1,…,m) will lead to zero. Therefore, if all the values of r_j within the chromatographic region where cluster Y_{test} is spectral correlative to the peak cluster X_{target}, one will conclude that cluster Y_{test} is spectral correlative to the cluster X_{target}, and these two chromatographic clusters contain of common components.

2.3.3 Similarity index (SI)

Similarity index (SI) is one of the most common methods used to comparing and contrasting spectra to identify unknown compounds, test the reproducibility of instruments, and check the reliability of methods under development. As the rapid growth of use of the chromatographic hyphenated technique for the quality control of CM, SI is also very useful to make judgments about similarity between chromatographic fingerprints of CM from different sources and / or different batches of productions.

$$SI = \frac{x^t \bullet \overline{X}}{norm\left|x^t\right| \bullet norm\left|\overline{X}\right|} \qquad [2.10]$$

The larger value of SI between the test fingerprint x and the mean vector of training fingerprint sets (X_A, X_B, or more) indicates that these fingerprints have more common

spectral features with each other. Then, the test fingerprint *x* can be discriminated based on comparing the magnitudes of the corresponding SI.

2.4 Bioassay of anti-cancer activity

As mentioned before, toxicity test is one of suggested tests from WHO for estimating the safety of CM. Generally, *in-vitro* toxicity test mainly looks for viable (living) cell number and proliferation of target cell lines treated with substances such as CM extracts by measuring with various bioassays. In many experimental situations, rapid and accurate assessment of viable cell number and cell proliferation is essential requirement to *in-vitro* studies. Typically, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide, a tetrazole) cell proliferation assay, trypan blue staining, BrdU (5-bromo-2'-deoxy-uridine) incorporation assay and flow cytometry are regarded as a fast and reliable *in-vitro* bioassay for cell proliferation study. Among these bioassays, MTT cell proliferation assay was chosen in this investigation on the safety and the anti-cancer activity of RDLP (Chapter 5), and it's the method is briefly given below.

2.4.1 MTT cell proliferation assay (MTT assay)

MTT assay is one of the popular colourimetric assays in a laboratory test for investigating cell proliferation by measuring the colour changes. It is commonly used to determine cytotoxicity of potential medicinal agents and other toxic materials.

In the assay, yellow MTT salt is reduced to purple formazan crystal in the mitochondria of living cells (Fig. 2.1). A solubilization solution such as dimethyl sulfoxide (DMSO) is

added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this coloured solution can be quantified by measuring at 570nm or a certain wavelength within the optimal range between 500 and 600 nm by a spectrophotometer (Mosmann, 1983).

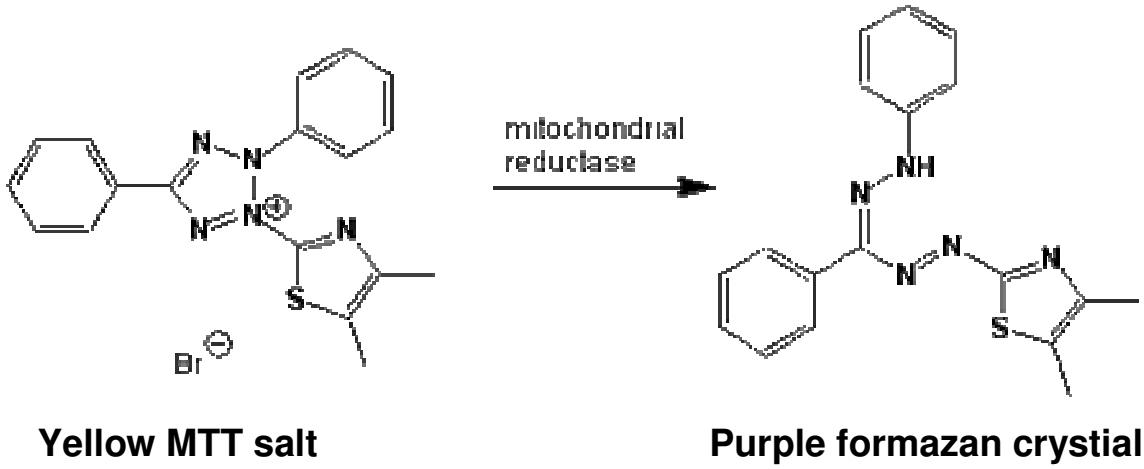


Fig. 2.1 Chemical reaction of MTT assay

This reduction reaction takes place when mitochondrial reductase enzymes are active to carry out the dehydrogenation of nicotinamide adenine dinucleotide (NADH), and therefore conversion is directly related to the number of living cells. When the absorbance of purple formazan produced by cell treated with an agent is compared with that of formazan produced by untreated cells, as control, the effectiveness of the agent in causing death of cells can be come up with by the plot of a dose-response curve where 50% inhibitory concentration can be determined (Wilson, 2000).

Chapter 3

Development of a Fast Procedure for Chemical Analysis of Commercial Products of Radix Ginseng and Radix Panacis Quinquefolli by High Performance Liquid Chromatography coupled with Diode Array and Evaporative Light Scattering Detectors (HPLC-DAD-ELSD)

3.1 Introduction

Oriental herbal products, including herbal and Chinese medicines, are a treasured legacy of Asian peoples. Multicomponent-processed natural products are used both as daily functional foods and as drugs to maintain health and treat disease states. Nowadays, oriental herbal products have a prominent role in nutraceutical market as dietary supplements for different health issues in the world. The passage of the U. S. Dietary Supplement Health Education Act of 1994 has mentioned the sales of dietary supplements have more than doubled (Richter, 2003), and the current growth rate of herbal products is around 11% per year (Blumenthal, 1999). Hence, international health organization and governmental agencies of World Health Organization (WHO) and the US Food and Drug Administration (FDA), respectively, have also recognized the consumer popularity of herbal products (Ho *et. al.*, 2003). As the fast growth rate of the herbal supplements in the nutraceutical market and no regulation as drugs is available, there are great concerns to regard their purity and potency (Angell and Kassirer, 1998). Some herbal products have also been reported to contain potential harmful adulterants (Ko, 1998; Slifman *et. al.,* 1998; Blumenthal, 1997), and have distinct variation contents of ingredients (Cui *et. al.*, 1994). In response, the safety, efficacy and quality of herbal products must be recognized through modern scientific research. Hence, the quality control of herbal products is one of the hot topics to chemist and pharmacologist.

Apart from research, education of the basic concept of quality control of herbal medicine is highly desirable for undergraduate students in order to encourage them to connect science with their daily life. Basically, the quality control in the chemical approach is the

identification and the quantitative study of the specific herbal marker for a herbal product with various chromatography. The commercial ginseng products were selected in the study as ginsengs are one of the most popular herbal medicines worldwide (Harkey *et. al.,* 2001; Hu, 1977). Also, there are a variety of plants belonging to the broad species of *Panax*. Each *Panax* species may, however, have slightly different bioactivities with the variation of their unique herbal markers, ginsenosides (Hobbs, C., 1996; Huang, K. C., 1993). For example, Oriental and American ginsengs have similar appearance, but their nature and some pharmacological activities are different.

In order to design laboratory experiments for undergraduate student, simple and quick experimental procedure is necessary. However, the chemical analysis procedures of ginsenosides in ginseng commercial products reported in the literature is quite time consuming and complicated, and are not suitable for education purpose (Fuzzati, 2004; Li and Fitzloff, 2002; Harkey *et. al.*, 2001; Liberti and Marderosian, 1978). Therefore, we tried to develop a fast and simple procedure including sample preparation and HPLC condition for analyzing the six major ginsenosides, Rf, Rg1, Rc, Rb1, Rb2 and Rd, in ginseng products. All these six ginsenoside standards were utilized to optimize the HPLC-DAD-ELSD chromatographic condition, and two commercial ginseng products prepared from Radix Ginseng and Radix Panacis Quinquefolii were selected. Finally, both products were qualified by comparing the total ginsenoside content obtained with that of labeled on the products. Although there are many different ginseng products available in the market, only two of them are selected in this work because of the limitation of experimental time period and differentiation of the samples with ginsenoside Rf.

3.1.1 Background

Oriental ginseng and American ginseng were the root part of *Panax ginseng* and *Panax quinquefolius* respectively. Two kinds of ginsengs belong to the same family of Araliaceae. Oriental ginseng derived from the cultivated form is known as "Yuanshen" (Garden Ginseng) and wild origin is known as "Shanshen" (Wild Ginseng). Their appearance is very similar: "Mainly roots fusiform, cylindrical or conical, 3-12cm in length, 1-2 cm in diameter; externally yellowish-brown, the upper part root exhibiting round and coarse transverse-striations and distinct longitudinal wrinkles; the lower part bearing several lateral roots. Texture hard, fracture yellowish-white, slightly starchy, bark exhibiting yellow-brown dotted resin canals, cambium ring brownish-yellow. Odour, slight and characteristic; taste slightly bitter and sweet" (Tu, 1995).

The chemical constituents of ginseng root have been investigated since the beginning of the 20th century. Several classes of compounds have been isolated and they are triterpene; saponins; essential oil; polyacetylenes; polysaccharides; nitrogen-containing compounds; and various ubiquitous compounds such as fatty acids, carbohydrates and phenolic compounds (Tang and Eisenbrand, 1992). Among the others, triterpene saponins are the major constituents of ginseng. The saponins in ginseng roots are known as ginsenosides. Ginsenosides can be divided into three categories (Li *et. al.*, 1998; Tang and Eisenbrand, 1992):

1. Protopanaxadiol, ginsenosides with 20*S*-protopanaxadiol as aglycone e.g. Rb1, Ra, Rb2, Rc and Rd;

2. Protopanaxatriol, ginsenosides with 20*S*-protopanaxatriol as aglycone e.g. Rg1, Re, Rf and Rg2;

3. Oleanolic acid e.g. ginsenoside R0.

The chemical structures of some selected ginseosides are listed in Figure 3.1.

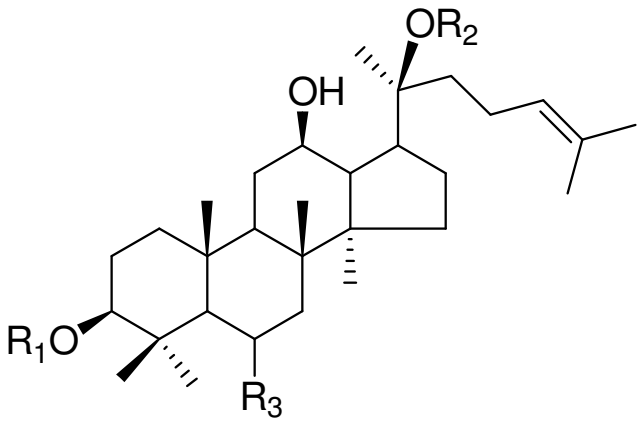


Fig. 3.1 Structures of six selected ginsenosides (Tang and Eisenbrand, 1992)

Ginsenoside	R_1	R_2	R_3	Molecular Formula	Molecular Weight
Rb_1	-Glc-Glc	-Glc-Glc	-H	$C_{54}H_{92}O_{23}$	1108
Rb_2	-Glc-Glc	-Glc-Ara(p)	-H	$C_{53}H_{90}O_{22}$	1078
Rc	-Glc-Glc	-Glc-Ara(f)	-H	$C_{53}H_{90}O_{22}$	1078
Rd	-Glc-Glc	-Glc	-H	$C_{48}H_{82}O_{18}$	94
Rf	-H	-H	-O-Glc-Glc	$C_{42}H_{72}O_{14}$	800
Rg_1	-H	-Glc	-O-Glc	$C_{42}H_{72}O_{14}$	800

Remarks:

Glc	: glucose	Ara (f)	: arabinose in furanose form
Ara (p)	: arabinose in pyranose form	Rha	: rhamnose

Each kind of ginseng would possess different composition of ginsenosides. As the ginsengs in this study, ginsenoside Rf is found in Oriental ginseng, but not American ginseng (Ho *et. al.*, 2003). This slightly difference in the ginsenoside composition of these ginseng can cause the ginseng having different nature and pharmacological actions. The pharmacological actions of Oriental and American ginsengs are listed in Table 3.1.

Table 3.1 Pharmacological actions of Oriental and American ginsengs (Guo *et. al.*, 2001; Tang, W. and Eisenbrand, G., 1992)

Pharmacological actions	
Oriental ginseng	American ginseng
➢ decrease the blood sugar levels ➢ stimulate the pituitary-adrenocortical system; ➢ affects on lipid metabolism; ➢ promote phagocytosis and enhancing lymphocyte-blastogenesis rate; ➢ stimulate the immunological function.	➢ promote secretion of body fluids; ➢ protective effect on liver injury; ➢ stimulate the lymphocyte and induce the interleukin activities; ➢ stimulate the cytokine production from spleen lymphocyte; ➢ decrease platelet aggregation rate; ➢ facilitates male copulatory behaviors; ➢ protect the low density lipoproteins from oxidation; ➢ immunostimulant activity.

3.2 Experimental

3.2.1 Samples of commercial ginseng products, chemicals and reagents

Six ginsenosides (Rf, Rg1, Rc, Rb1, Rb2 and Rd) standards were employed in the present study on the commercial products of Radix Ginseng and Radix Panacis Quinquefolii. Ginsinosides Rf and Rg1 were purchased from International Laboratory (USA), and ginsinosides Rc, Rb1, Rb2 and Rd were acquired form Wako (Japan). Two commercial ginseng products which were Ginseng tablet and American Ginseng capsule were obtained from a local health food store. Details of the samples are listed in Table 3.2. Chromatographic grade methanol (MeOH) and acetonitrile (ACN) were obtained from Tedia (USA). Analytical grade formic acid was obtained from Sigma-Aldrich (USA). Double deionized water utilized from preparing mobile phase was purified by Milli-Q water system (Millipore Corp., Bedford, MA, USA).

Table 3.2 Information of the two commercial ginseng products in this study

No. (Symbol)	Ginseng product (Lot No.)	Brand	Description	Country	Percentage of ginsenosides labeled	Appearance
1 (AGS)	American White Ginseng (35851D4366)	GNC	➢ 500 mg per capsule ➢ Made from American white ginseng root powder (*Panax quinquefolium*)	USA	5% (around 15mg per capsule)	
2 (OGA)	Ginseng (4DA0394)	YourLife	➢ 100 mg per tablet ➢ Made from *Panax gineng* root extract (*Panax ginseng*)	USA	7%	

3.2.2 Optimization of experimental condition

3.2.1.1 HPLC-DAD-ELSD Chromatographic conditions

An Agilent Series 1100 HPLC system (Agilent Technologies, Inc., CA), coupled with a HP1100 diode array detector (Aglient Technologies, Inc., CA) and an Alltech ELSD 2000 evaporative light scattering detector (Alltech Associates, Inc., USA) serially was used. All separations were performed on a C_{18} reversed-phase HPLC column (Hypersil ODS, 250 x 4.6 mm I.D., 5 μm, Thermo Fisher Scientific, Inc., USA). The associated computer software of the Hewlett Packard (HP) HPLC-DAD system, HP ChemStation, was utilized for the processing the chromatographic data obtained.

Optimization of HPLC-DAD-ELSD chromatographic condition involved two parts which were the peak separation of the six ginsenosides and the operation condition of ELSD. The peak separation was dependent on the mobile phase composition and flow rate of elution. The operation condition of ELSD was including nitrogen gas flow rate drift temperature and a mode of impactor. The working principle of ELSD can find the details in Section 2.2.1.2. The stepwise processes carried out to optimize the peak separation and the ELSD parameters.

The mobile phase for HPLC separation of the six ginsenosides was a binary mixture of ACN and 0.1 % formic acid in double deionized water. The gradient of the mobile phase composition with time are shown in Table 3.3 (Li *et. al.*, 2005), and the elution system was labeled as C1. The peaks separated with C1 was then evaluated with peak resolutions, R_s, between two ginosensides nearby each other by equation 3.1 (Skoog *et al.*,

1996), and five resolutions were considered. Here, each R_s should be not less than 1.5; otherwise the elution system would be modified. The flow rate of C1 was 0.7 ml/min. The flow rate was also adjusted repeatedly till the elution completed within 30 minutes, and the peak resolutions reached the above criterion as well. The UV absorption of ginsenosides was measured at 203nm. The HPLC column was kept at room temperature.

$$R_s = \frac{2[(t_R)_2 - (t_R)_1]}{W_1 + W_2} \qquad [3.1]$$

Where $(t_R)_1$: retention time of an analyte 1

$(t_R)_2$: retention time of an analyte 2 neighboring analyte 1

W_1 : peak width of an analyte 1

W_2 : peak width of an analyte 2 neighboring analyte 1

Table 3.3 Gradient of the mobile phase composition of elution system C1

Elution time (min)	ACN (%)	0.1% Formic acid (%)
0	25	75
8	26	74
10	33	67
30	45	55

Five ELSD operation conditions were selected from the ELSD operation manual (Alltech, 2003) and are listed in Table 3.4. Two sets of drift tube temperature (T_d) and nitrogen gas flow rate (R_{N2}) were estimated by calculating the mobile phase composition at the beginning and the end of the optimized elution system. The calculations of T_d and R_{N2} are shown in Appendix 3.1. Each set of T_d and R_{N2} was applied in both ON and OFF impactor modes. One of five operation condition in ON impactor mode was

recommended by the operation manual. The baseline of each set of the operation condition was observed and, a set of condition chosen was the most flatten baseline achieved.

Table 3.4 Five sets of the operation condition of ELSD for detecting the six ginsenosides

Set	Nitrogen gas flow rate (R_{N_2}, L/min)	Drift tube temp. (T_d, ^oC)	Impactor mode
E1	2.8	103.8	OFF
E2	2.7	99.3	OFF
E3	2.8	103.8	ON
E4	2.7	99.3	ON
E5*	1.5	40.0	ON

*recommended by Alltech Associates, Inc. (Alltech, 2003)

3.2.1.2 Sample preparation

0.5 g of the powder of each ginseng sample was mixed with 5 ml methanol and extracted by the sonication or homogenization method. The extracting durations sonication method were 15 minutes (Method 1A) and 30 minutes (Method 1B), while those by homogenization method were 5 minutes (Method 2A) and 10 minutes (Method 2B). The extracts were centrifuged in 500 rpm for 2 minutes. The supernatants were collected and filtered through 0.45 μm Nylon-66 filters (Alltech, USA). Eight extracts were obtained from the two commercial samples. The extracts from OGS were labeled as O1A, O1B, O2A and O2B, while those from AGS were marked as A1A, A1B, A2A and A2B. The whole optimization scheme of the six ginsenosides from the ginseng commercial

products can be found in Figure 3.2. The total peak areas of the six ginsenosides (Rg1, Rf, Rb1, Rc, Rb2 and Rd) in each extract were studied after the extracts were analyzed by HPLC-DAD under the optimized chromatographic condition as mentioned Section 3.3.1.1.

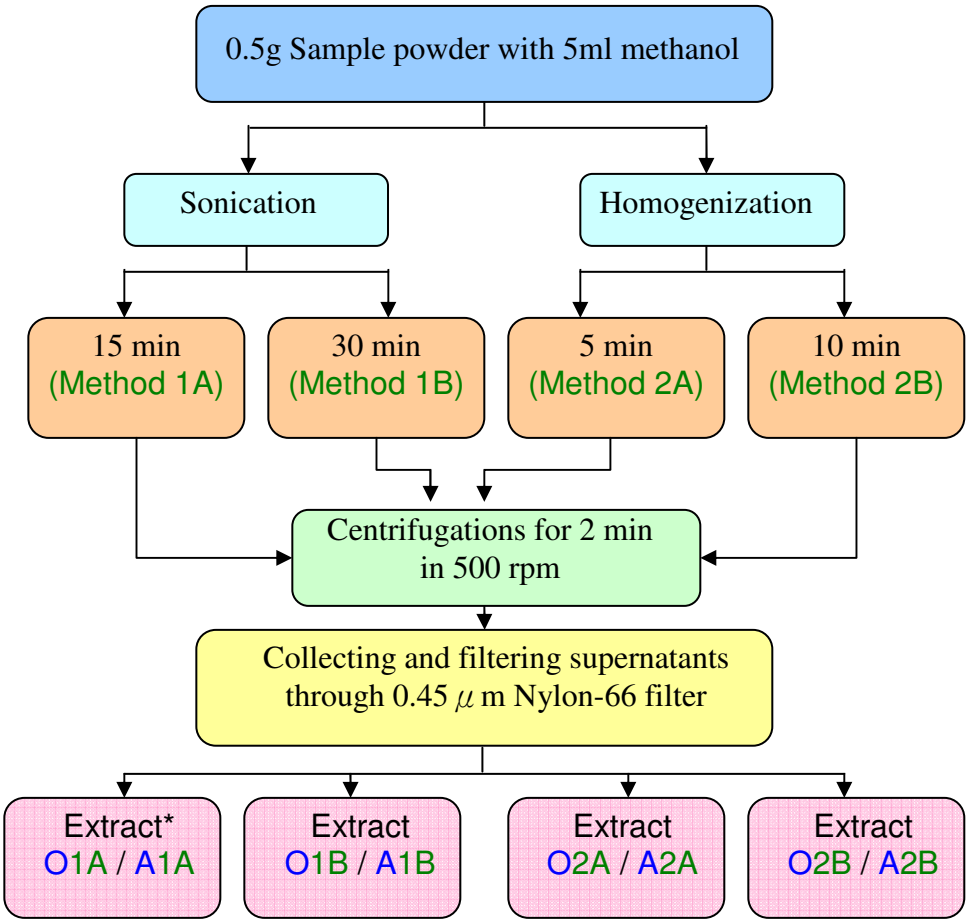


Fig. 3.2 Optimization scheme for the extraction of the six gensinosides from the two ginseng commercial products. *Labeling of extracts indicates the samples and the method used; for example, O1A represents the Oriental ginseng (O) extracted by Method 1A.

3.3 Results and Discussion

3.3.1 Optimization of experimental condition

3.3.1.1 HPLC-DAD-ELSD chromatographic condition

Figure 3.3 showed the chromatogram of the six ginsenosides by using the elution system C1 (Table 3.3) found in the literature (Li *et. al.*, 2005). Most ginsenosides, Rg, Rc, Rb2 and Rd, were well separated except two Rf and Rb1 with two resolutions (R_s) less than 1.5 (Table 3.5). In order to separate all the six ginsenosides, the elution system C1 was modified by trial-and-error approach.

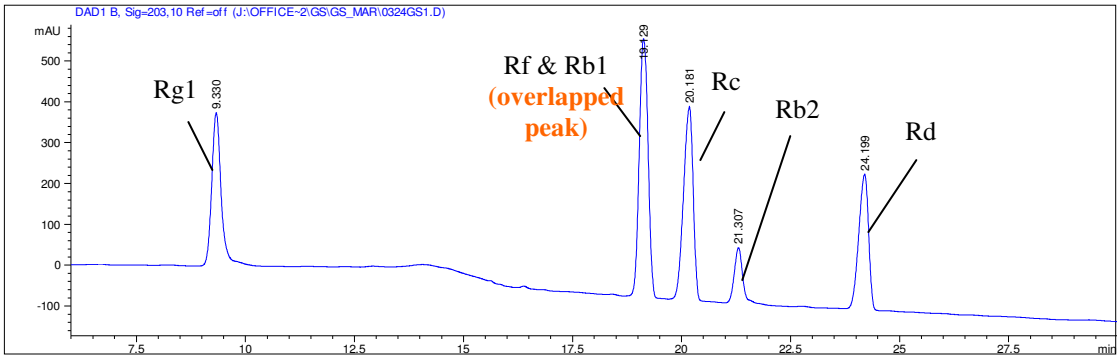


Fig. 3.3 HPLC-DAD chromatogram of the six ginsenoside standards obtained by using elution system C1 (Table 3.3)

Then, the gradient of the mobile phase composition and the flow rate of the elution system C1 (Table 3.3) were modified, so as to resolve the overlapped peak, ginsenosides Rf and Rb1. Seven other elution systems were tried also, and their resolutions and time of complete elution can be found in Table 3.5. Based on the criteria specified in Section 3.2.1.1, elution system C8 achieved that all the resolutions were over 1.5 and the separation was completed within 30 minutes. The HPLC-DAD chromatogram of elution system C8 (Table 3.6) and its mobile phase composition are given in Figure 3.4. The flow rate of elution system C8 was 1.2 ml/min. Hence, the elution system C8 was employed for subsequent study on the commercial ginseng products, AGS and OGS. The

chromatograms and mobile phase compositions of the other elution systems can be found in Appendix 3.2.

Table 3.5 Peak resolutions of the six ginsenoside standards, and the time of complete elution of the eight elution systems

Elution system	R_{s1}	R_{s2}	R_{s3}	R_{s4}	R_{s5}	Time of complete elution (min)
C1	42.7	0.0	4.5	5.0	12.7	25
C2	61.5	8.3	5.9	7.2	16.3	31
C3	45.8	3.5	5.2	6.2	15.0	35
C4	62.9	5.3	4.6	5.4	14.1	34
C5	47.9	10.0	5.6	6.5	15.5	30
C6	57.0	10.4	4.9	5.8	13.4	33
C7	58.0	13.3	5.1	6.2	13.8	30
C8	**59.0**	**15.3**	**5.1**	**6.1**	**13.5**	**29**

Remark:

R_{s1}: Peak resolution between Rg1 and Rf

R_{s2}: Peak resolution between Rf and Rb1

R_{s3}: Peak resolution between Rb1 and Rc

R_{s4}: Peak resolution between Rc and Rb2

R_{s5}: Peak resolution between Rb2 and Rd

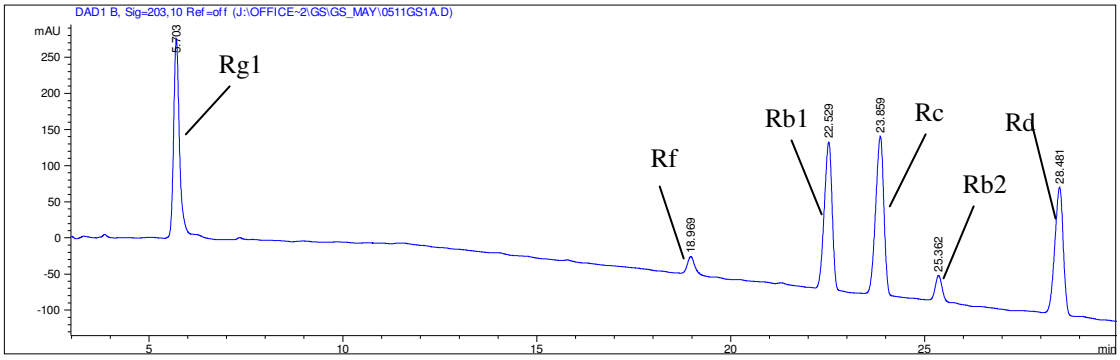


Fig. 3.4 HPLC-DAD chromatogram of the six ginsenoside standards obtained by using elution system C8 (Table 3.6)

Table 3.6 Gradient of the mobile phase composition of elution system C8 at *1.2 ml/min*

Elution time (min)	ACN (%)	Formic acid (0.1%) (%)
0	25	75
8	26	74
20	33	67
30	36	64

The HPLC-ELSD chromatograms obtained by Set E1 and E2 (Table 3.4) are presented in Figure 3.5 and their baselines suddenly rise up rapidly and flatten at the maximum intensity. This phenomenon was found in both sets of condition operated in the impactor OFF mode as the effluent from HPLC was not completely evaporated. Impactor OFF mode was not applicable in this study. Actually, the conditions of Sets E1 and E2 were not run through the analyses because the accumulation of the effluent would cause poor sensitivity of the detector. As under this situation, the tube temperature and the nitrogen gas flow rate were increased, and the impactor mode was changed to ON immediately in

order to evaporate the effluent rapidly and allowed the baseline of the chromatogram going back to zero.

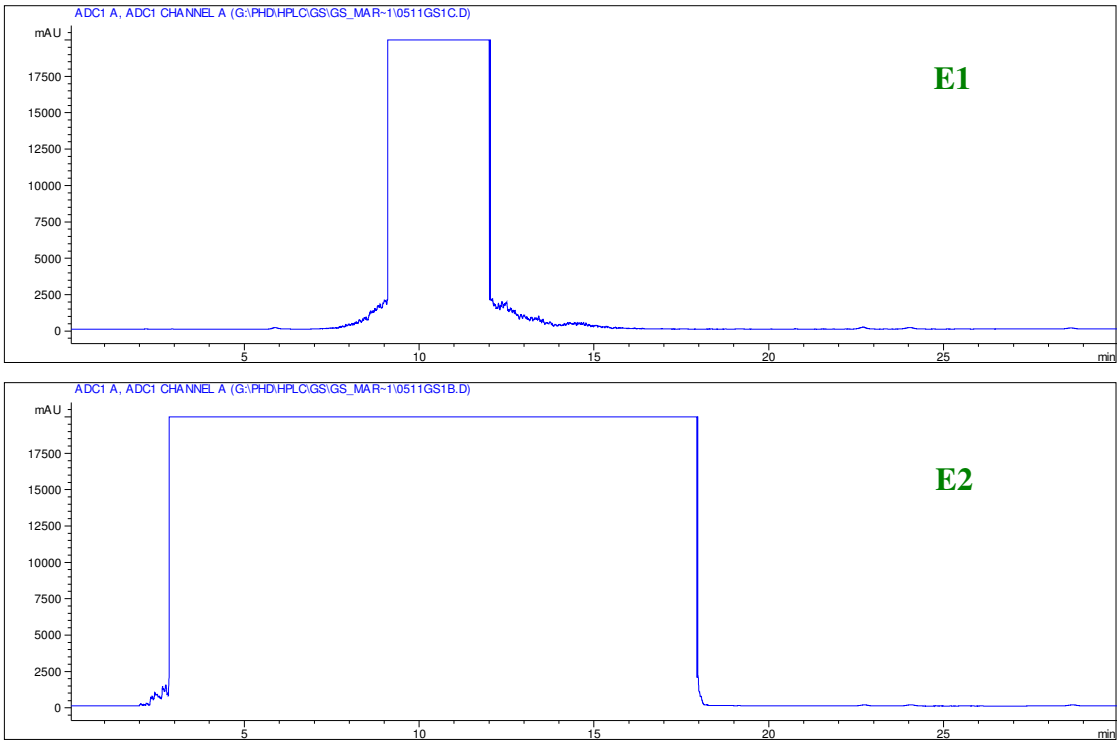


Fig. 3.5 HPLC-ELSD chromatograms of Set E1 and Set E2 (Table 3.4)

The ELSD conditions of Set E3, E4 and E5 (Table 3.4) were applied successfully to detect the six ginsenosides and their chromatograms (Fig. 3.6). This indicated the ON mode of impactor is important to evaporate the effluent efficiently under different tube temperature (T_d) and nitrogen gas flow rate (R_{N_2}). The T_d and R_{N_2} were also important to affect the baseline of the chromatogram. Among the Regions 1 from Set E3, E4 and E5 (Fig. 3.6), the baselines of Set E5 was the flattest resulting from the low T_d and the slow R_{N_2} performed steadily in ELSD. Hence, the ELSD operation condition of set E5 was employed in this work.

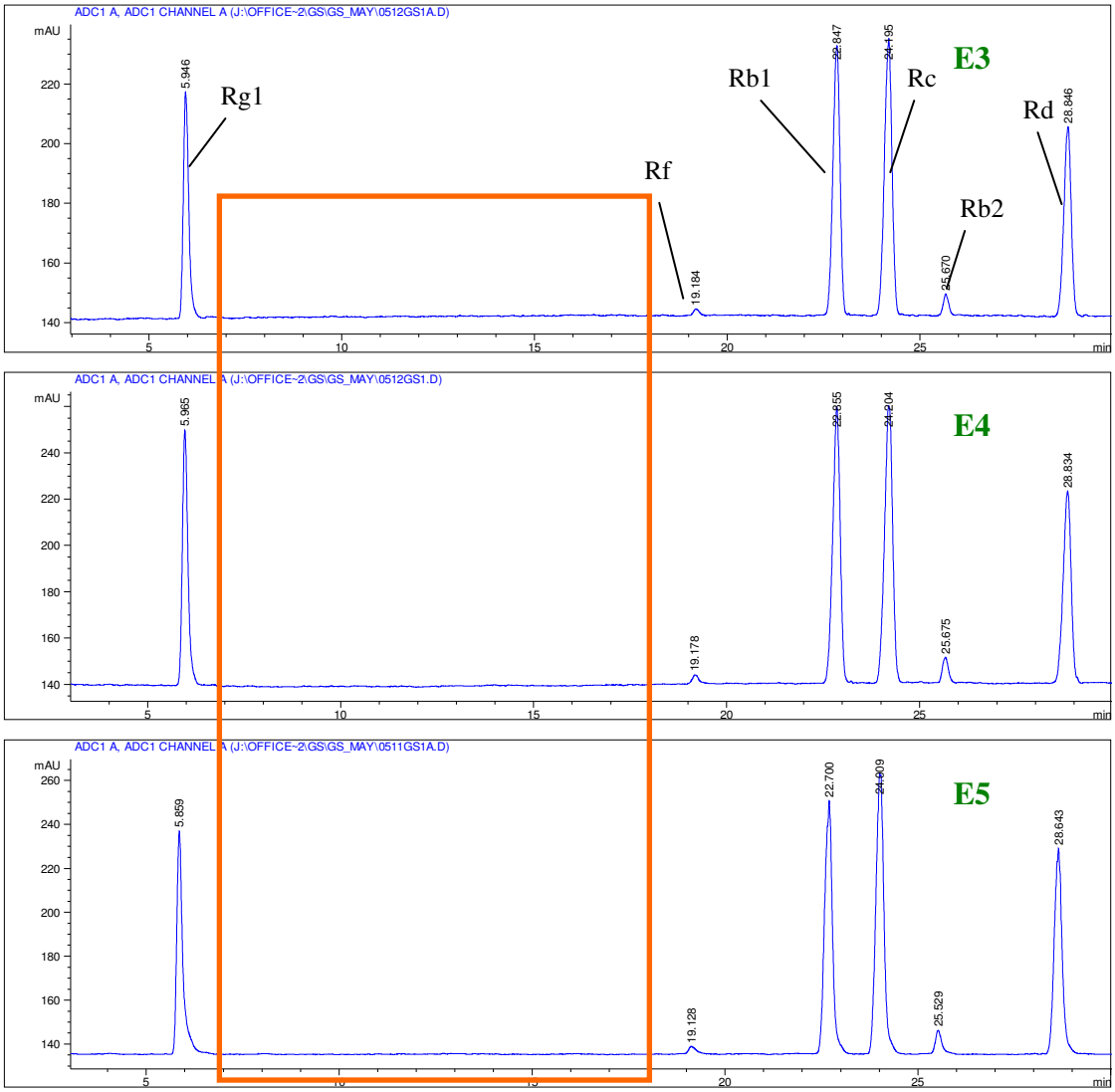


Fig. 3.6 HPLC-ELSD chromatograms as obtained under ELSD settings of Set E3, Set E4 and Set E5 (Table 3.4)

3.3.1.2 Sample preparation

Sample preparation has been reported to be the bottleneck of most of the analytical procedures, as it is one of the least evolved parts of the whole experimental process, and it evolves many procedures to obtain a herbal extract (Wan *et. al.,* 2006). Two simple extraction techniques, sonication and homogenization, were selected to compare for

extraction of the six ginsenosides from the commercial ginseng products in this study as these techniques is simply and allow no heating process. Heating process, like reflux and soxhlet extractions, should be avoided to use for extracting the finished products because of the unknown manufacture procedures. The sample preparation was carried out under room temperature in this study.

By comparing different extracting durations of the same technique (Methods 1A and 1B, or Methods 2A and 2B in Section 3.2.2.2), sonication and homogenization could increase the total peak areas of the six ginsenosides from AGS (Fig. 3.7) and OGS (Fig. 3.8) with time, and AGS was increased to higher extent (Fig. 3.7). By comparing the performance of Method 1A, 2A, 1B and 2B, homogenization was found to be more efficient to extract the six ginsenosides from AGS and OGS. Method 2B was the most efficient one among all for both commercial ginseng products and hence it is the best method of sample preparation in this work.

Table 3.7 Total peak areas of the six ginsenosides with standard deviation (S.D.) obtained from Method 1A, 1B, 2A and 2B

Extraction Method	1A		1B		2A		2B	
	AGS	OGS	AGS	OGS	AGS	OGS	AGS	OGS
Total peak area (mAU)	6791.57	2501.08	7467.50	2874.56	6961.27	2789.62	7657.54	3099.87
S.D.	298.82	89.29	123.21	137.69	93.98	50.49	166.17	190.95

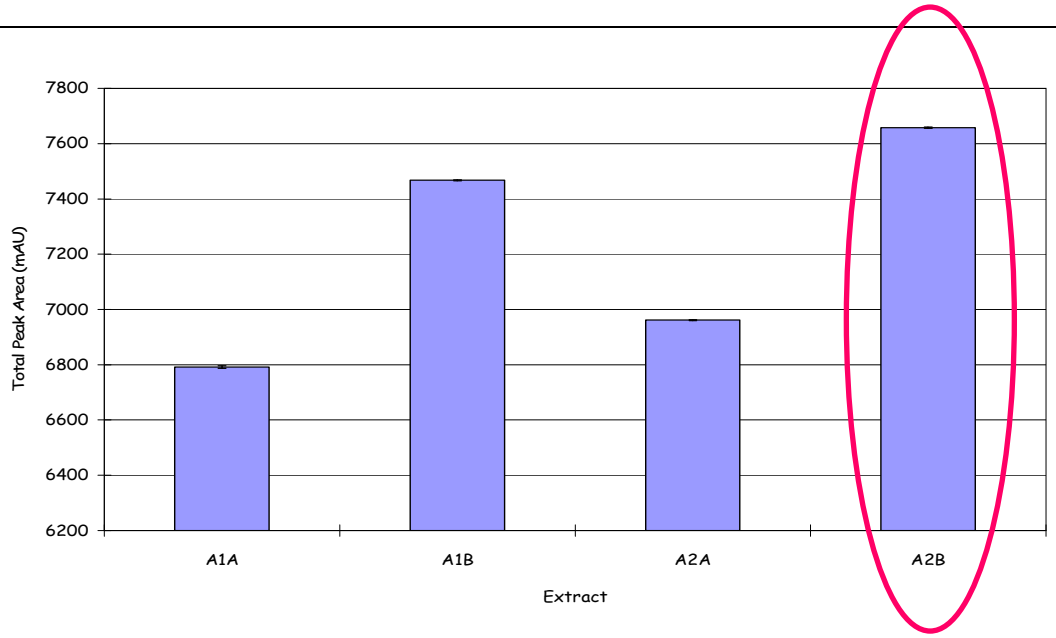


Fig. 3.7 Total peak areas of the six ginsenosides from AGS as extracted by different methods

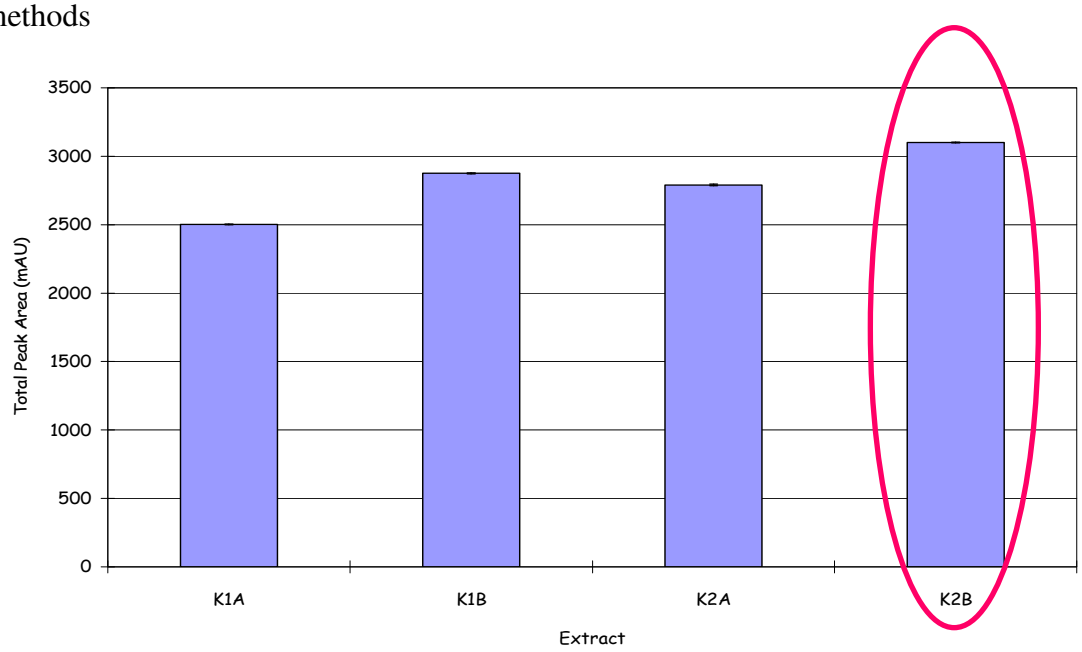


Fig. 3.8 Total peak areas of the six ginsenosides from OGS as extracted by different methods

3.3.1.3 Summary

In HPLC-DAD-ELSD chromatographic condition, the modified elution system, elution system C8, was developed (Table 3.6), the flow rate was increased to 1.2 ml/min, and Set

E5 (Table 3.4) of the ELSD detection parameters was chosen. The sample preparation Method 2B was applied to extract the six ginsenosides from the ginseng commercial products. The experimental conditions were employed for the chemical analyses and quality control of these two products.

3.3.2 Chemical analyses of the commercial ginseng products

3.3.2.1 Qualitative analysis

For qualitative analysis uisng HPLC-DAD-ELSD, the six ginsenosides were identified in all samples by comparing their retention times with those of the standards.

Table 3.8 shows the retention times of the six ginsenosides obtained from ELSD were a little bit slower than those from DAD as both detectors were coupled in series, i.e. HPLC-DAD-ELSD. The mean derivation of retention time between DAD and ELSD was in the range of 0.04 – 0.18 min in this work.

According to the experimental findings (Table 3.8), ginsenosides Rg1, Rb1, Rc, Rb2 and Rd were identified in the commercial products of AGS and OGS, while ginsenoside Rf was found in OGS only. Thus Rf can be used to distinguish AGS and OGS that were prepared from Radix Panacis Quinquefolii and Radix Ginseng respectively. The HPLC-DAD and HPLC-ELSD chromatograms of AGS and OGS are given Appendix 3.4.

Table 3.8 Retention times of the six ginsenosides of AGS and OGS in HPLC-DAD-ELSD analysis

	Retention time (min) of Ginsenosides					
	Rg1	Rf	Rb1	Rc	Rb2	Rd
American ginseng capsule (AGS)						
DAD	5.41	---	22.61	23.88	25.15	28.50
ELSD	5.57	---	22.76	24.05	25.30	28.66
Oriental ginseng tablet (OGS)						
DAD	5.67	18.82	22.40	23.80	25.37	28.45
ELSD	5.79	18.99	22.56	23.96	25.53	28.67

3.3.2.2 Quantitative analysis

Quantitative analysis of AGS and OGS were carried out based on the results acquired from HPLC-DAD-ELSD analysis. Due to the distinct variation on contents of the six ginsenosides in the commercial ginseng samples, ten concentrations (20, 40, 60, 100, 200, 300, 400, 500, 750, 1000 ppm) of the ginsenosides mixture were prepared and injected in triplicate, and then the calibration curves obtained by DAD and ELSD were constructed by plotting the peak area against the concentration of each ginsenoside (Fig. 3.9 (a) and (b) respectively).

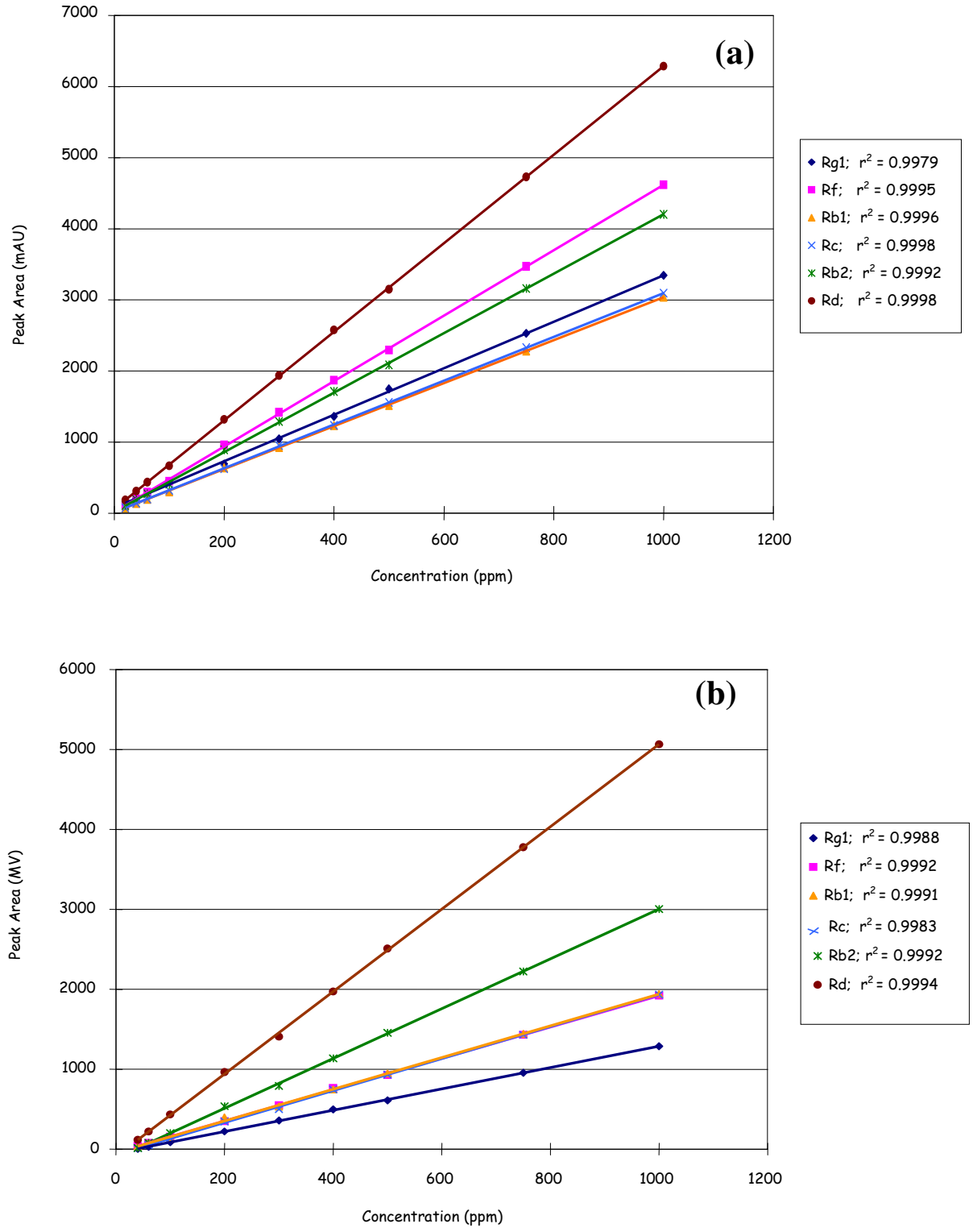


Fig. 3.9 Calibration curves of the six ginsenosides standards based on (a) DAD and (b) ELSD

The peak areas and concentrations of ginsenosides concerned in AGS and OGS are listed in Table 3.9. In general, the concentrations of ginsenosides detected by ELSD were less than those detected by DAD. This might because the impactor ON operation mode of ELSD split out a part of the ginsenosides inconsistently. In order to eliminate the circumstance, internal standard would be used. Therefore, the concentrations of the ginsenosides obtained by DAD were relatively accurate, and were applied for comparing the ginsenoside content in AGS and OGS. Most ginsenoside contents in AGS except ginsenosides Rf and Rb2 were higher than those in OGS. In the same way, the total concentration of these six ginsenosides in AGS (2.14 ± 0.09%) was higher that in OGS (0.64 ± 0.02%) as well. By comparing with the total ginsenosides labeled (Table 3.2), AGS was less a half total ginsenoside (2.14%) than its labeled (5%), while OGS was much more less (0.64%) than its labeled (7%). This would be owing to the labeled total ginsenoside is counted more than six ginsenosides.

3.3.3 Development of HPLC separation procedure for undergraduate students

Owing to the rapid growth of the herbal supplements in the market, introduction of the basic concept of its quality control is important in tertiary student. This is not only able to encourage them to connect science with everyday life; this is also good for them to know what the quality control of herbal supplements in work place is. In fact, the experimental technique of tertiary students and the time of the experiment session are limited. Therefore, they need a set of simple and fast experiment procedure for their study, and we successfully developed it as mentioned in Sections 3.3.1 and 3.3.2.

The experiment of the quality control of the two different ginseng products, Radix Ginseng and Radix Panacis Quinquefolii, are designed for one triple period of experiment lesson and overnight injection for HPLC analysis. This experiment is performed by a group of three to four students. Here, it is separated into three sections of sample preparation, establishment of calibration curves and the qualitative and quantitative analyses of the sample concerned. In sample preparation, each sample is extracted for one experiment. Next, ten data points of each of the six ginsenosides (Rg1, Rf, Rb1, Rc, Rb2 and Rd) are prepared from 1000 ppm stock solution of the ginsenosides mixture which has been prepared earlier. The sample extracts and the standard mixtures are injected into the in HPLC instrument triplicate in the third section. The first two sections and HPLC are designed to complete in one session, and the third one is carried out by HPLC automatically for an overnight.

The experiment designed is flexible for tertiary institute with different instruments. For example, in sample preparation, the homogenization method can be replaced by the sonication, and either one of two detectors, DAD and ELSD, can be applied. The manuscript of this study is under preparation for and will be submitted to Journal of Chemical Education for publication.

Table 3.9 Concentrations of ginsenosides with standard deviation (w/w ± S.D. %) of the commercial ginseng products as investigated by HPLC-DAD-ELSD

Ginsenoside	American Ginseng (AGS) [1] capsule				Oriental Ginseng (OGS) [1] tablet			
	DAD		ELSD		DAD		ELSD	
	Peak Area (mAU)	Conc. (w/w ± S.D.%) [2]	Peak Area (MV)	Conc. (w/w ± S.D.%) [2]	Peak Area (mAU)	Conc. (w/w ±S.D.%) [2]	Peak Area (MV)	Conc. (w/w ±S.D.%) [2]
Rg1	2163.7	0.64 ±0.02	675.63	0.54 ± 0.02	599.01	0.16 ± 0.00	169.94	0.16 ± 0.01
Rf	---	---		---	138.91	0.02 ± 0.00	11.0	0.03 ± 0.00
Rb1	3258.9	1.07 ± 0.02	1567.13	0.81 ± 0.02	561.72	0.19 ± 0.01	231.63	0.14 ± 0.01
Rc	608.97	0.20 ± 0.01	109.73	0.10 ± 0.00	359.33	0.11 ± 0.01	105.63	0.10 ± 0.00
Rb2	308.97	0.05 ± 0.00	29.35	0.04 ±0.00	442.37	0.10 ± 0.00	163.05	0.09 ± 0.00
Rd	1110.60	0.17 ± 0.01	252.7	0.07 ± 0.00	381.73	0.05 ± 0.30	111.45	0.04 ± 0.00
Total	7487.53	2.14 ± 0.09	2634.55	1.56 ± 0.05	2471.57	0.64 ± 0.02	792.70	0.55 ± 0.01

Remarks:

[1] : mean value of triplicate experiment

[2] : w/w (mg/mg); per 500 mg product

3.4 Conclusion

In the foregoing, both the sample preparation procedure and HPLC-DAD-ELSD chromatographic condition were optimized to analyze the six ginsenosides (Rg1, Rf, Rb1, Rc, Rb2 and Rd) in the commercial ginseng products. The optimized experimental condition could help to complete the qualitative and quantitative analyses of the ginseng sample in a relative simpler and faster than the procedures recommended by previous investigations. The characteristics of the developed method fit into the theme about the quality control of the ginseng commercial products to tertiary students. Also, the flexible design of the experiment is easy to adapt in different tertiary institutions.

In the qualitative analysis, Radix Ginseng (OGS) tablet and Radix Panacis Quinquefolii (AGS) capsule were able to be distinguished as the ginsenoside Rf is absent in AGS. On the other hand, the quantity of the six ginsenosides, OGS and AGS were also studied with HPLC-DAD-ELSD. This was shown that the concentrations of ginsenosides determined by ELSD was less than those found by DAD as the impactor ON operation mode of ELSD. The total ginsenoside content in AGS is more that that in OGS. However, both samples concerned did not reach the total ginsenoside content labeled by their manufacturers.

Chapter 4

Determination of Nucleosides in Ganoderma amboinense with High Performance Liquid Chromatography coupled with Diode Array Detector (HPLC-DAD) and Evolving Window Orthogonal Projections (EWOP) Method

4.1 Introduction

4.1.1 Background

Mushrooms have been widely recognized to have the nutritional and medicinal values over the world for thousand of years (Borchers*et. al.*, 1999; Chang and Hayes, 1978). In the point of view in the nutritional value, mushrooms contain relatively high amounts of protein and essential amino acids (Chang, 1980), low in total fat and a high percentage of polyunsaturated fatty acids, abundance in carbohydrates and fiber (Miles and Chang, 1997), and significant amount of the water soluble vitamins and minerals (Chang *et. al.,* 1993). On the other hand, at least 270 species of mushrooms are considered to possess a wide range of medical properties including antitumor, antiviral, antibiotic, anti-inflammatory, cardiovascular system action and tonic activities (Sullivan *et. al.*, 2006; Johnston, 2005; Wasser, 2002; Miles and Chang, 1997; Jong and Birmingham, 1992), and these kinds of mushrooms is categorized as "medical mushroom". Among hundreds of species of medical mushrooms, the Ganodermataceae, one of the fungi families, is the most significant one and is studied extensively worldwide due to their pharmacological activities. In the family, over 200 species have been found (Mau, 2005; Kawagishi, 1997; Keller, 1997; Steyaert, 1980). Ganoderma amboinense which is known as Deer Horn Lingzhi (DH Lingzhi) is one of Ganodermataceae and was studied here.

Over 100 reports have been published concerning the chemical composition of Ganodermataceae from its fruit body with the help of chromatography and spectrochemistry systems for twenty years (Su, 1991), and several kinds of chemical including polysaccharides, triterpenoids, amino acids, polypeptides, nucleosides, nucleic

acids, sterols and alkaloids have been found and their biological activities have been investigated (Kim and Kim, 1999; Jong and Birmingham, 1992; Kino *et. al.*, 1989; Franz, 1989; Hou *et. al.*, 1988; Yu *et. al.*, 1979). Researchers focus on these chemicals present in different parts of the fruit body (cap, stipe, mycelium and spore) related to their corresponding pharmacological activities in Ganodermataceae (Di *et. al.*, 2003; Yu *et. al.*, 1997; Horner *et. al.*, 1993). Among them, polysaccharides and triterpenoids are of great concern because of their richness in Ganodermataceae and their well-known pharmacological activities. Other chemical components, however, possess important medical activities also. For example, some nucleosides in Ganodermataceae have been reported to have anticancer activity and suppress the platelet aggregation (Wu *et. al.*, 1997; Shimizu *et. al.*, 1985), while alkaloids isolated from the cultured extract of *Gandoerma lucidum* have proven to increase coronary flow and lower coronary resistance (Chang and But, 1986).

In this investigation, six nucleoside components, adenine, adenosine, inosine, guanosine, uracil and uridine, in different parts of Ganoderma amboinense were analyzed qualitatively and quantitatively. The fruit body of Gandoerma amboinense sample, as well as its two portions, the cap and stipe, were studied by using analyzed with analytical high performance liquid chromatography equipped with diode array detector (HPLC-DAD). Then the chemometric method, evolving window orthogonal projections (EWOP), was employed to analyze the data acquired to extract useful chemical information. A brief report on this mushroom is given below.

4.1.2 Ganoderma amboinense

Ganoderma amboinense is an antler form of *Ganoderma lucidum* (PolyU Modern TCM Research Institute Ltd., 2005; Upton, 2000). Due to its special appearance, it is also called as Deer Horn Lingzhi (DH Lingzhi). DH Lingzhi originates from China, Japan, Indonesia, Philippines and other countries (Wu *et. al.,* 1997). Apart from the appearance, it has no direct connection to the actual Deer Antler Velvet (PolyU Modern TCM Research Institute Ltd., 2005). The shape of DH Lingzhi (Fig. 4.1(a)) differs a lot from *Ganoderma lucidum* (Fig. 4.1 (b)). DH Lingzhi is predominantly differentiated by its lack of a well-formed cap, its mostly branched and gnarled appearance, and brown to reddish-brown in colour. On the other hand, *Ganoderma lucidum* has circular to semicircular cap shape, less branches and no gnarled and is dark red to reddish-brown in colour. But other features of DH Lingzhi are more or less the same as *Ganoderma lucidum* including woody and fibrous texture, musty aroma, and intended bitter taste with high triterpenes. These variations result from the habitat of DH Lingzhi under little light and high level of carbon dioxide. Therefore, wild DH Lingzhi in nature is very rare, but it has been extensively cultivated under controlled conditions nowadays.

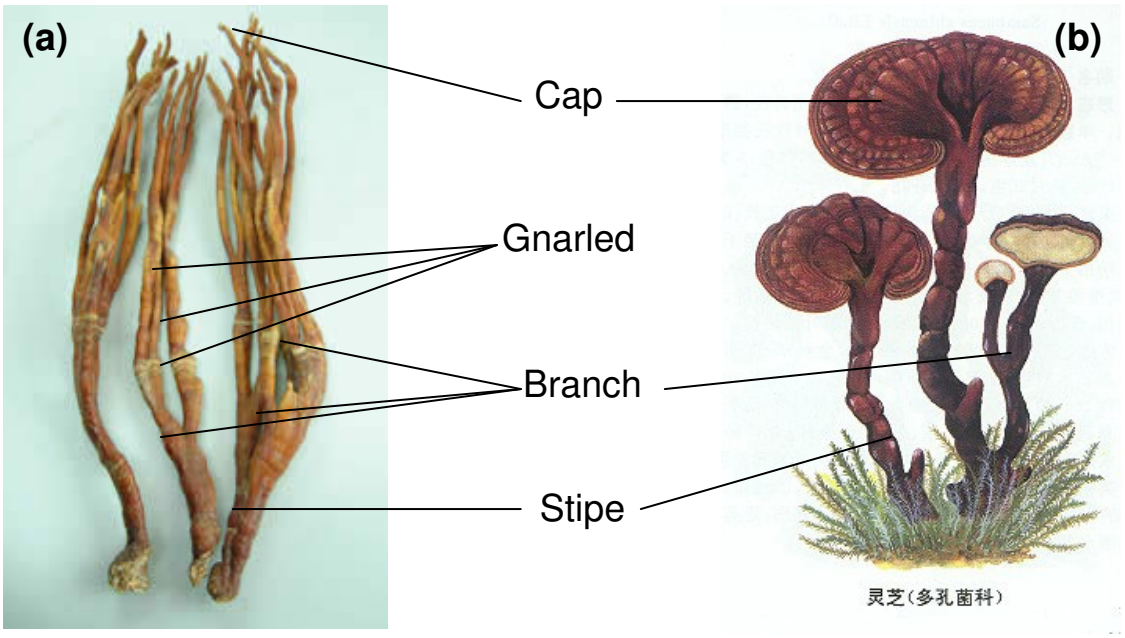


Fig. 4.1 Appearance of (a) Ganoderma amboinense and (b) *Ganoderma lucidum* (Lingzhi Cultivation Site, 2005)

DH Lingzhi is reported as rare species and was utilized as a luxury offering to emperors owing to it uses. In China, it is usually administered in the form of liquid extract or tablets made of extract. It is used as a sedative and tranquilizer for dizziness and insomnia due to neurasthenia and hypertension, a tonic for symptoms of weakness or debility and utilized for chronic bronchitis and asthmatic condition (Xie and Huang, 1998). In modern research, DH Lingzhi has been studied trying to find out the effective chemical ingredients. Polysaccharides, triterpenes and antioxidants obtained from DH Lingzhi contain much higher level than those from other *Ganoderma* as it does not release any spore at maturity and valuable ingredients remain in its fruit body (PolyU TCM Research, 2005). A polysaccharide, β-D-glucan, was reported having higher concentration yield in DH Lingzhi and stronger anti-tumor effects than other *Ganoderma*

(Upton, 2000). Also, DH Lingzhi is considered as a natural source of steroid, lanostanoid and lucidone A (Casteel, 1999; Lin, 1993). The pharmacological activities of DH Lingzhi with related chemical constituents are listed in Table 4.1. It should be noted that most medical activities of DH Lingzhi are contributed from polysaccharides and triperpenes.

Table 4.1 Pharmacological activities of the chemical constituents of DH Lingzhi (Li, 2005; Upton, 2000; Casteel, 1999; Zhu *et. al*., 1999)

Medical activity	Chemical constituents
Anticancer	Polysaccharides: β-D-glucan
	Triterpenes: Ganoderic acid X
	Steroid: Ergosterol peroxide; steroidal endperoixides
Antioxidative	Triterpenes and related compounds
Antiviral	Polysaccharide bound with acidic protein
	Triterpenes
	Steroid: Ergosterol peroxide
Hepatoprotective	Polysaccharides
	Triterpenes and related compounds
Hypoglycemic	Polysaccharides
Immunomodulatory	Polysaccharides
Anti-inflammatory	Polysaccharides
	Triterpenes
	Steroid: Ergosterol peroxide; steroidal endperoixides

Besides, nucleosides in *Ganoderma lucidum* were studied and found to have important medical activities. But, there is no report of this kind for DH Lingzhi. Here, we focused on six nucleoside components, and their chemical structures and pharmacological activities can be found in Table 4.2.

Table 4.2 Chemical structures and pharmacological activities of the six nucleosides studied (Lazarowski *et. al.*, 2000; Mesnil and Yamasaki, 2000; Starling *et. al.*, 1996; Williams *et. al.*, 1990; Yamamoto *et. al.*, 1981; Farme and Farrar, 1976)

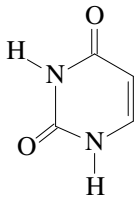
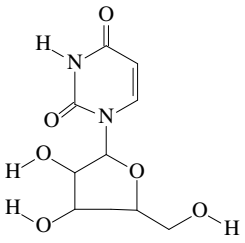
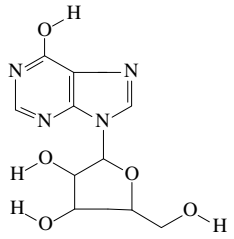
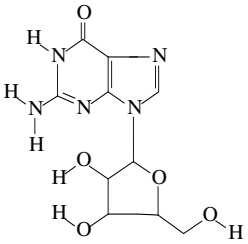
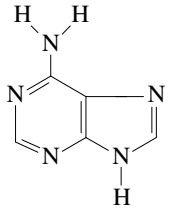
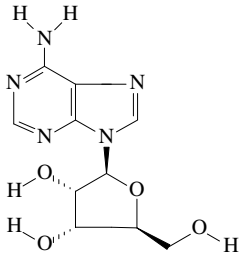
Nucleoside	Chemical Structure	Pharmacological activity
Uracil	 $C_4H_4N_2O_2$	➢ Muscular restorative activity ➢ Anticancer activity combined with 1-(2-tetrahydrofuryl)-5-fluorouracil (FT) in a molar ration of 1:4
Uridine	 $C_9H_{12}N_2O_6$	➢ Muscular restorative activity ➢ Regulatory effect of an airway epithelia mucociliary clearance

Table 4.2 Chemical structures and pharmacological activities of the six nucleosides studied (Continued)

Nucleoside	Chemical Structure	Pharmacological activity
Inosine	 $C_{10}H_{12}N_4O$	➢ Exercis-boosting effects ➢ Metabolic activation effect
Guanosine	 $C_{10}H_{13}N_5O_5$	➢ Cytotoxic activity ➢ Anticancer activity
Adenine	 $C_5H_5N_5$	➢ Muscular restorative activity ➢ An integral part of DNA, RNA and ATP
Adenosine	 $C_{10}H_{13}N_5O_4$	➢ Muscular restorative activity ➢ Anticancer activity ➢ Inhibitory effect of platelet aggregation

4.2 Method of Investigation and Chemometric Data Treatment

Modern analytical chemistry has benefited from the developments of second- and higher-order instruments which are capable of providing bi- and multidimensional data arrays (Booksh and Kowalski, 1994). During the past decades, second-order instruments and techniques, especially the so-called hyphenated chromatography such as gas chromatography-mass spectroscopy (GC-MS), high performance liquid chromatography-diode array detector (HPLC-DAD) and liquid chromatography-mass spectroscopy (LC-MS) have been extensively used in the analysis of the multi-component system of Chinese medicine (CM). This kind of instruments is especially important to analyze the complex chemical composition of CM as it provides chemical separation with chromatography and identification with spectrometry simultaneously (Gong *et. al.*, 2004; Mjøs, 2003; Gong *et. al.*, 2001; Manne and Grande, 2000; Fleming *et. al.*, 1999; Manne, 1995). However, CMs often contain chemical components with similar chemical and physical properties making separation of the components difficult and, the qualitative and quantitative analyses not easy as well (Zhang *et. al.*, 2005; Jalali-Heravi and Vosough, 2004; Li *et. al.*, 2003; Lee *et. al.*, 1997).

Fortunately, combination of hyphenated chromatography and chemometric data processing provides powerful tools to deal with such complex systems of CM. In mathematics data collected from hyphenated chromatography can be expressed in the matrix form as X(m × n). Here, $X_{m\times n}$ represents an absorbance matrix expressing the chromatographic profiles of m retention time measured at n consecutive wavelengths. The quantitative information is good to take advantage of resolving this matrix of CM by

relevant chemometric methods such as evolving factor analysis (EFA), heuristic evolving latent projections (HELP) and evolving window orthogonal projections (EWOP). Here, HPLC-DAD combined with EWOP method was applied to analyze the nucleoside content in different parts of DH Lingzhi because of the complexity and the low contents of the nucleosides in the DH Lingzhi extract.

4.2.1 High performance liquid chromatography-diode array detector (HPLC-DAD)

HPLC-DAD is the most common hyphenated chromatography. Here, reversed-phase HPLC-DAD is used for the analysis of nucleosides in DH Lingzhi. HPLC can separate components within a mixture in solution into its individual components ideally. The mobile phase is mechanically pumped through a column containing stationary phase. Under an optimum chromatographic condition, the separation occurs when each component in a mixture interacts with the two phases differently from the others (Weston and Brown, 1997). In reversed-phase chromatography, the stationary phase is nonpolar and the mobile phase is a relatively polar solvent. The least polar component is adsorbed on the stationary phase with covalent bond; while the most polar component is eluted and detected first (Weston and Brown, 1997).

DAD is a spectrophotometric detector and is widely used for liquid chromatography based upon absorption of ultraviolet and visible radiation. It is equipped with filter wheels that contain several interference filters that can be rapidly switched into place during detection resulting in giving the entire spectrum of an analyte eluted from the

column (Skoog *et. al.*, 1996). The spectra of the six nucleosides are detected here in the range from 240 to 400nm here.

4.2.2 Evolving window orthogonal projections (EWOP) method

EWOP method is one of the resolution methods to resolve two-way bilinear multi-component data into relating spectra and peak profile of the pure constituents (Xu *et. al.,* 1999). The approach of the method is based on the evolving character of chromatogram. The chromatogram character obtained from HPLC-DAD is the ultraviolet spectrum at each data point of the retention time. To explain the methodology, a selected region of a chromatogram (Fig. 4.2(a)) is screened with the pure spectrum of a target component point by point to establish a zero-concentration graph (ZCG) (Fig. 4.2(b)), and then the ZCG is employed to directly and accurately recognize the selective region. The region represents that the spectrum of the region matches that of the target component. In this way, the component concerned in the chromatogram is identified. The chromatographic profile of the component (Fig. 4.3) is obtained from the ZCG, and its peak area can be used for quantitative analysis. The elaborated theory of EWOP can be found in Section 2.3.1.

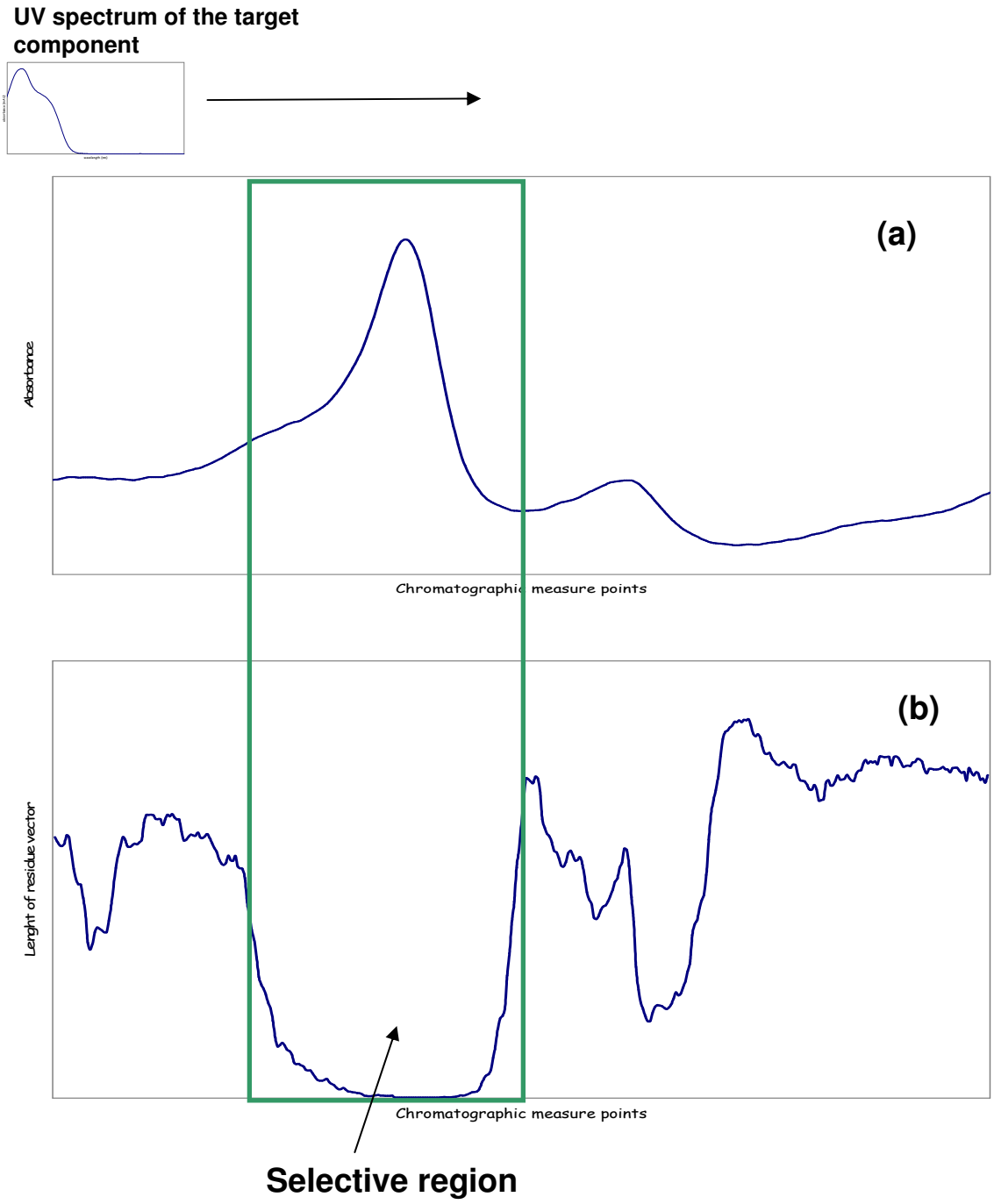


Fig. 4.2 Illustration of the working procedure of EWOP method in applying to a complex chromatogram

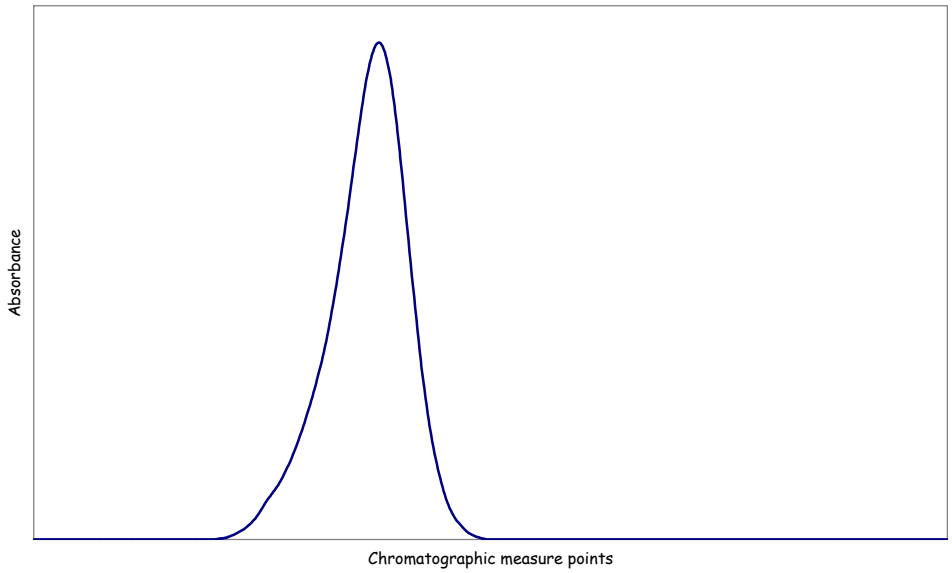


Fig. 4.3 Chromatographic profile of the target component in the selected region of the chromatogram

4.3 Experimental

4.3.1 Samples of Ganoderma amboinense (DH Lingzhi), chemicals and reagents

Two DH Lingzhi samples were obtained from Conserving & Breeding Centre of Microorganism and Fungus, Edible Fungi Research Laboratory and Agricultural Institute, Xi'an, P. R. China in spring 2004. One was the whole DH Lingzhi and the other one was the sliced DH Lingzhi. In this study, the whole DH Lingzhi sample was divided into two parts, the head and body. Then, each of the three samples of Ganoderma amboinense was ground and mixed well.

Six nucleoside standards of adenine, adenosine, inosine, guanosine, uracil and uridine were purchased from Wako, Japan. Double deionized water was utilized for the

extraction and preparing the buffer solution was purified by Milli-Q water system (Millipore Corp., Bedford, MA, USA). Ammonium phosphate ($(NH4)H_2PO_4$) in analytical grade for preparing the buffer solutions was obtained from Sigma (USA). Chromatographic grade methanol (MeOH) was obtained from Tedia (USA).

4.3.2 Sample preparation

The water-soluble constituents, nucleosides, were extracted by the following procedure (Leung *et. al.*, 2000). 0.5g of the ground sample was firstly mixed with 20mL of deionized water and the solution was sonicated for 2 hours. After this, the liquid layer was filtered off and dried by using a rotary evaporator. The residue was dissolved in 10mL methanol. The sample solution was then passed through a SEP-PAK C_{18} cartridge (Waters Assoc., USA) being pre-washed with 2ml methanol and then 5ml of water. After loading, the sample solution was directed through the cartridge and collected in a vial. Then, the resulting solution was filtered through a 0.45μm Nylon-66 filter (Alltech, USA). In the HPLC analysis, 20 μL of the standards and sample solutions were used were.

4.3.3 HPLC-DAD analysis

The chromatographic separation was carried out using an Agilent Series 1100 HPLC system (Agilent Technologies, Inc., CA), which consisted of a degasser, the pumps, an autosampler and a photodiode array detector (DAD), equipped with a C_{18} reversed-phase HPLC column (Hypersil ODS, 5 μm particle size, 4.6 × 250mm) for separation and detection of the DH Lingzhi samples. The associated computer software HP

ChemStation of the Hewlett Packard (HP) was utilized for processing the chromatographic HPLC-DAD data.

The mobile phase for HPLC separation of the nucleosides was a mixture of buffer solution. The Buffer solution A was the mixture of 2.5% methanol and 97.5% 0.01M $(NH_4)H_2PO_4$; while buffer solution B was the mixture of 20.0% methanol and 80.0% 0.01M $(NH_4)H_2PO_4$. The variations of the mobile phase composition with time are listed in Table 4.3 (Shiao *et. al.*, 1994). The flow rate was 1.0 ml/min. The absorbance of the sample extract was monitored from 240 to 400 nm. The HPLC column was kept at room temperature.

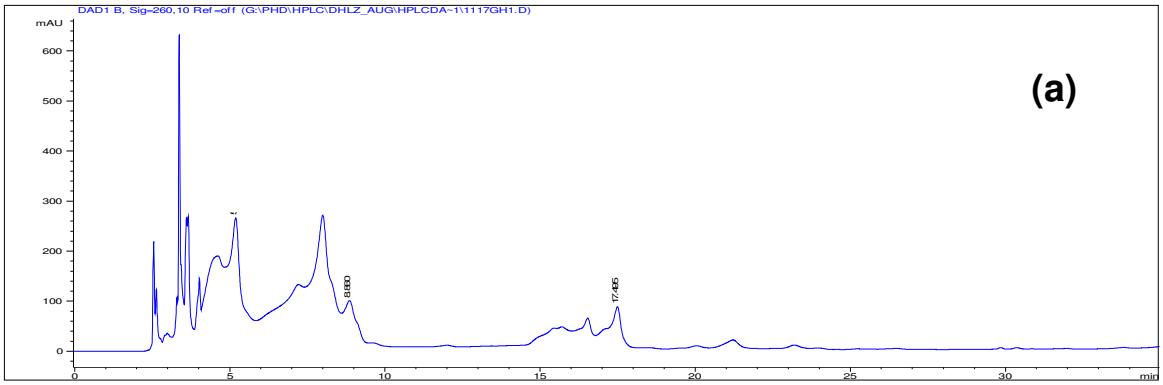
Table 4.3 Gradient of the mobile phase composition

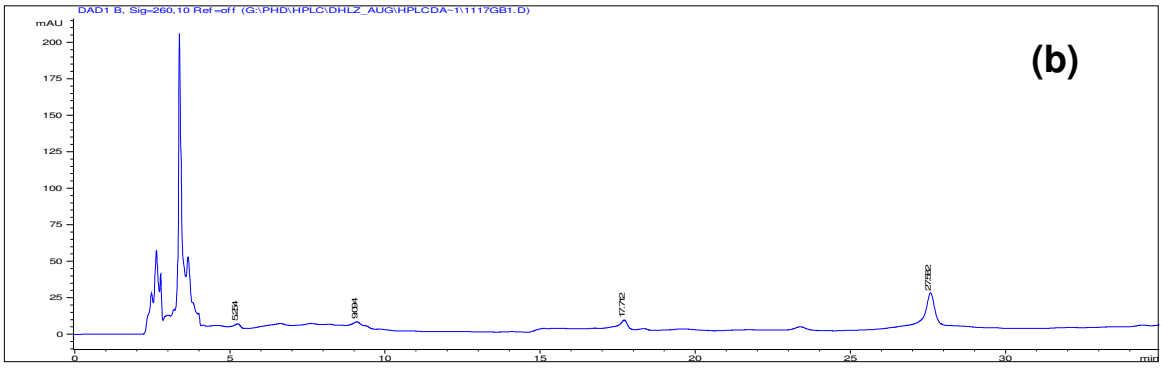
Elution time (min)	Buffer solution A (%)	Buffer solution B (%)
0.0	100	0
10.0	100	0
10.5	75	25
20.0	60	40
30.0	0	100

4.4 Results and Discussion

4.4.1 Identification of the six nucleoside compounds in DH Lingzhi with HPLC-DAD

Figure 4.4 showed the HPLC-DAD chromatograms at 260 nm of the cap, stipe and fruit body of DH Lingzhi. It was found that there are many variations among chromatograms obtained. The chromatogram of cap (Fig. 4.4 (a)) shows that its chemical compositions is the most complicated among the three type of samples studied and several peak clusters were overlapped seriously. On the other hand, profiles of the chromatograms of stipe and fruit body were similar and are less intense after 5th minute, but overlapped peaks still exist.





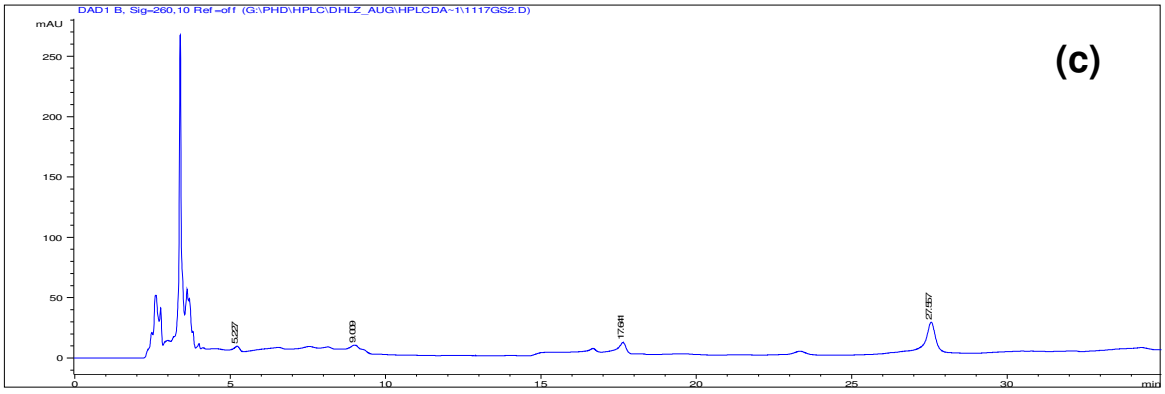


Fig. 4.4 HPLC-DAD chromatograms of (a) cap, (b) strip and (c) fruit body of DH Lingzhi (Table 4.3)

With the retention time and the UV spectra of the nucleoside standards (Table 4.4), the nucleoside compounds in the cap, stipe and fruit body were identified. Among the six nucleosides studied, only four nucleosides of uracil, uridine, guanosine and adenosine were found in the DH Lingzhi samples in general. By only comparing with the retention times of the standards, it seem that all the six nucleosides could be found in each portion of DH Lingzhi, as they have retention time closed to the corresponding standards (Table 4.4). In order to confirm this, matching their UV spectra with those of the standards was necessary. It can be seen that the UV spectra obtained from the cap that the spectra of uridine and guanosine of the cap were the same as those of the standards, and hence uridine and guanosine were identified. The UV spectra of uracil, uridine, guanosine and adenosine from stipe and fruit body were identical to the standards, and hence the four necolesides were confirmed. Inosine and adenine could not be found in each portion of DH Lingzhi. This might be due to the contents of inosine and adenine in the samples are below their detection limits. The detection limits of inosine and adenine were 0.16 and 0.2 μg/g respectively.

The absolute content of the four identified nucleosides can further be determined by their calibration curves, but the chromatographic peaks of some nucleosides were too small, and some overlapping peaks representing the nucleosides did exist in the profiles investigated. It is clear that quantitative analysis of the nucleosides based upon their peak areas obtained from the HP ChemStation software was not reliable. Hence, the chemometric method of EWOP was applied and the results are reported in the following section (Section 4.4.2).

Table 4.4 UV spectra and retention times of the nucleosides in the cap, stipe and fruit body of DH Lingzhi and the standards

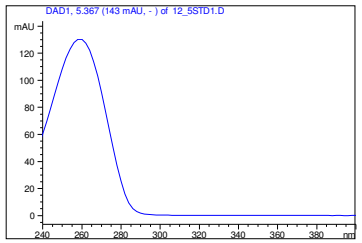
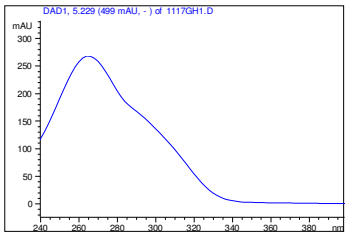
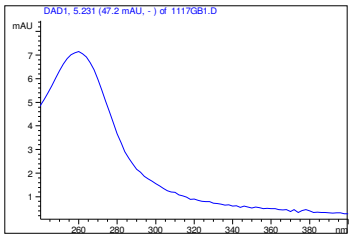
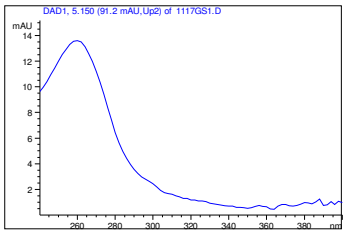
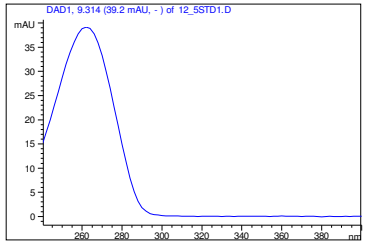
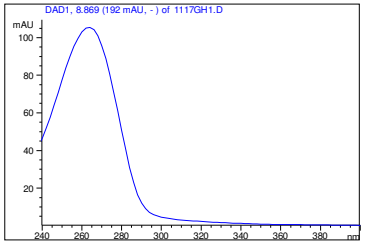
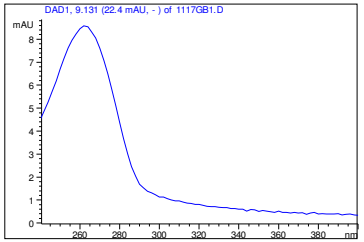
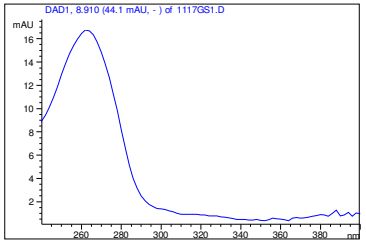
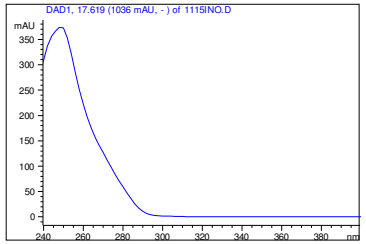
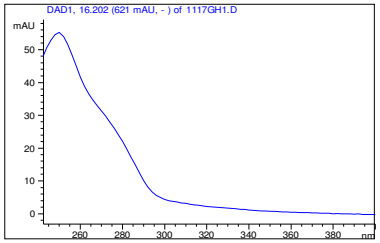
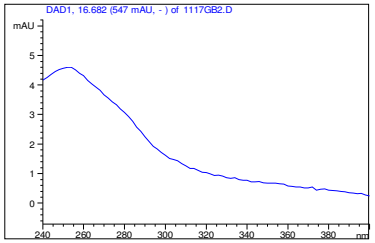
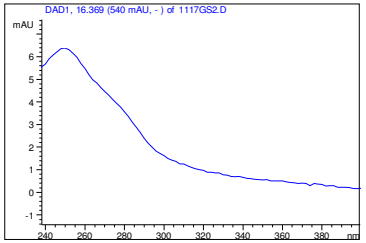
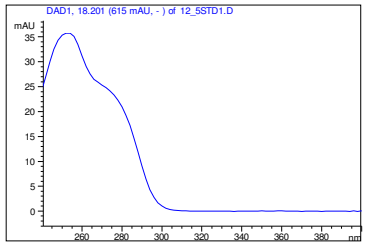
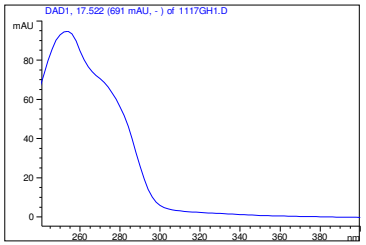
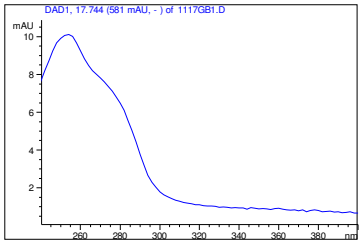
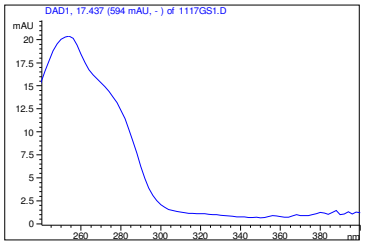
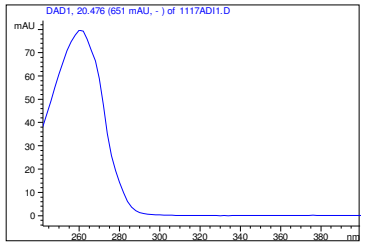
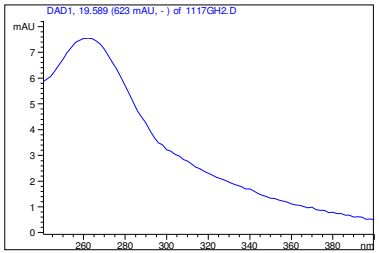
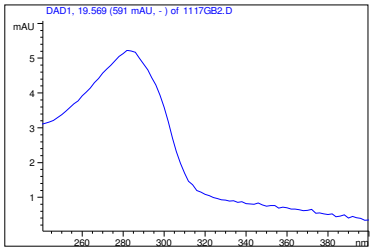
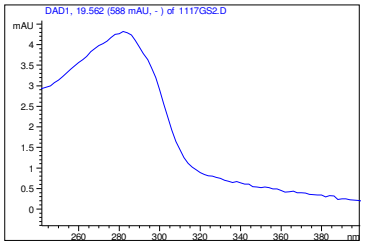
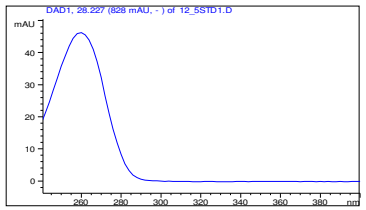
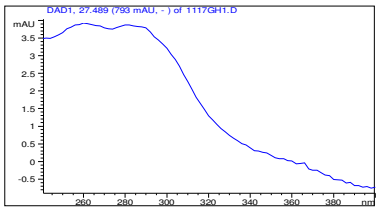
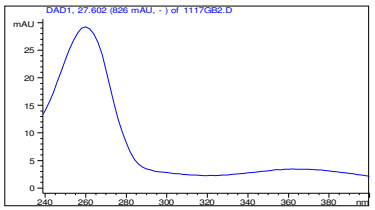
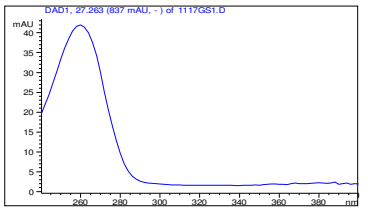
Nucleoside	UV spectrum and retention time			
	Standard	Cap	Stipe	Fruit body
Uracil	 **5.36 min**	 **5.23 min**	 **5.23 min**	 **5.15 min**
Uridine	 **9.35 min**	 **8.89 min**	 **9.13 min**	 **8.91 min**
Inosine	 **17.57 min**	 **16.20 min**	 **16.68 min**	 **16.37 min**

Table 4.4 UV spectra and retention times of the nucleosides in the cap, stipe and fruit body of DH Lingzhi and the standards (Continue

Nucleoside	UV spectrum and retention time			
	Standard	Cap	Stipe	Fruit body
Guanosine	 **18.19 min**	 **17.52 min**	 **17.74 min**	 **17.44 min**
Adensine	 **20.11 min**	 **19.59 min**	 **19.57 min**	 **19.56 min**
Adenosine	 **28.31 min**	 **27.49 min**	 **27.60 min**	 **27.26 min**

4.4.2 Quantitative analysis of the six nucleoside compounds in the DH Lingzhi samples with the EWOP method

Owing to the complexity of the samples studied, evolving window orthogonal projections (EWOP) method was employed to determine the peak areas of the four nucleosides in order to achieve the quantitative analysis. As mentioned in Section 4.2.2, before determination of the peak area of a particular component, the selected chromatographic region is screened by the pure spectrum of a particular component as a result the chromatographic profile and peak area can be found to achieve the purpose. Here, the selected regions of the chromatograms of the cap, stipe and fruit body were inspected with the spectra of the nucleoside (Section 4.4.1) to determine the peak areas of pure nucleosides peaks in the chromatogram.

The zero-concentration graph (ZCG) of adenosine from the stipe of DH Lingzhi as obtained by EWOP method in this work was shown in Figure 4.5. Its selective region was identified, and the pure chromatographic profile of adenosine was obtained (Fig. 4.6). With the pure chromatographic profile and UV spectrum of adenosine available, the total absorbance of the chromatogram and UV spectrum response, so-called two-way response, was determined. The total peak area of adenosine is then proportional to the overall volume of its two-way response. By using the same way of adenosine, the peak areas of the other nucleosides can be determined (Section 4.4.1) and the results are summarized in Table 4.5. The other ZCGs and chromatographic profiles of the nucleosides from the cap, stipe and fruit body of DH Lingzhi can found in Appendix 4.1, 4.2 and 4.3 respectively.

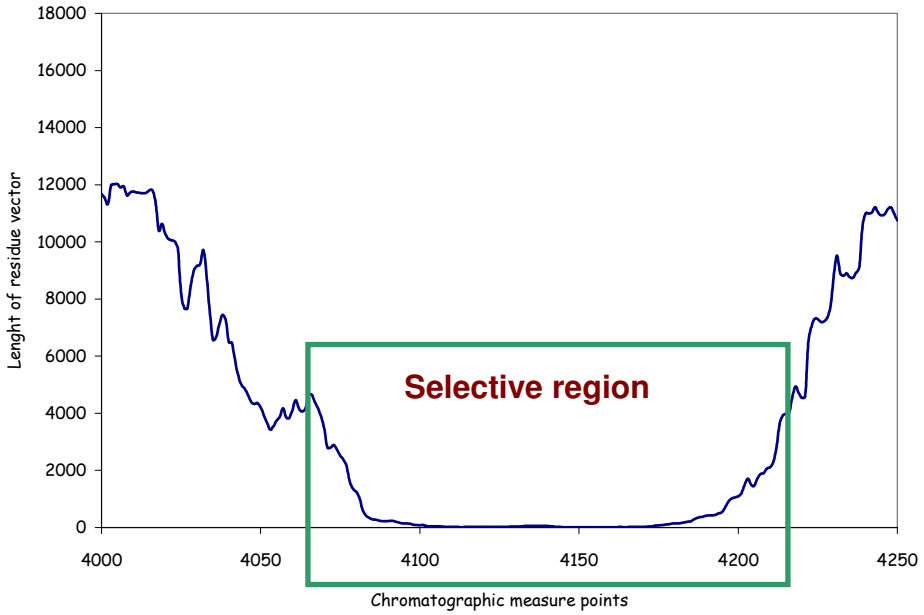


Fig 4.5 Zero-concentration graph (4000 to 4250 point) of adenosine from the stipe of DH Lingzhi and the selective region bracketed

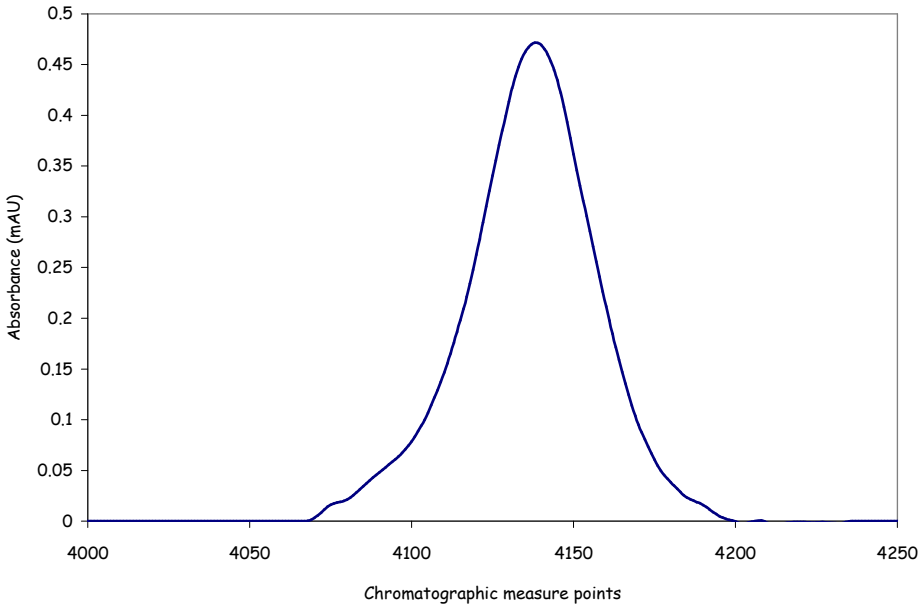


Fig. 4.6 The resolved chromatographic profile of adenosine from the stipe of DH Lingzhi

In the zero-concentration graph of uridine in fruit body (Fig. 4.7) and the strip (Appendix 4.3) of DH Lingzhi, two selective regions were overlapped with each other because of having the same or the similar UV spectrum. Under this situation, the resolved chromatographic profile (Fig. 4.8) and the peak area obtained not come from the pure uridine, and the concentration of uridine in these portions could not be determined.

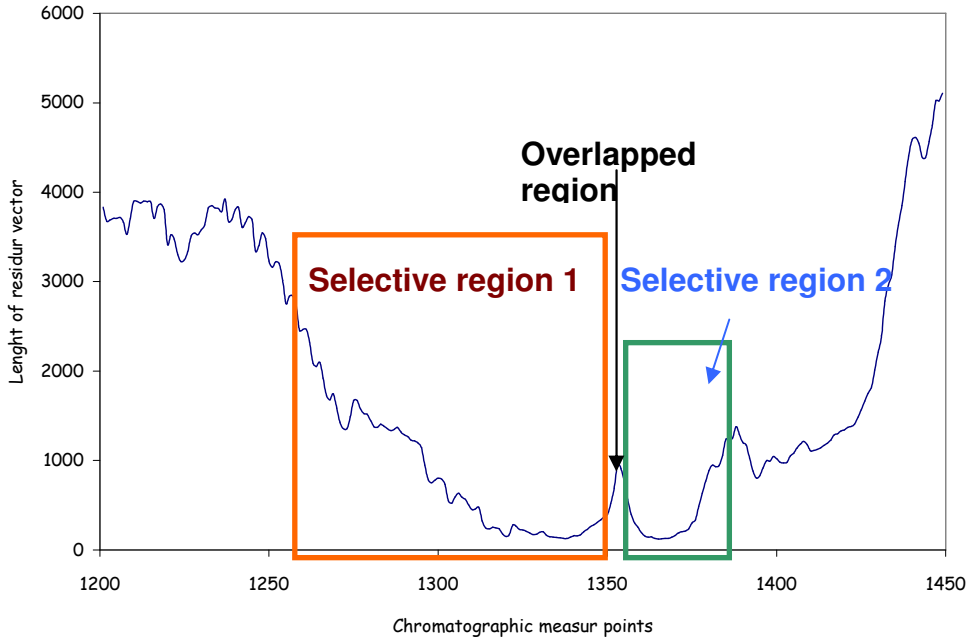


Fig. 4.7 Zero-concentration graph of uridine from the fruit body of DH Lingzhi

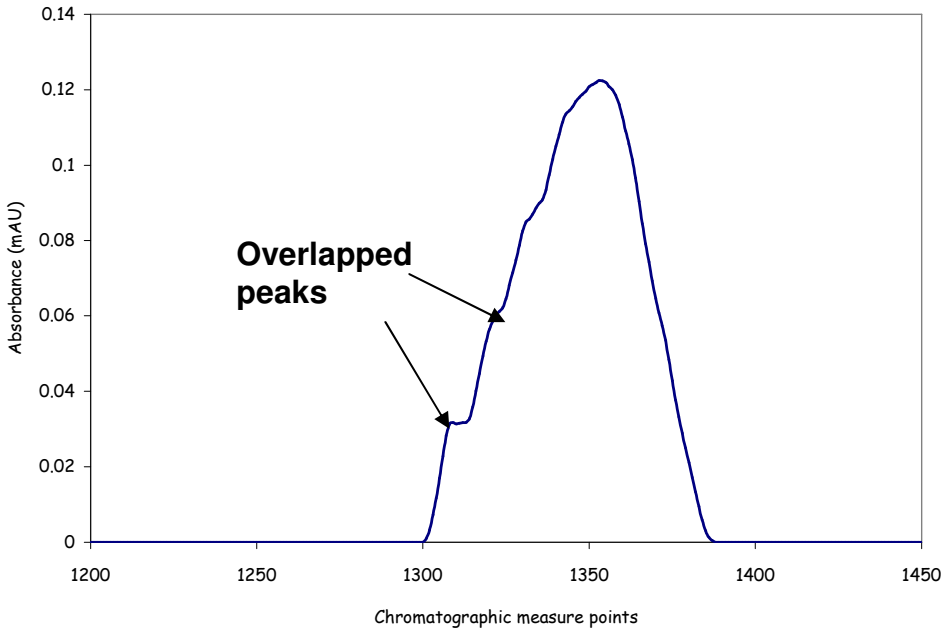


Fig. 4.8 The resolved chromatographic profile of uridine from the fruit body of DH Lingzhi

The calibration curves of the standards uracil, uridine, guanosine and adenosine being set up through the HP ChemStation software could not be used to calculate directly the concentrations of these components in the samples because their peak areas were determined by EWOP method. Here these calibration curves have to be treated by EWOP as well before for usage and they are showed in Figure 4.9. In this way, the concentrations of the nucleosides in samples were determined and listed in Table 4.5. It can be seen that the cap was found to have much higher contents of uridine and guanosine than the body and fruit body. As the fruit body of DH Lingzhi was composed by different proportions of the cap and stipe of DH Lingzhi, its nucleoside contests were expected to be between those of the cap and stipe. The results given in Table 4.5 verify it.

The large difference in the contents of these nucleosides found in different parts of DH Lingzhi should lead to variations in their therapeutic properties and even the extent of treatment of disease concerned though they were just different parts of the same DH Lingzhi. Further studies through biological test and clinical trial are needed to get more detail.

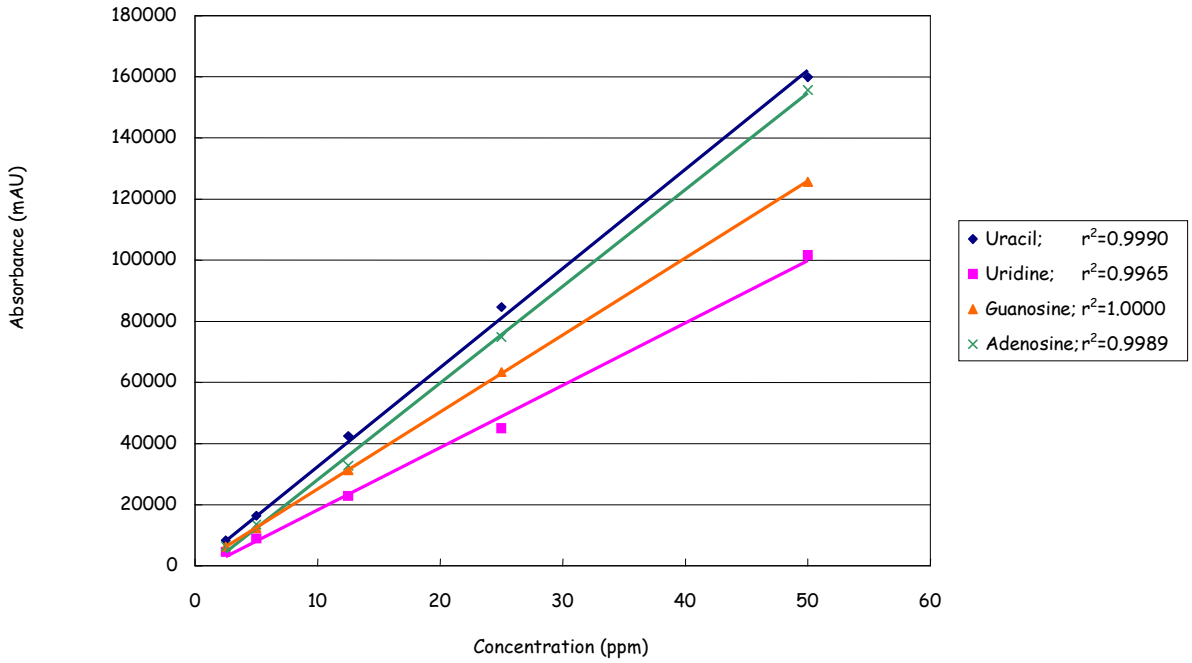


Fig. 4.9 Calibration curves of the four nucleoside standards after EWOP treatment

Table 4.5 Peak areas and concentrations of the four nucleosides with standard deviations (Conc. ± S.D.) as determined by EWOP method

Nucleoside	Cap[1]		Stipe[1]		Fruit body[1]	
	Peak area (mAU)	Conc.± S.D. (ppm)	Peak area (mAU)	Conc.± S.D. (ppm)	Peak area (mAU)	Conc.±S.D. (ppm)
Uracil	---[2]	---[2]	1155.36	7.12 ± 0.256	2751.96	16.96 ± 0.07
Uridine	76887.06	776.09 ± 4.42	---[3]	---[3]	---[3]	---[3]
Guanosine	48801.88	452.57 ± 17.06	6275.06	58.34 ± 0.21	8247.92	76.62 ± 0.63
Adenosine	---[2]	---[2]	17988.56	135.26 ±1.16	19788.11	148.46 ± 9.46

Remarks:

[1] : mean value of triplicate experiment

[2] : no peak identified via UV spectrum

[3] : identified but impure component resolved found by the EWOP method

4.5 Conclusion

In this investigation, the nucleoside composition of cap, stipe and fruit body of the sample of Ganoderma amobinenese (DH Lingzhi) were determined by HPLC-DAD and EWOP methods. During the chromatographic analysis, the peaks of nucleosides of the samples seriously overlapped with one another and only qualitative determination could be conducted with the UV spectra of the six nucleoside standards, uracil, uridine, guanosine, inosine, adenine and adenosine. The stipe and fruit body portions were found to possess only four nucleosides of uracil, uridine, guanosine and adenosine, while the cap portion was found to have uridine and guanosine only.

In order to determine the concentrations of nucleosides in different parts of DH Lingzhi, the combined approach of HPLC-DAD analysis with EWOP method was employed. It successful determined the nucleosides contents of the samples, but the concentration of uridine in the stipe and fruit body portions could not be found owing to overlapped peaks of the components with the similar UV spectrum. The variation of the nucleoside concentrations in different parts of DH Lingzhi indicates that each of its parts would give different therapeutic activities.

Chapter 5

Study of the Chemical Composition and Bioactivity of a Chinese Medicine formulation RDLP using Chromatographic method and *in-vitro* Bioassay

5.1 Introduction

Liver cancer is the fifth most common cancer in the world. Liver cancer, so-called a deadly cancer, kills almost all patients within a year who they get it. From the estimation of the World Health Organization (WHO) in 1990, there were about 430,000 new cases of liver cancer worldwide each year, and a similar number of patients died from this disease. Most of these cases are more frequently found in East Asia (China, Hong Kong, Taiwan, Korea, and Japan) and South Africa (Fong, 2003). Owing to the high death rate, many different kinds of chemotherapies and radiotherapies have been developed for the treatment in modern medicine. Some of them are effective to great extent. But they may cause undesirable side effects on the body, and the treated patients often feel very tired and weak, and suffer from stress, anxiety and fear, insomnia, and loss of appetite (McNamara and Ke, 1995). Complementary and alternative medicine (CAM) therefore becomes an acceptable way throughout the world to handle the disease and side effects (Xu, and Wang, 2002; Eisenberg *et. al.*, 1998; MacLennan *et. al.* 1996; Thomas *et. al.*, 1991).

Chinese medicine has been studied to be regarded as the most popular CAM (Harmsworth and Lewith, 2001). There is consensus that the treatments of CM are concerned about strengthening the physical health and psychology of the patient. In physical health, various herbal formulations are used to help in bolstering the immune system and increasing the body's own defence mechanisms to kill the cancer cell. CM remedies can help reduce or eliminate the side effects from chemotherapy and radiotherapy. However, western medical community is still concerned about the efficacy

and the safety of CM because of its complicated chemical composition, lack of concrete evidence of biological activity (Wiseman, 2002), and the herbal prescriptions developed based on the experience of practitioners (Xu and Wang, 2002). Unlike modern drug in the form of a single active chemical ingredient, herbal medicine especially CM is usually prepared from aqueous extracts of a few herbs and contains hundreds of chemical compounds (Yuan and Lin, 2002). Some chemical components are useless or even toxic and should be eliminated. Thus, discovering active compound is benefited to ensure the safety and efficacy in CM.

A selected CM formulation, RDLP, is an instant CM formulation. It was developed by a famous CM practitioner in Hong Kong and has been used to treat many liver cancer patients for more than 20 years with high success rate. The chemical compositions and pharmacological effects of RDLP have not been studied in detail before. In this work, RDLP was examined with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide) cell proliferation assay (MTT assay) to study its safety and efficacy through *in-vitro* tests of two normal human cell line (MRC-5 and WRL-68) and two human hepatoma cell lines (Hep3B and HepG2). With regard to the RDLP chemical composition, it was investigated by high performance liquid chromatography (HPLC) with diode array detector (HPLC-DAD) and preparative HPLC (PHPLC). In addition, we tried to find the RDLP active compounds by cooperating (PHPLC) and MTT assay, called bioassay-guided fractionation. Details of the works done are given in the following sections.

5.2 Methods of Investigation

5.2.1 Bioassay-guided fractionation

Bioassay-guided fractionation is one most commonly used approach for finding active compounds. It has gained definite success in discovering new biologically active structure (Bagchi and Preuss, 2005, Fang *et. al.*, 2005; Abdel-Wahab et. al., 2002; Harvey, 2000). In this process, herb medicine is separated into many fractions. Then, bioassay of each fraction is performed to determine which one should be subjected to further fractionation. Through the cycles of fractionation and bioactivity study, active compounds may be discovered and are isolated for further investigation if possible. In this work, the fractionation process was performed by PHPLC, while MTT assay was utilized to assess the bioactivity of the extract of RDLP and its fractions on two human hepatoma (Hep3B and HepG2), liver (WRL-68) and lung (MRC-5) normal cell lines.

5.2.2 Preparative high performance liquid chromatography (PHPLC)

The fractions obtained in bioassay-guided fractionation are commonly carried out on mother liquor with a sequence of solvent-solvent separation processes. Each process is trying to be simple and easy to handle, but it is quite labour intensive to collect a beach-scale of fractions for bioactivity assay. Firstly, the extracts of RDLP obtained by sample preparation as mentioned in the next section were investigated by HPLC-DAD and *in-vitro* bioassay. Then, PHPLC was applied to collect the fractions from a selected extract of RDLP via the automatic devices of PHPLC and fraction collector. The

chromatographic condition of PHPLC was optimized by analytical high performance liquid chromatography (ALC) before carrying out the process.

The working principle of ALC and PHPLC are the same. The differences come from scale and size of the column utilized. The internal diameter of PHPLC column is usually twenty five times bigger than that of ALC column. Thus, the column size determines the applications of ALC and PHPLC. The major application of ALC is to separate a mixture of compounds into individual components by chromatographic separation for qualitative and quantitative determination; while the usage of PHPLC is to isolate the pure substances from a mixture for subsequent studies such as structural determination, bioassays, pharmacological studies, standards for quantitative analysis, etc.

Running the separation of a mixture like CM on ALC should be the initial step to determine the feasibility of the PHPLC because of the advantages of short running time and much less amount of solvent needed in this small scale of analytical system. Although the chromatographic condition obtained from ALC does not give the same results as those by the large scale of PHPLC system, this information is very useful for optimizing the PHPLC separation. In order to have a successful scale-up, it is desirable to maintain kinetic (particle size, pore size, temperature, mobile phase) and dynamic (bed height, flow rate, packing density) equivalence between the chromatography columns of the ALC and PHPLC system (Rathore and Velayudhan, 2003).

The common method of scaling up preparative chromatography is to keep the plate count constant, and increase the feed volume and column volumes proportionately. This was originally based on the assumption of linear adsorption. In this case, the length and

efficiency of PHPLC column are the same as those of ALC column, and then the elution profiles produced should be identical. Equation 5.1 is the scale-up formulas related to the volumetric flow rate and the injection volume (Rathore and Velayudhan, 2003; Heue *et. al.*, 1996).

$$\frac{X_P}{X_A} = \frac{F_P}{F_A} = \frac{d_P^2 L_P}{d_A^2 L_A} \qquad [5.1]$$

Where X_P and X_A : injection volumes of PHPLC and ALC columns respectively

F_P and F_A : volumetric flow rates of PHPLC and ALC columns respectively

d_P and d_A : diameters of PHPLC and ALC columns respectively

L_P and L_A : lengths of the PHPLC and ALC columns respectively

5.2.3 MTT cell proliferation assay (MTT assay)

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was employed in this work to investigate the bioactivities of the water and the methanol extracts and fractions of RDLP on human cell lines. In the bioactivity study, the safety and efficacy of the two RDLP extracts were determined via Hep3B, HepG2, WRL-68 and MRC-5 cell lines. It was desirable to select an effective and safe extract from them for further study. Then the bioassay-guided fractionation method as described earlier was applied to find the bioactive compounds from the selected extract on Hep3B and HepG2 to be mentioned.

In the MTT assay, the optical density (O.D.) of cells treated with medium only and the extracts are measured at 595nm. The O.D. percentage (O.D. %) was calculated using Equation 5.2. The value of O.D. % is directly proportional to the quantity of cell survival, and the cells incubated with medium only is served as a control with 100% survival. The antiproliferation curve was set up by plotting the O.D. % against a series of concentration of the extracts, and the 50% inhibitory concentration (IC50) was determined.

$$O.D.\% = \frac{O.D.\ \ of\ \ cell\ \ treated\ \ with\ \ sample}{O.D.\ \ of\ \ cell\ \ treated\ \ with\ \ medium} \times 100\% \qquad [5.2]$$

5.3 Experimental

5.3.1 Sample, chemicals and reagents

The RDLP sample used in this study was a sachet containing powdered herbal formulation prescribed by a famous traditional CM practitioner in Hong Kong. Chromatographic graded methanol and analytical graded dimethyl sulfoxide (DMSO) were purchased from Tedia (USA). MTT salt was obtained from Sigma (USA). The reagents for cell culture including phosphate buffered saline (PBS) buffer, fetal bovine serum (FBS), Penicillin-Streptomycin liquid, Dulbecco modified Eagle's medium (DMEM) , RPMI medium 1640 (RPMI) and minimum essential medium (MEM), were purchased from Gibco (USA). Double deionized water utilized for extraction and preparing mobile phase was purified by Milli-Q water system (Millipore Corp., Bedford. MA, USA).

5.3.2 Sample preparation

5 g RDLP powder was mixed with 300ml 100% water and 100% methanol, and sonicated for 30 minutes. The mixture was centrifuged at 3000 rpm for 20 minutes. The supernatants were collected and evaporated with rotary evaporator until dryness. It was then dissolved with 120mL of 50% methanol solution. The extract was filtered with nylon pre-cut membranes (0.45μm pore size, Millipore) before chromatographic analysis.

RDLP extracts and fractions were powdered before subjecting to the MTT assay. The extract and fractions were dried with rotary evaporator and then lyophilized by freeze dryer to get the dry powder. The dry powder was stored at -18^{o}C and dark place until use. The concentrations of the extracts and fractions used in the bioassay were based on the dry weights of the powder extracts (mg/ml).

5.3.3 Chromatographic conditions

5.3.3.1 Analytical high performance liquid chromatography (ALC)

The RDLP extract was analyzed by using a C_{18} reversed-phase HPLC column (Hypersil ODS, 250 x 4.6 mm I.D., 5μm, Thermo Fisher Scientific, Inc., USA) conjunct with a HP 1100 series HPLC Pump and HP 1100 series photodiode array detector (DAD). The associated computer software of the HP HPLC-DAD system, HP ChemStation, was utilized for the chromatographic data acquired.

The mobile phase utilized to analyze the RDLP extract was under isocratic condition of methanol – water (1 : 1 v/v) and the flow rate was 0.5 ml/min. The detection wavelength was set at 225 nm.

5.3.3.2 Preparative high performance liquid chromatography (PHPLC)

The extracts were fractionated by Hypersil ODS C18 preparative column (250 x 21.2 mm I. D., 5 µm, Thermo Fisher Scientific, Inc., USA) with HP 1100 preparative series HPLC Pump, preparative autosampler, and DAD with detection at 225nm. A solvent saver, Solvent Recycler Junior (Alltech, USA), was connected with the preparative system. The mobile phase was the same as those in ALC with the flow rate 10 ml/min and injection volume 400 µl which were calculated by Equation 5.1 and 5.2 respectively. The fractions of RDLP extract were obtained by the bioassay-guided fractionation and collected with a Foxy® Jr. fraction collector (ISCO, Inc., USA).

5.3.4 Cell Culture

Human hepatoma cell lines (HepG2 and Hep3B), human normal lung cell line (MRC-5) and HeLa cell line (WRL-68) used here were obtained from Department of Biochemistry in The Chinese University of Hong Kong. These cells were cultured with their appropriate media (Table 5.1) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) Penicillin-Streptomycin liquid (containing 100 unit/ml penicilline and 100ug/ml streptomycin) in 75cm^2 tissue culture flasks at a humidified 5% carbon dioxide incubator at 37^{o}C.

Table 5.1 Culture media for the cell lines used

Cell line	Medium
Hep3B	Dulbecco modified Eagle's medium (DMEM)
WRL-68	Dulbecco modified Eagle's medium (DMEM)
HepG2	RPMI medium 1640 (RPMI)
MRC-5	Minimum Essential Medium (MEM)

5.3.5 MTT assay

The cells were first seeded in 96-well micro-plates (1 x 10^4 cells/well in 100µl appropriate medium) and cultured in a humidified incubator at 37°C with 5% carbon dioxide for 24 hours. Then the medium was discarded, and the cells were re-incubated for 3 days with a serial concentration of the extract dissolved in culture medium. Afterwards, the extract was removed, and each well of the micro-plate was rinsed with 200µL phosphate-buffered Saline (PBS) at pH 7.4. 30µL pale yellow MTT salt solution (3 mg/mL PBS) was added to each well and incubated for 3 hours at 37°C CO_2 incubator to allow the formation of purple formazan crystals. 100µL DMSO was added to dissolve the formazan crystals. The amount of the resulting formazan was measured in terms of optical density (OD) at 595nm in a microplate reader (Model 550, Bio-Rad) (American Type Culture Collection (ATCC), 2004). The cells incubated in medium were served as a control with 100% survival. Three independent experiments were performed in triplication, and the OD% and 50% IC50 were presented as average value ± standard derivation (S.D.) (Chow, et al, 2002).

5.4 Results and Discussion

5.4.1 Qualitative analysis of RDLP extract by ALC

Trying ALC separation is the first step for collecting a quantity amount of the purified material from herbal medicine by PHPLC. This step is very helpful to optimize the PHPLC chromatogram condition and analyze the chemical composition of herbal medicine qualitatively. As no information of the herbal composition was provided for RDLP, the sample extraction and the optimal HPLC chromatographic condition were developed by in the trial-and-error manner.

The chemical components of RDLP were extracted with water, methanol, ethanol, chloroform and hexane. These solvents with different polarity were chosen in order to get the RDLP chemical components with different polarity. Finally, we found that its chemical components could be extracted successfully by polar solvents, water and methanol, only. The water and methanol extracts of RDLP were then examined by MTT assay to compare their efficacy and safety on human hepatoma and normal cell lines respectively. The effectiveness of the growth inhibition effect of these two extracts was similar on the human hepatoma cell lines, extract was found less extent to inhibit the growth of normal cell (Section 5.4.3). Based on the safety issue, the water extract was further analyzed.

The mobile phase selected for PHPLC fractionation is restricted to non-buffer solvent as the salt in mobile phase collected with fractions and purified material does not evaporated out. Our signal pump system of PHPLC is also limited to the isocratic mode of the

mobile phase system. Therefore, the water extract was analyzed by analytical HPLC with different ratios of methanol – water, ethanol – water, isopropanol – water and acetonitrile - water under isocratic condition with flow rate 0.5 ml/min. It was found that the optimized condition was methanol - water (1 : 1 v/v) in 0.5 ml/min with sample injection of 20 µl. Figure 5.1 gives the HPLC chromatogram of the RDLP water extract under the optimized ALC condition. The chromatograms of other solvent systems can be found in Appendix 5.1.

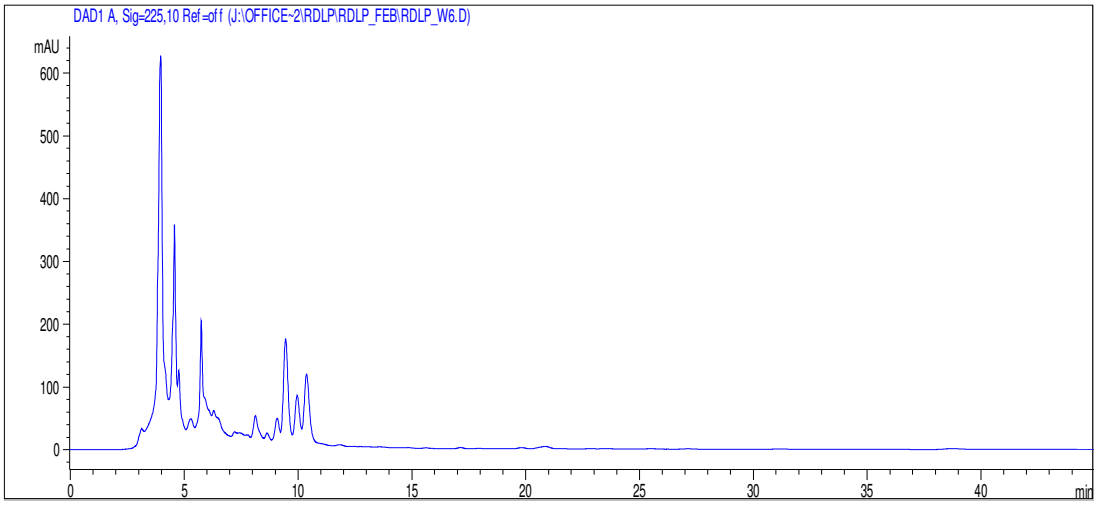


Fig. 5.1 ALC chromatograms of the RDLP water extract analyzed with the solvent system of methanol – water (1 : 1 v/v) in 0.5 ml/min

5.4.2 Antiproliferation effect of the water and the methanol extracts of RDLP

The active chemical compounds of RDLP should be most likely to be extracted by polar solvents, water and methanol, as the patients take the water dissolved RDLP. We tried this by the trial and error process. These extracts were then evaluated by their

performance on antiproliferation on two human cancer cell lines (Hep3B and HepG2), liver (WRL-68) and lung (MRC-5) normal cell line with MTT assay. The antiproliferation effect on Hep3B and HepG2 represented the anticancer effect, while that on WRL-68 and MRC-5 related to safety. Ideally, an extract is good if it gives positive growth inhibition on human cancer cell lines, but no any inhibitory effect on human normal cell lines. This is usually impossible as *in-vitro* cell lines are very sensitive to the strange substance in culture medium. Hence, we attempted to prove that the extracts of RDLP are effective in inhibiting the proliferation of the cancer cell lines, but less harmful to normal cell lines.

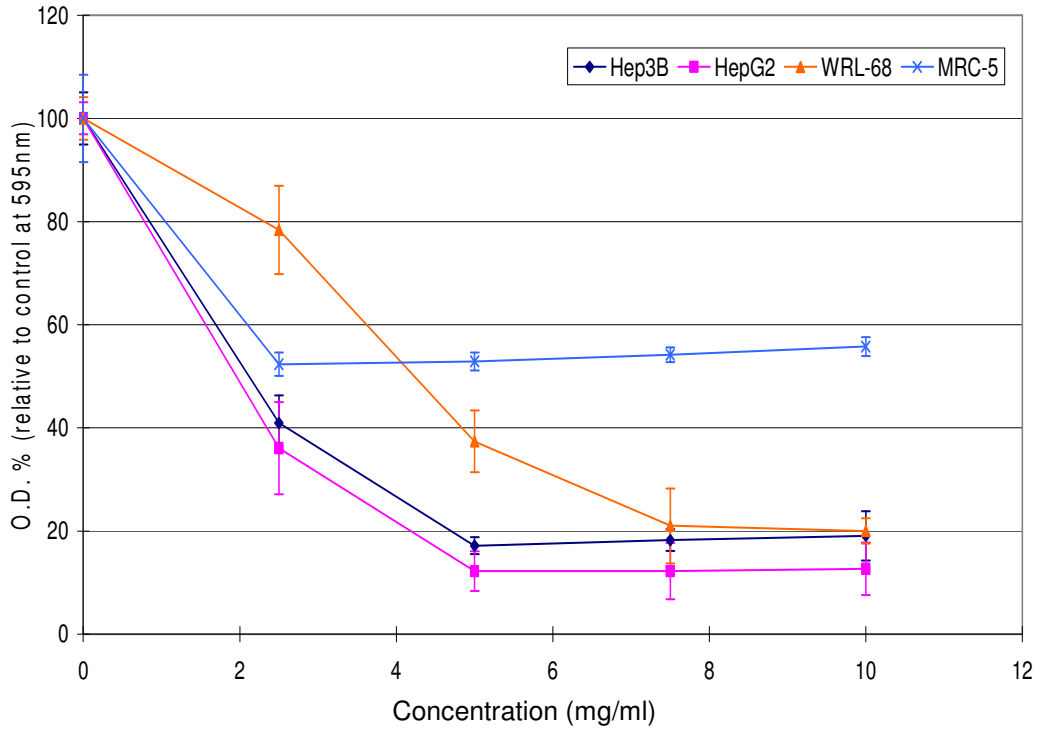


Fig. 5.2 Plots showing the antiproliferation effect of the water extract of RDLP (in the concentration range of 0 – 10 mg/ml) on Hep3B, HepG2, WRL-68 and MRC-5

Figure 5.2 depicts the antiproliferation effect of the water extract of RDLP on the human cell lines. The growth of the human cell lines except MRC-5 could be inhibited in a concentration-dependent manner. From the plots, the IC50 values of Hep3B and HepG2 were determined to be 2.32 ± 0.07 and 2.19 ± 0.28 mg/ml respectively; while that of WRL-68 was 4.32 ± 0.35 mg/ml. As for WRL-68, its IC50 has value around 2-fold greater than that of Hep3B and HepG2. This indicates that the RDLP water extract has potent anticancer effect on liver cancer, and is relatively less harmful to the growth of normal liver cell. In MRC-5, the plot of antiproliferation effect of the water extract (Fig. 5.2) is somewhat unusual at relatively high concentration. More detailed biological screening tests are needed in order to understand the changes of the MRC-5. No explanation is provided here. However, the result indicated that the water extract might attacks cells in liver selectively, especially, liver cancer cells.

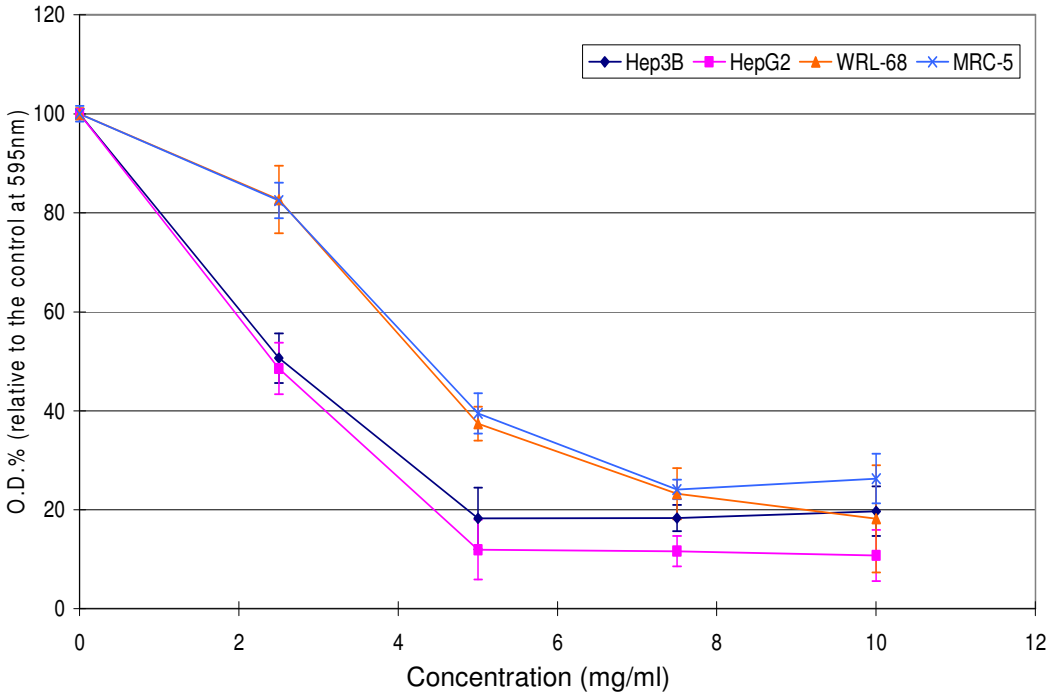


Fig. 5.3 Plots showing the antiproliferation effect of the methanol extract of RDLP (in concentration range of 0 – 10 mg/ml) on Hep3B, HepG2, WRL-68 and MRC-5

The antiproliferation effect of the methanol extract of RDLP on human cell lines is depicted in Figure 5.3. This extract was found to be able to inhibit the proliferation of the human hepatoma and the normal cell line as well. The IC50 of Hep3B and HepG2 were 2.68 ± 0.08 and 2.54 ± 0.16 mg/ml respectively, while the IC50 of WRL-68 and MRC-5 were 4.41 ± 0.32 and 4.58 ± 0.14 mg/ml respectively. The 2-fold larger values of IC50 of the normal cell lines indicate that the methanol extract has potent to inhibit the growth of the hepatoma cell lines and anticancer effect.

Table 5.2 IC50 ± S. D. of the water and the methanol extracts of RDLP on Hep3B, HepG2, WRL-68 and MRC-5 cell lines

RDLP extract	IC50 ± S. D. (mg/ml) of the human cell lines[1]			
	Hep3B	HepG2	WRL-68	MRC-5
Water	2.37 ± 0.07	2.19 ± 0.28	4.32 ± 0.35	>10
Methanol	2.68 ± 0.08	2.54 ± 0.16	4.41 ± 0.32	4.58 ± 0.14

Remark:

[1]: mean value of triplicate experiment

In general, the water and the methanol extracts of RDLP were found to be more effective to inhibit the proliferation of Hep3B and HepG2, and less potent to WRL-86 and MRC-5. A comparison of the IC50s of the water and the methanol extract of RDLP indicated that the water extract was a little bit more effective to inhibit the proliferation of Hep3B and HepG2 as it gives slightly lower values (Table 5.2). In WRL-68, these two extracts gave the same antiproliferation effect. It should be mentioned that the IC50 of the water extract on MRC-5 was more than 10 mg/ml, while that of the methanol extract was 4.58 mg/ml. This might indicated that the RDLP water extract were more selective to affect the cells in liver during the treatment of liver cancer. In view of this, the water extract of RDLP was further studied by PHPLC fractionation.

5.4.3 Fractionation of the RDLP water extract by PHPLC

The RDLP water extract was analyzed by PHPLC by using the identical stationary phase, Hypersil ODS C18 column, and mobile phase system to those of ALC analysis. The flow rate and injection volume for the bench-scale PHPLC were scale-up according to Equation 5.1 and 5.2. The flow rate and the injection volume of the ALC and PHPLC were 0.5 ml/min and 10 ml/min, and 20µl and 400µl respectively. The elution pattern of the PHPLC chromatogram was successful reproduced to that of ALC. Four fractions, F-1, F-1A, F-1B and F-2 (Table 5.3), were fractionated and isolated by the scale-up chromatographic condition from the RDLP water extract. For each fraction, its profile assigned for collection and the collection time are given in Figure 5.4 and Table 5.3 respectively. The fractions to be collected were decided by the bioassay-guided fractionation approach, and details can be found in Section 5.4.4.

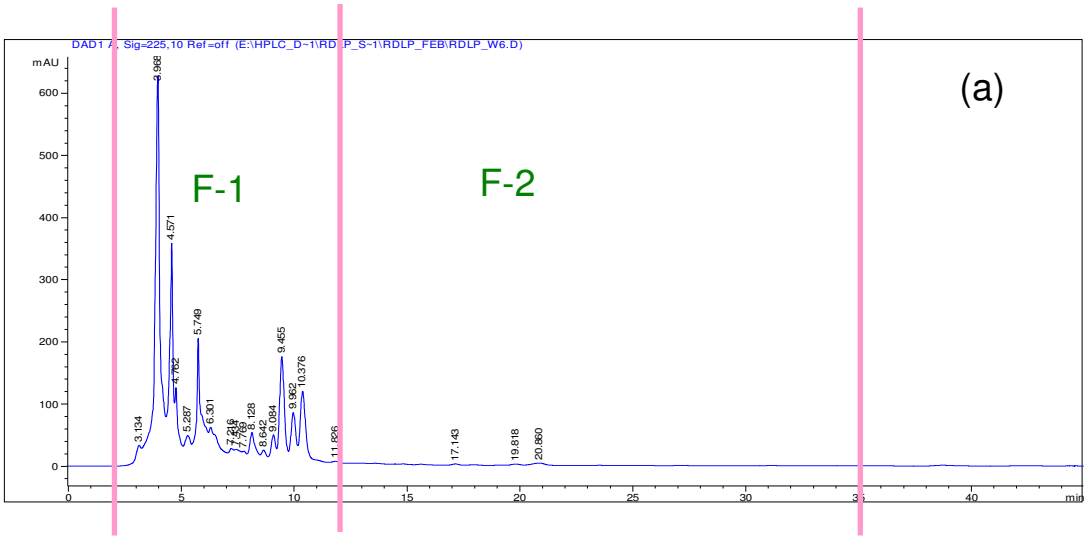


Fig. 5.4 Chromatographic distribution of fractions (a) F-1 and F-2, and (b) F-1A and F1-B as collected by PHPLC

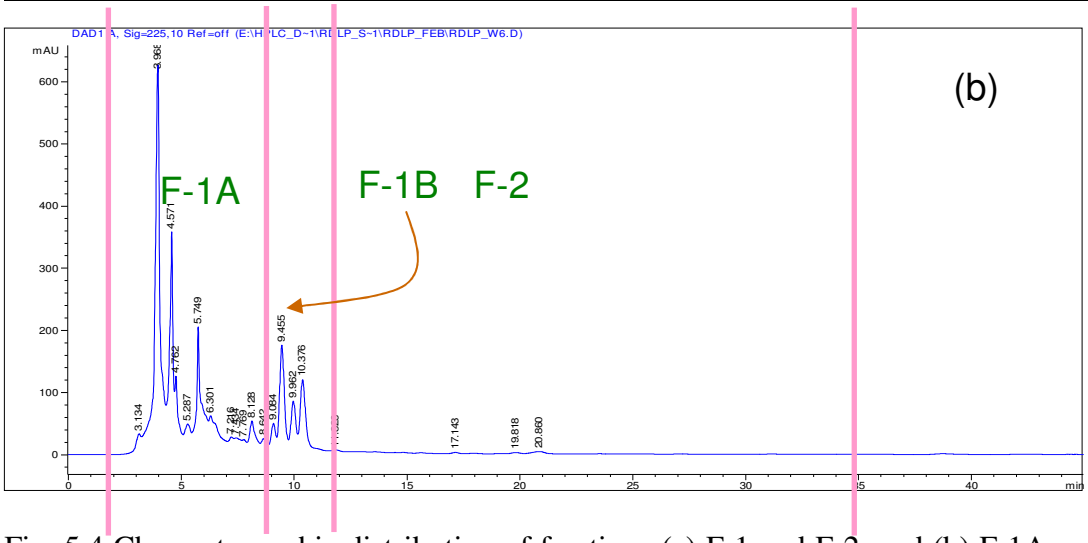


Fig. 5.4 Chromatographic distribution of fractions (a) F-1 and F-2, and (b) F-1A and F1-B as collected by PHPLC (Continued)

Table 5.3 Collection time of the four fractions of the RDLP water extract

Fraction	Collection time (min)
F-1	2.0 ~ 12.0
F-1A	2.0 ~ 9.0
F-1B	9.1 ~ 12.0
F-2	12.1 ~ 35.0

5.4.4 Antiproliferation effect of the fractions from the water extract of RDLP

The water extract of RDLP was fractionated into different fractions by two cycles of bioassay guided fractionation (Fig. 5.5). Each cycle of bioassay guided fractionation produced two fractions. The chemical compounds within the extract were separated by

the HPLC mobile phase (Section 5.4.1), and also fractioned by the scaled up PHPLC chromatographic condition (Section 5.4.2). The fractions of the water extract were then studied by MTT assay on Hep3B and HepG2. Afterward, antiproliferation effect on Hep3B and HepG2 of the fractions were accessed by the water extract. In the first cycle, F-1 was found to be an active fraction, while F-2 is not. Then, it was further fractioned to give F-1A and F-1B and examined by MTT assay.

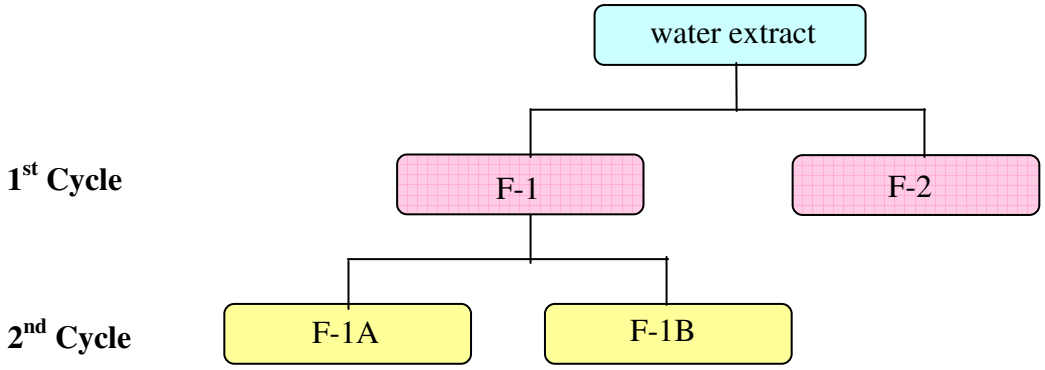


Fig. 5.5 Scheme of fractionation and isolation of the bioactive fractions of the water extract of RDLP

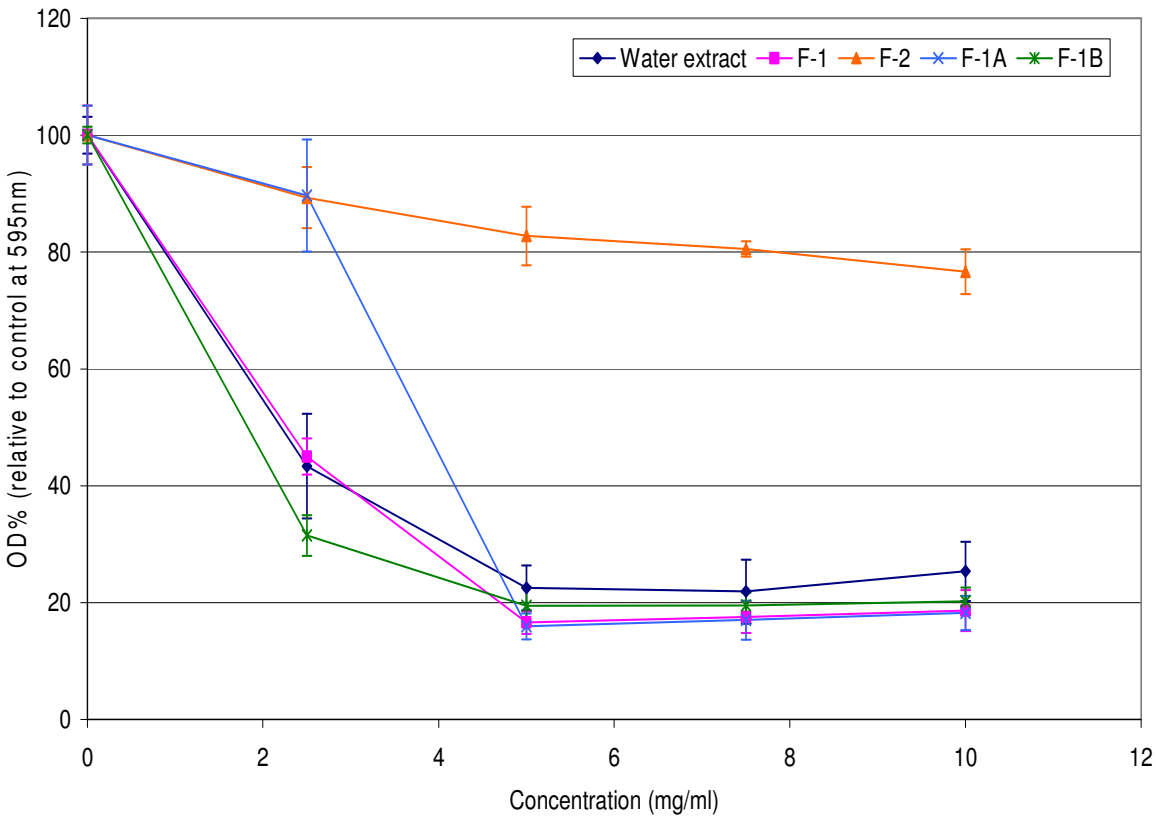


Fig. 5.6 Antiproliferation effect of the four fractions collected and the water extraction of RDLP (in concentration range of 0 – 10 mg/ml) on Hep3B

Figure 5.6 shows the antiproliferation effect of the four fractions generated from two successive cycles (Fig. 5.5) and the water extract of RDLP on Hep3B; obtained by the mean values of triplicate experiment

It was found that the antiproliferation effect of F-1 is much stronger than that of F-2. Comparison of the antiproliferation effect of F-1 with the water extract shows that they gave similar effect on Hep3B, as the IC50 of F-1 and the water extract were 2.38 ± 0.07 and 2.45 ± 0.11 mg/ml respectively, which are very close to each other. This strongly indicates that active compounds of RDLP water extract are in F-1. Hence, F-1 was

further fractioned into two, F-1A and F-1B by PHPLC under the same chromatographic condition. In the second cycle (Fig. 5.5), both F-1A and F-1B could also inhibit the growth of Hep3B, but the potency of F-1A was significantly weakened when compare with the water extract. In contrast, the potency of F-1B was stronger than that of the water extract. This means that F-1B was concentrated on the active chemical compounds of the water extract. With time limitation, no further bioassay-guided fractionation were carried out to get more details.

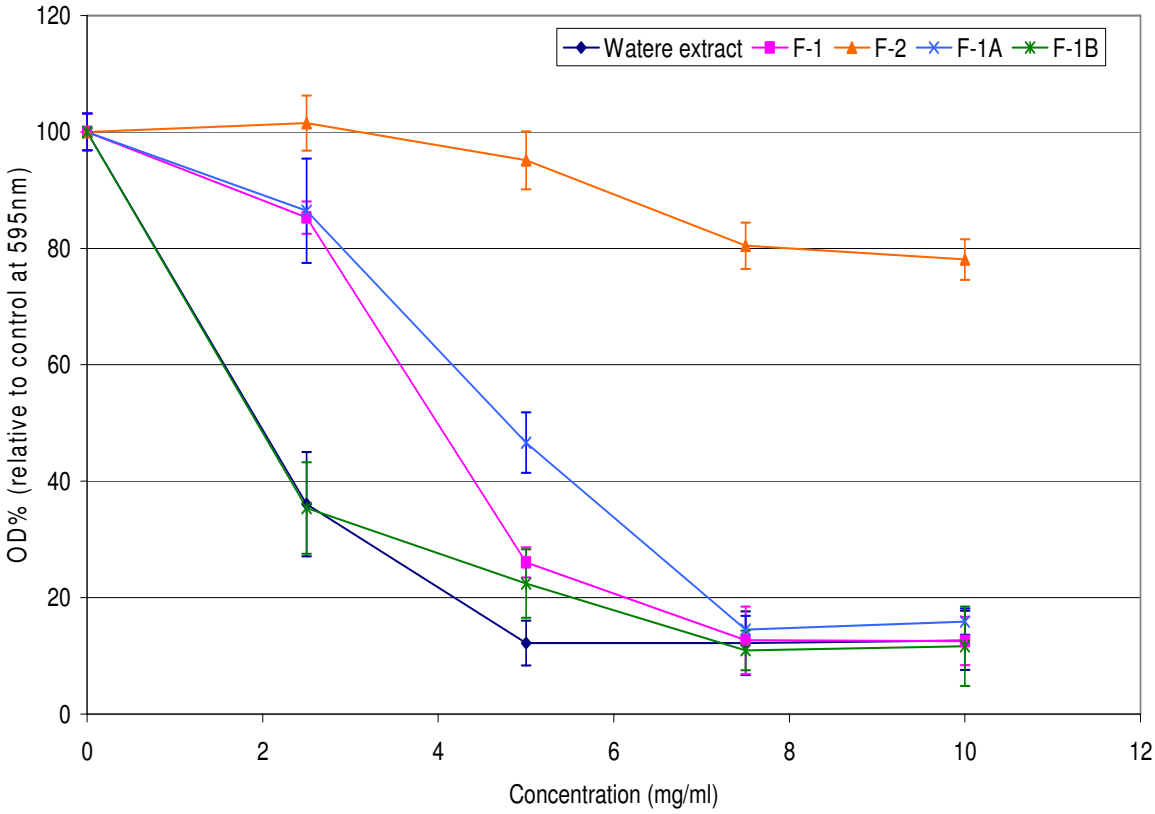


Fig. 5.7 Antiproliferation effect of the four fractions collected and the RDLP water extract (in concentration range of 0 – 10 mg/ml) on HepG2

The first cycle of the fractionation showed that F-1 and F-2 gave different effect on in HepG2 (Fig. 5.7). As mentioned above, F-1 was more extensively to inhibit the growth

of HepG2 than F-2. Similar result was found on Hep3B. However, the IC50 of F-1 on HepG2 was around two times greater than that of the RDLP parent water extract. As for the fractions of F-1, F-1A and F-1B, the antiproliferation effect of F-1A was significantly weaker than that of the water extract, while that of F-1B was almost the same as that of the water extract. This indicates that the active components of the water extract could be found in F-1B as well.

Table 5.4 Summary of the IC50 ± S.D. of the water extract of RDLP and its four fractions on Hep3B and HepG2

Extract/ Fraction	IC50 ± S.D. (mg/ml) of the human hepatoma cell lines[1]	
	Hep3B	HepG2
Water extract	2.32 ± 0.07	2.19 ± 0.28
F-1	2.45 ± 0.11	3.97 ± 0.16
F-2	>10	>10
F-1A	3.86 ± 0.57	4.02 ± 0.71
F-1B	1.79 ± 0.14	2.05 ± 0.07

Remark:

[1]: mean value of triplicate experiment

Based on the above finding, F-1B was found as to be bioactive fraction in the RDLP water extract on Hep3B and HepG2 cell lines, and the other fractions might seem to be eliminated for liver caner treatment. Yet, as a CM formulation, its chemical components come from the component herbs with different roles, King, Minister, Assistant and / or Messenger, according to the working principle of CM compound formula. In this work, the fractions collected gave different results on the antiproliferation effect on the human

hepotoma cell lines, in which might result from their different biological function in RDLP. Here, the biological function of the fractions was not confirmed by using this simple *in-vitro* bioassay.

5.5 Conclusion

In this study, we successful found that the polar solvents, water and methanol, could extract the active chemical compounds a CM compound formula RDLP with proven efficacy on liver cancer. Also, the HPLC chromatographic condition was developed via ALC and PHPLC for chemical study and fractionation. The RDLP water extract was found to be not only possessing the anticancer effect on the human hepatoma cancer lines (Hep3B and HepG2), but also it was safe for liver (WRL-68) and especially lung (MRC-5) normal cell lines. This evidence supports why the RDLP formulation has been used successfully to treat liver cancer for more than twenty years by the Chinese medicine practitioner.

We also attempted to discover the bioactive compounds in the water extract of RDLP via bioassay-guided fractionation to collected four fractions, F-1, F-2, F-1A and F-1B. F-1B was found to have the strongest antiproliferation effect on both hepatoma cancer cell lines, and should contain bioactive chemical components responsible for the cancer treatment of the RDLP water extract. The other fractions of the water extract also performed different extent of antiproliferation effects on the cancer cell lines. The other biological functions of F-1, F-2 and F-1A were not further studied here.

Chapter 6

Characterization of Danggui Buxue Tang by High Performance Liquid Chromatography and Chemometric Techniques

6.1 Introduction

6.1.1 Background

Among thousands of different TCM formulations, Danggui Buxue Tang (DBT; an herbal decoction) is one of the simplest ones. The use of DBT was first recorded in *Neiwaishang Bianhuo Lun* by Li Dongyuan in China in 1247 A.D. It stated this formulation containing two component herbs, Radix Astragali (Huangqi, HQ) and Radix Angelicae Sinensis (Danggui, DG), which are boiled with water to prepare the DBT decoction. DBT is prescribed to women in China to improve menopausal symptoms, and it is recommended to drink the decoction daily to raise the "*qi*" (the vital energy) and nourish the "*blood*" (the body circulation) of the individual.

In recent years, some studies revealed that water extract of DBT exhibited stronger biological activities (Dong *et. al.,* 2006; Gao *et. al.,* 2006; Mak *et. al.,* 2006; Song *et. al.,* 2004). In order to get better understanding on the pharmacokinetics and mechanism involved of the water extract of DBT, different *in-vivo* and *in-vitro* tests were carried out. Promising and convincing results were obtained (Chiu *et. al.,* 2007; Dong *et. al.,* 2006; Gao *et. al.,* 2006; Mak *et. al.,* 2006; Song *et. al.,* 2004). In addition, the chemical properties of water extract of DBT were investigated by HPLC-DAD. Five chemical components of DBT, astragaloside IV, calycosin, formononetin, ferulic acid, and *Z*-ligustilide, were considered as markers in qualitative and quantitative analysis for quality control. This way is regarded as the "marker approach" (Mok and Chau, 2006). There were also other studies trying to correlate these five components to the efficacies of DBT

(Dong *et. al.,* 2006; Gao *et. al.,* 2006; Song *et. al.,* 2004). However, it is better to consider more chemicals of the herbal preparation. Therefore, other new approaches of "pattern approach" and "multi-component approach" should be considered to get more chemical information in order to provide better understanding on the relationship between the chemical components and biological activities of DBT.

In this study, the combination of HPLC-DAD and chemometrics was applied for analyzing the chemical compositions of the extracts of DBT and its components, HQ and DG. All, the "marker", "multi-component" and "pattern" approaches were utilized to interpret the data acquired. As mentioned above, studying just a few specific herbal markers in samples is regarded as "marker approach". Here, five chemical components of DBT, astragaloside IV, formononetin, ferulic acid, *Z*-ligustilide and n-butylidenephthalides, were investigated in the boiled DBT extract by using their own retention times and UV spectra for HPLC-DAD only. Through this, only five chemical components in the extract were considered, but a pool of unknown chemical constituents in the formulation is ignored. In order to know more chemical information of the complex extraction of DBT, chemometric data processing methods of multicomponent spectral correlation chromatogram (MSCC) and similarity index (SI) were employed in our "multi-component" and "pattern" approaches on the chromatogram of DBT respectively. In "pattern approach", similarity index (SI) was applied to study the entire chromatographic profiles between the boiled and soaked DBT extracts. On the other hand, the clusters of chemical constituents in the boiled DBT extract were characterized by MSCC attempting to identify what chemical components of DBT can be found in its components herbs. Details of the works done are given in the following sections.

6.1.2 Danggui Buxue Tang

DBT is a Chinese medicinal decoction used commonly for treating women's ailments. It comprises two component herbs Radix Astragali (Huangqi, HQ) and Radix Angelicae Sinensis (Danggui, DG). Water extracts of DBT obtained from different ratios of DG and HQ were found to give different extents of effect in cell proliferation on MG-63 cells and MCF-7 cells, anti-platelet aggregation and immunomodulatory effects onto cultured T-lymphocytes and macrophages (Dong *et. al.,* 2006; Gao *et. al.,* 2006). The mass ratio of 5:1 in HQ to DG was found to give the best effect and this verified the best ratio recommended in the ancient literature.

Pharmacological results indicated that DBT has the ability to promote hematopoetic function, stimulate cardiovascular circulation, prevent osteoporosis, and possesses anti-oxidation activity and immune stimulatory activity (Gao *et. al.,* 2006; Ning *et. al.,* 2002; Wu *et. al.,* 1999; Han *et. al.,* 1998). Besides, a study found that water extracts of DBT obtained under different extraction conditions showed marked effects in stimulating osteoblast proliferation, estrogen promoter activation and anti-platelet aggregation activity (Song *et. al.,* 2004). In addition, the cardioprotective activity of DBT investigated by the H9c2 cell line and rats were also carried out (Chiu *et. al.,* 2007; Mak, *et. al.,* 2006). The results indicated that DBT treatment helped to protect against oxidant injury in H9c2 cells (Chiu *et. al.,* 2007) and DBT pretreatment could enhance myocardial mitochondrial and red blood cell glutathione status and thereby increasing their resistance to oxidative stress-induced injury in rats (Mak *et. al.,* 2006).

It is worthwhile to note that the stronger biological activities of DBT extracts comparison to that of its components herbs was accompanied by the higher levels of active chemcial ingredients, namely astragaloside IV, calycosin, formononetin, ferulic acid, and Z-ligustilide (Chiu *et. al.,* 2007; Dong *et. al.*, 2006; Song *et. al.*, 2004). The first three present in HQ while the last two in DG. The chemical structures of these five active components are shown in Table 6.1. Since they are all water-soluble, so high-performance liquid chromatography (HPLC) is commonly utilized to analyze their chemical properties (Chiu *et. al.,* 2007; Dong *et. al.,* 2006; Gao *et. al.,* 2006; Mak *et. al.,* 2006; Song *et. al.,* 2004).

Table 6.1 Chemical structures of five active components of DBT

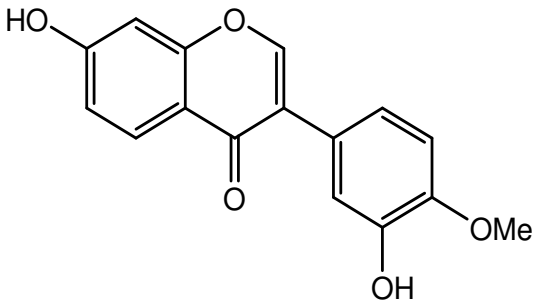
Active component	Chemical Structure
Calycosin	 $C_{16}H_{12}O_5$

Table 6.1 Chemical structures of five active components of DBT (Continued)

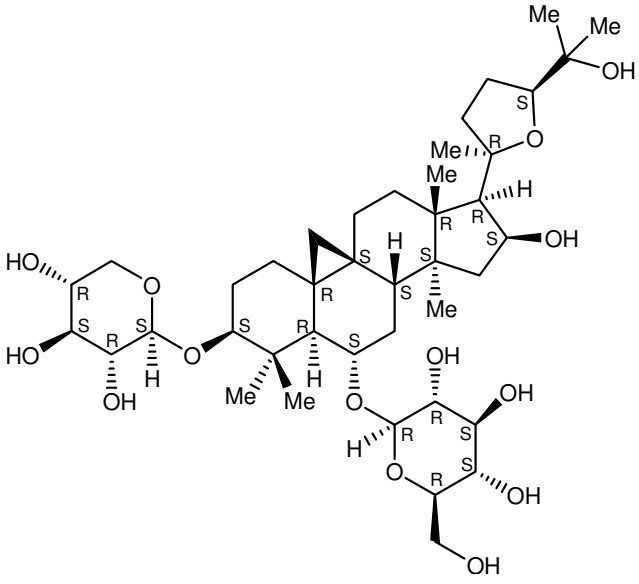
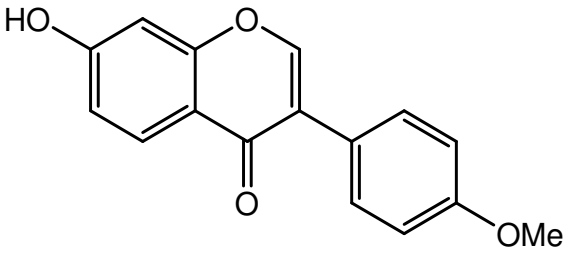
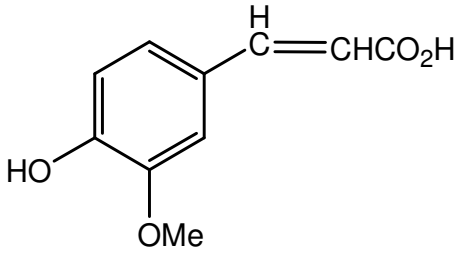
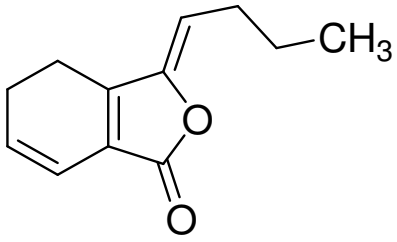
Active component	Chemical Structure
Astragaloside IV	 $C_{41}H_{68}O_{14}$
Formononetin	 $C_{16}H_{12}O_4$

Table 6.1 Chemical structures of five active components of DBT (Continued)

Active component	Chemical Structure
Ferulic acid	 $C_{10}H_{10}O_4$
Z-ligustilide	 $C_{12}H_{14}O_2$

6.2 Methods of Investigation

Two types of DBT, HQ and DG extracts, boiled (B) and soaked (U), were studied in this work. As mentioned, three approaches were applied to characterize their chemical compositions. These three approaches are "marker approach", "pattern approach" and "multi-component approach". Besides, chemometric techniques are also applied to retrieve more chemical information from the data obtained and to improve the quality of fingerprints for future chemical characterization of DBT.

"Marker approach" refers to making use of one or very few markers or active components to identify herbal extracts (Mok and Chau, 2006). This approach is widely used in Chinese Medicine (CM) pharmacopoeias and related regulatory agencies as measurement of the amount of markers can be carried out rapidly and at low cost. Here, five markers as mentioned before were utilized to study chemical compositions of boiled DBT, HQ and DG extracts. However, this approach has its limitations. The first one is that the number of standards available is very limited and in reality, some markers or active components are not unique to a particular herb but also present in other herbs (Mok and Chau, 2006). In addition, a few markers cannot reflect completely the whole biological activities of the CM concerned as the pharmaceutical actions are contributed by different components working together. As a result, the outcome from "marker approach" is only acceptable.

"Multi-component approach" is a natural extension of the "marker approach". It uses the relative compositions of many or even all identified components of the sample through their chemical profiles, to represent the sample. This approach has been employed for some complicated herbal medicines (Mok and Chau, 2006). However, the complex herbal medicine system usually contain hundreds or even thousands of chemicals and so its chromatogram acquired by hyphenated instrument almost always contains overlapping peaks that could hinder the identification of chemical components present as the exact retention times and pure spectra of the corresponding components cannot be acquired. Here, a powerful chemometric tool called multi-component spectral correlation chromatogram (MSCC) was introduced so as to detect whether the overlapping peaks were spectrally correlated with each other with consideration of their retention times also.

In this way MSCC can facilitate the comparison of common chromatographic peak clusters among fingerprints of different samples. This makes "multi-component approach" possible and more convenient, and we applied it in this way to identify the chemical components of boiled DBT from its components herbs, HQ and DG.

Other than "marker approach" and "multi-components approach", the "pattern approach" is an alternative way to characterize herbal samples (Mok and Chau, 2006). In general, this approach can be classified as the "all-information based approaches" as it makes use of the whole chromatograms as a pattern or an image to scrutinize the complicated herbal extract. This approach is the kind of fingerprint that the US Food and Drug Administration (FDA) adopted in the regulation of TCM injection products and supposed to be implemented in year 2004 (Mok and Chau, 2006). The advantage of using the entire chromatogram over certain number of selected peaks in the other two approaches is that for complex systems such as herbal extracts or preparations, subjective integration parameters and peaks selection are avoided. Most importantly, the biological or the pharmaceutical activities of CM are due to more than one marker or one active component. Thus, the whole pattern of chromatogram in this approach can provide another picture on pharmacological activity of CM in the real situation. Similarity index (SI) is usually applied in order to quantify how similar are the chromatograms of the CM samples concerned. Here, the SI tool was utilized to compare the chromatographic fingerprints of the boiled and soaked sets of DBT, DG and HQ.

6.3 Experimental

6.3.1 Samples of Radix Astragali and Radix Angelicae Sinense, chemicals and reagents

The dried roots of Radix Astragali (HQ) and Radix Angelicae Sinense (DG) were obtained from Institute of Chinese Medicine, Chinese University of Hong Kong, P. R. China in summer 2007. Both samples were grounded and mixed well individually. Danggui Buxue Tang (DBT) was the mixture of HQ and DG (5:1 w/w).

Five standards were applied to be markers of HQ and DG. Formononetin and astragaloside IV as the markers of HQ were obtained from LKT Laboratories (USA) and NICPBP (China) respectively. Two essential oil markers of DG, *Z*-ligustilide and n-butylidenephthalide, were purchased from Wako (Japan) and Lancaster Synthetic (USA) respectively. Ferulic acid, the DG marker, was bought from Acros Organics (USA). Double deionized water for the extraction and the mobile phase was purified by Milli-Q water system (Millipore Corp., Bedford, MA, USA). HPLC grade methanol was purchased from Tedia (USA).

6.3.2 Sample preparation

The extraction procedure of DBT following the traditional preparation was reported to be the optimal extraction condition by Tsim, *et. al.* of Hong Kong University of Science and Technology (Song, 2004). DBT (mixture of 5g HQ and 1g DG) and the component herbs were boiled with 48 ml double-deionized water under refluxing for 2 hour. Afterwards,

the extract was filtered, and the residue was extracted repeatedly twice. All the liquid extracts were combined and concentrated with rotary evaporator until to one-third of its original volume. The concentrated extract was then dried by lyophilization to obtain the powder extract of DBT. The same condition was applied to prepare the powder extracts DG and HQ as well.

DBT, HQ and DG were also extracted by soaked in water at room temperature twice for 2 hours, and the drying process was the same as above. Totally, six extracts were obtained in this study and their labels are given in Table 6.2. In HPLC-DAD measurement, 50 mg/ml of the extracts were filtered with 0.45μm nylon filter (Alltech).

Table 6.2 Water extracts of DBT, HQ and DG and their labels

Extracts studied	Label
Boiled Danggui Buxue Tang	B-DBT
Boiled Huangqi	B-HQ
Boiled Danggui	B-DG
Soaked Danggui Buxue Tang	U-DBT
Soaked Huangqi	U-HQ
Soaked Danggui	U-DG

6.3.3 HPLC-DAD analysis

All the chromatographic separation were carried out using an Agilent Series 1100 HPLC system (Agilent Technologies, Inc., CA), which consisted of a degasser, the pumps, an autosampler and a photodiode array detector (DAD), and equipped with a C_{18} reversed-phase HPLC column (Hypersil ODS, 5 μm particle size, 4.6 × 250mm) for separation and detection of the DBT, DG and HQ extracts. The associated computer software of the Hewlett Packard (HP) HPLC-DAD system, HP ChemStation, was utilized for processing the chromatographic data.

The mobile phase for HPLC separation of the chemical constituents of the sample extracts was a mixture of water and methanol. The variations of the mobile phase compositions with time used are listed in Table 6.3 (Wang, 2006). The flow rate was 0.8 ml/min. The UV absorbance of the sample extracts was monitored from 200 to 400 nm. The reverse phase column was kept at room temperature.

Table 6.3 Gradient of the mobile phase composition

Elution time (min)	Methanol (%)	Water (%)
0.0	1	99
10.0	25	75
20.0	50	50
30.0	70	30
50.0	90	10
60.0	90	10

6.4 Results and Discussion

6.4.1 Study of chemical components of the water extract of DBT by "marker approach"

Astragaloside IV, calycosin, formononetin, ferulic acid, and *Z*-ligustilide are widely accepted and used as standards for DBT for chemical analysis in the "marker approach" (Chiu *et. al.,* 2007; Dong *et. al.,* 2006; Gao *et. al.,* 2006; Mak *et. al.,* 2006; Song *et. al.,* 2004). The first three markers present in HQ also while the last two in DG. However, calycosin was not used here and it was replaced by another standard called n-butylidenephthalide that presents in DG.

Before quantitative determination of these markers in the water extract of DBT, HQ and DG, qualitative analysis was required to carry out first. However, it was found that the chromatogram of astragaloside IV at 100ppm concentration at 203nm (Appendix 6.1), which is the wavelength suggested in examination of astragaloside IV according to some literatures, showed no absorption (Chiu*et. al.,* 2007; Dong *et. al.,* 2006; Gao *et. al.,* 2006; Mak *et. al.,* 2006; Song *et. al.,* 2004). Therefore, the standard astragaloside IV cannot be examined under this chromatographic condition and cannot be used for qualitative analysis of DBT. Besides, in order to examine the other four markers, formononetin, n-butylidenephthalide, ferulic acid, and *Z*-ligustilide, by one fingerprint of DBT extract, one selected wavelength must be chosen first. In this study, 254nm was chosen although ferulic acid and *Z*-ligustilide have λ max at 313 nm. Table 6.4 lists the retention properties and UV spectra of these four markers, and the chromatograms of these five

markers were shown in Appendix 6.1. Splitted peaks were found in formonentin, n-butylidenephthalide and *Z*-ligustilide. This phenomenon might cause by various issues including instrumental problem, the purity and the stability of herbal markers. Essential oil standards of *Z*-ligustilide and n-butylidenphthalides were very unstable, and their isomers formed rapidly at room temperature. So, their splitted peaks were most likely coming from their isomers with much closed retention time. The splitted peak of formonentin might result from impure compound.

Table 6.4 UV spectra and retention times of formononetin, n-butylidenephthalide, ferulic acid, and *Z*-ligustilide at 254nm in DBT for qualitative analysis

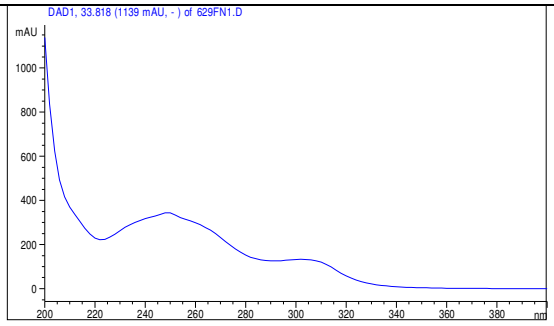
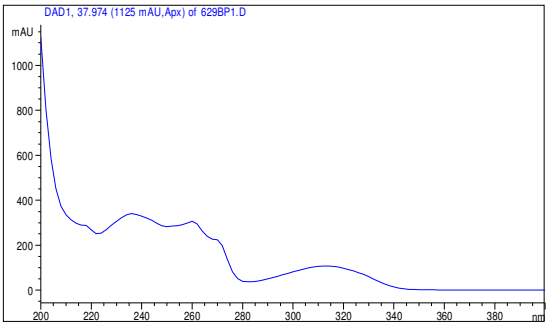
Chemical marker	UV spectrum and retention time
Formononetin	 **33.59 + 33.82 min (splitted peak)**
n-butylidenephthalide	 **37.97 + 38.26 min (splitted peak)**

Table 6.4 UV spectra and retention times of formononetin, n-butylidenephthalide, ferulic acid, and Z-ligustilide at 254nm in DBT for qualitative analysis (Contiuned)

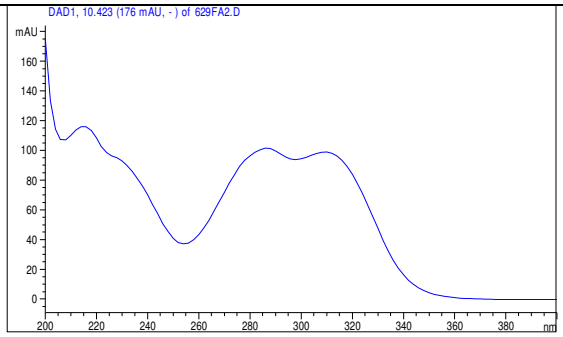
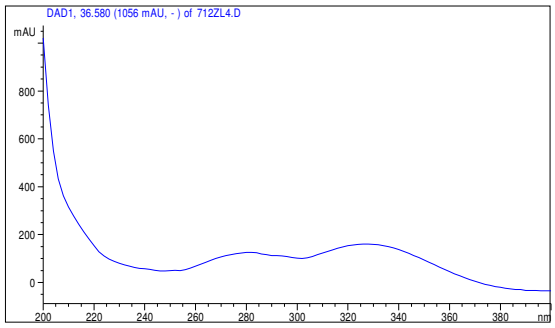
Chemical marker	UV spectrum and retention time
Ferulic acid	 **10.42 min**
Z-ligustilide	 **36.579 + 37.183 min (splitted peak)**

Figure 6.1 shows the chromatogram of B-DBT, B-DG and B-HQ (Table 6.2) at 254nm. No peaks from the standards were detected under this chromatographic condition. This tells us that B-DBT, B-DG and B-HQ did not contain formononetin, n-butylidenephthalides, ferulic acid, and *Z*-ligustilide. It may be due to the sources of HQ and DG contains no or very little these four markers to be detected under our chromatographic condition when the background spectra became more significant. In addition, these markers may be probably heat-sensitive so that they may be easily decomposed under boiling extraction condition. Besides, CMs including DBT, DG and

HQ are a complicated multi-component chemical system. It is inevitable that their chromatograms usually contains overlapping peak clusters which mean that the detected peaks from different components are close to one another or even group together. This leads to difficulty in using individual peaks to carry out qualitative determination by using conventional methods.

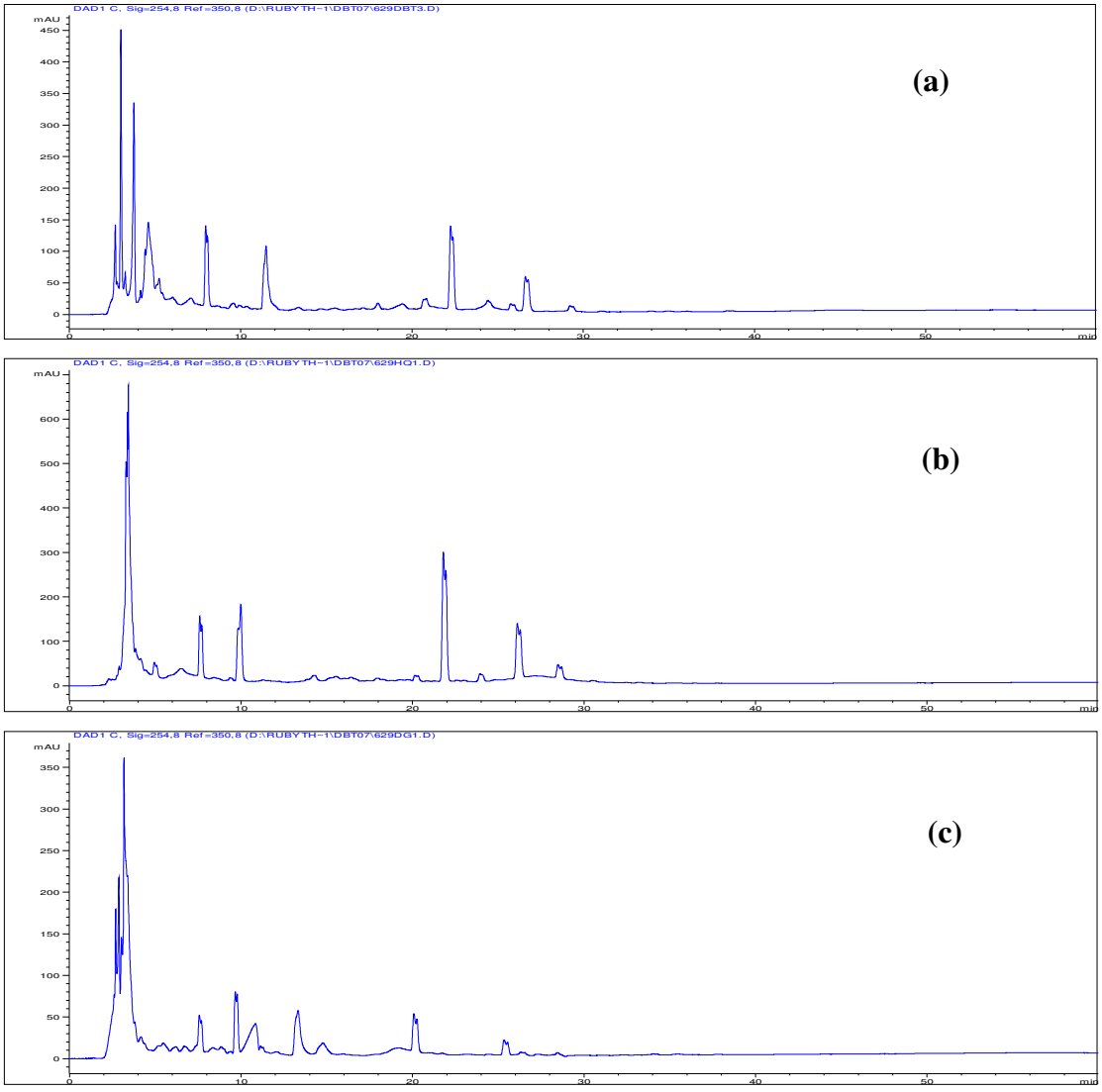


Fig. 6.1 HPLC-DAD chromatograms of (a) B-DBT, (b) B-HQ and (c) B-DG at 254nm (Table 6.3)

From the above results, we can see that the sample of B-DBT, B-DG and b-HQ cannot be studied appropriately by using the five markers only for characterization and identification of their chemical compositions because of mainly the extraction method affect what chemical composition to be obtained. Therefore these samples are not suitable to be studied purely based on experimental separation. Hence, chemometric methods are required to process the chromatographic data to study the chemical constituents of these samples.

6.4.2 Study of chemical components of the water extract of DBT by "pattern approach"

In the traditional extraction method of DBT, boiling in water by moderate heat is recommended and the best extraction condition from chemist point of view (Chiu *et. al.,* 2007; Dong *et. al.,* 2006; Gao *et. al.,* 2006; Song *et. al.,* 2004). However, no other extraction conditions are provided in the literature for DBT formulation. Soaked is also commonly used as extraction condition of CM or CM formulation. Therefore, we attempted to compare the DBT, DG, HQ extracts obtained from boiled and soaked extraction method. HPLC was applied to obtain their chromatographic data and to estimate how similar are the boiled and soaked set data with similarity index (SI). Here, the pattern of chromatograms acquired from wavelengths between 200 and 400 nm was considered.

Figure 6.2 and 6.3 show the 3D chromatograms and 2D total chromatograms of boiled and soaked of DBT, DG and HQ, respectively Table 6.5 lists the SIs between boiled and soaked DBT, HQ and DG extracts.

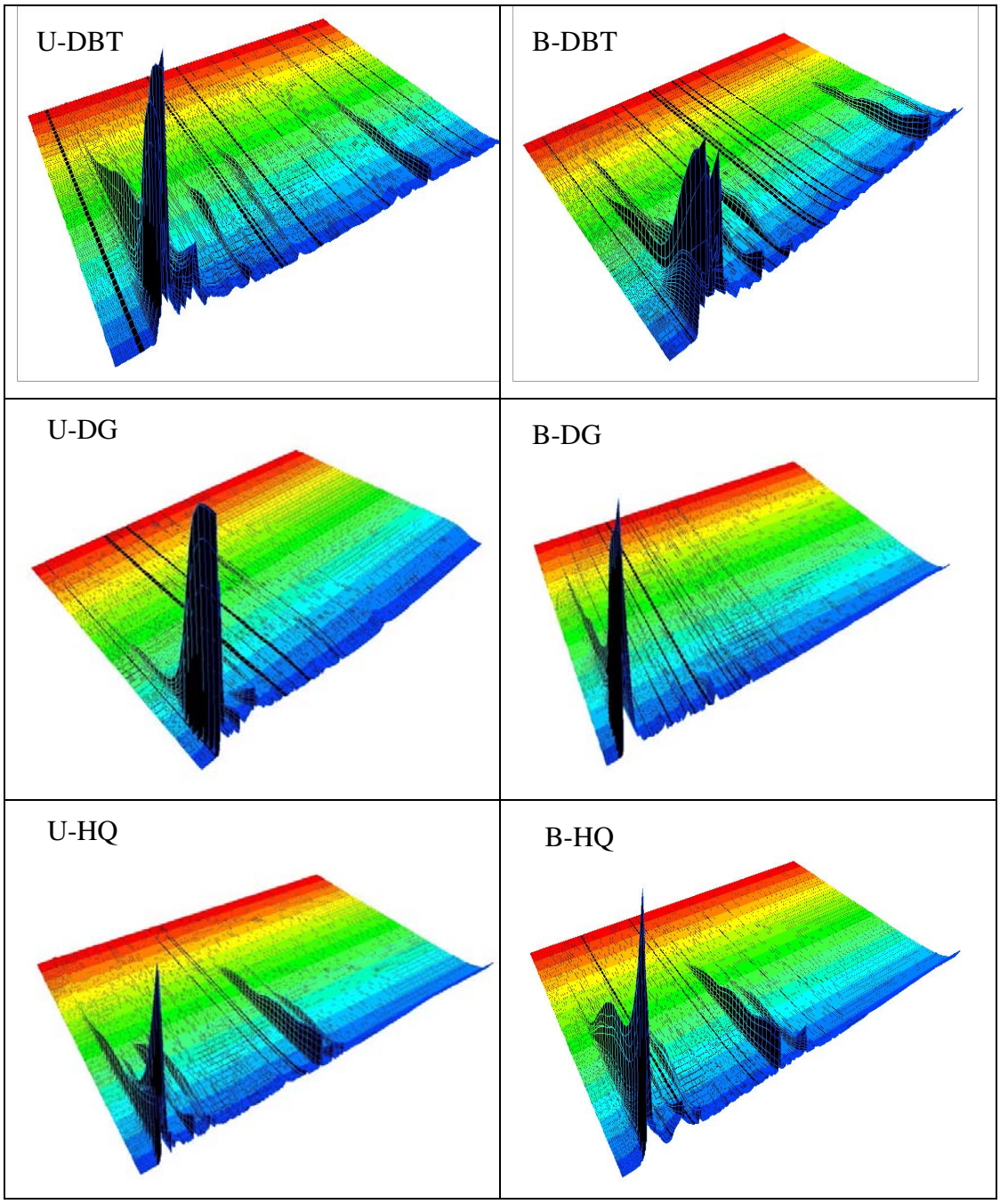


Fig. 6.2 3D Chromatograms of U-DBT, U-DG, U-HQ, B-DBT, B-DG and B-HQ

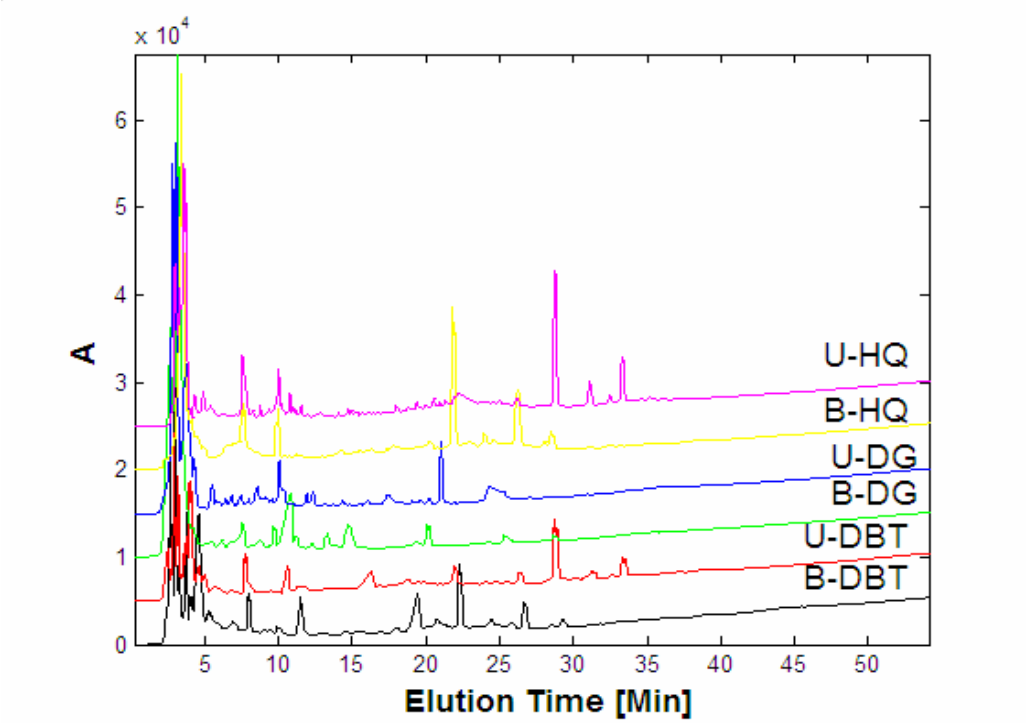


Fig. 6.3 2D chromatograms of boiled and soaked DBT, HQ and DG

Table 6.5 Similarity indices of the water extracts of boiled and soaked DBT, HQ and DG.

Extracts	*Similarity index
U-DBT and B-DBT	86.0%
U-HQ and B-HQ	93.6%
U-DG and B-DG	85.6%

* SI was obtained by comparing the two chromatograms of boiled and soaked samples under study. 100% represents that the two chromatograms are identical to each other

From Table 6.5, it can be seen that SI value of U-DBT against B-DBT is just 86%. This implies that the chromatograms of U-DBT and B-DBT are different in the number of peaks observed and individual peak areas. In other words, the chemical composition of U-DBT is not the same as that of B-DBT. The differences can be attributed to that some chemical components of DBT are heat-sensitive and may change under boiling. In

addition, reactions among these components upon heating in the boiling process can give rise to new compounds. In summary, the boiled and soaked DBT should lead to different biological activities. These variations are also observed in the samples of U-DG and B-DG, U-HQ and B-HQ.

6.4.3 Study of chemical components of water extract of DBT by "multi-component approach"

As mentioned before, the "multi-component approach" is an extension of the "marker approach" as it uses much more or over all identified components from the CM samples concerned from chemical analysis. Chemical structure of some of them may not be known. Yet, their retention times, the parameter usually used by analytical chemists as well as their pure spectra are known obtained through chromatographic and chemometric studies. The former parameter is usually the one used by analytical chemists for identification while the later one for the same purpose through not easy to get from overlapping peak clusters. In here, the "multi-component approach" was applied to treat the chromatographic data sets of all the B-DBT, B-DG and B-HQ samples obtained.

Chromatograms of complicated DBT, DG and HQ systems contain overlapped peak clusters that may lead to incorrect identification of the components present if no date processing is carried out. Therefore, data treatment together with data pre-treatment is necessary to tackle this problem. The chemometric data processing tool called multicomponent spectral correlation chromatogram (MSCC) was utilized for comparing the well resolved spectrochromatograms and even overlapped spectrochromatograms to resolve overlapped peaks in order to help identification of the chemical components of B-

DBT. Figure 6.4 depicts total chromatograms with elution time points of B-DBT, B-DG and B-HQ.

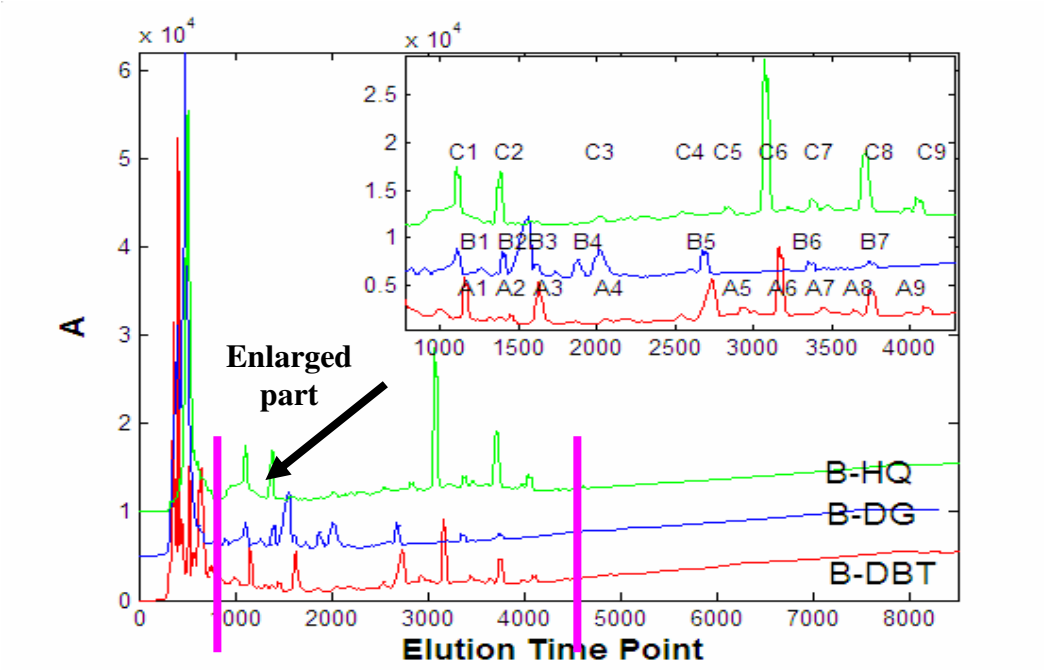


Fig. 6.4 2D Chromatograms of B-HQ, B-DG and B-DBT in elution time points and the enlarged part represents the region with elution time point of 1000-4500

From the above chromatograms, we can see that the elution time point 0-1000 displayed many peaks clusters with serious overlapping that were not easy to analyze. Here, the peaks in the range of 1000-4500 were chosen for identification. Figure 6.5 shows the spectrachromatograms (200-400nm) of peak cluster A1, B1 and C1 (Fig. 6.4), and Figure 6.6 depicts the MSCC results of data sets A1 and B1, A1 and C1 as well. Appendix 6.2 gives spectrachromatograms of all peak clusters in B-DBT, B-DG and B-HQ and Appendix 6.3 shows all the MSCC results.

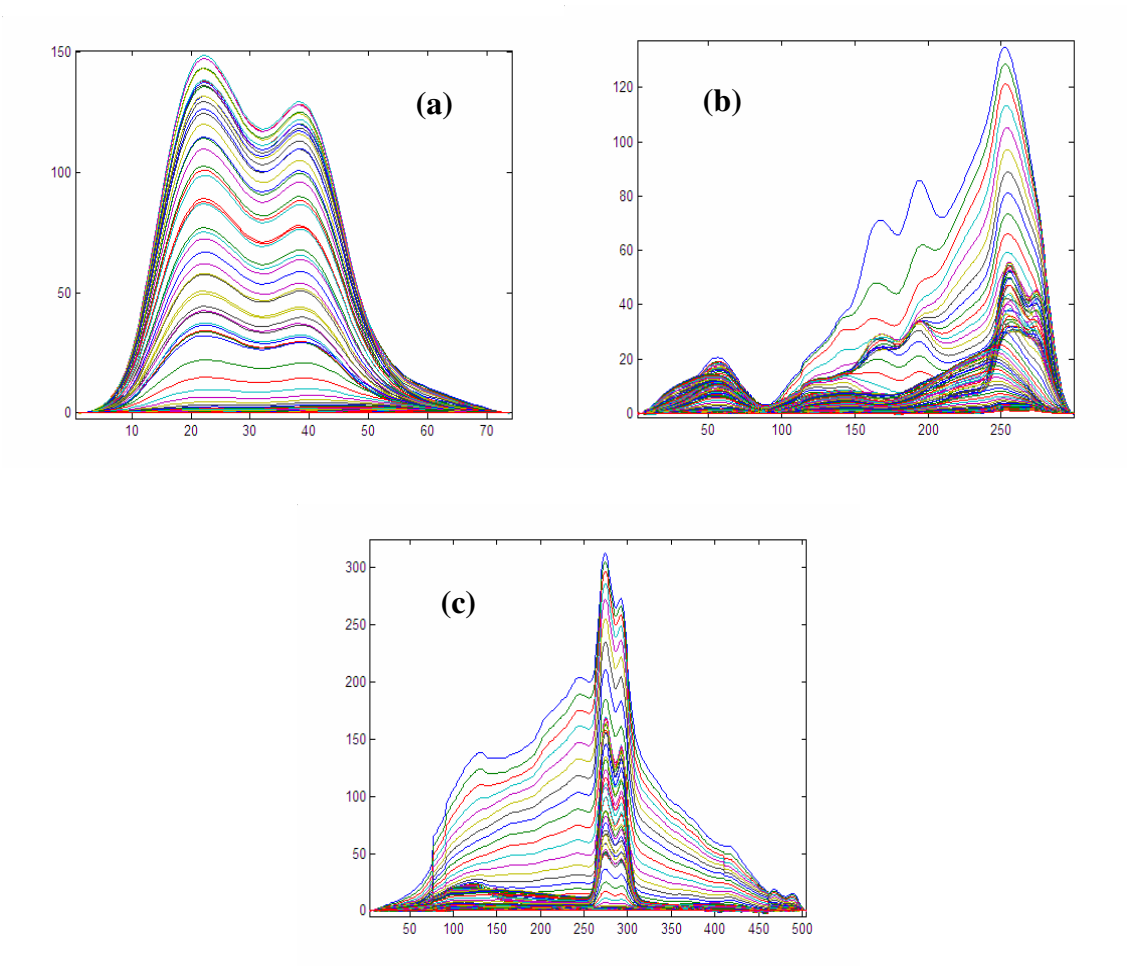


Fig. 6.5 Spectrachromatograms (200-400nm) of peak cluster (a) A1, (b) B1 and (c) C1 of B-DBT, B-DG and B-HQ respectively

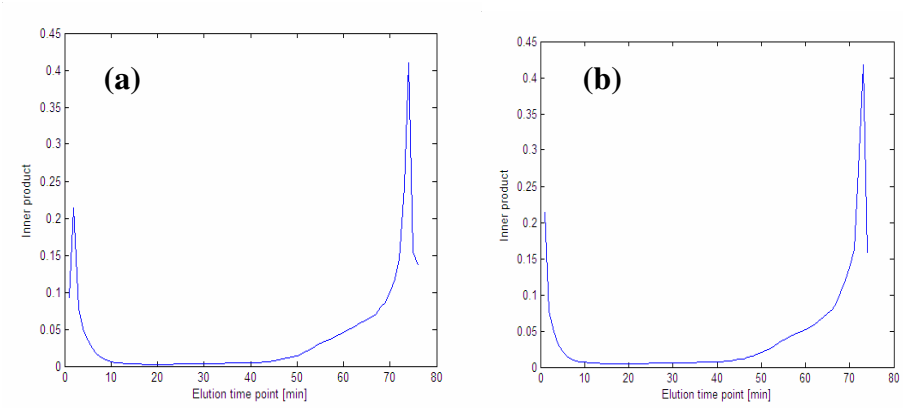


Fig. 6.6 MSCC results of data set (a) A1 and B1 and (b) A1 and C1.

As can be seen that the UV spectrachromatograms of A1, B1 and C1 (Fig 6.5) are different, though their retention times are closed to one another. This implied that the corresponding components are not exactly the same components. But the result obtained from MSCC (Fig. 6.6) shows that the spectral features of B1 and C1 are similar as that of A1 as the inner product (Fig. 6.6) is near to zero. Therefore, component A1 of B-DBT can be concluded to be contributed by both B1 from B-DG and C1 from B-HQ. Similarly, MSCC treatment was done on other parts of the chromatograms (Fig. 6.3). Table 6.5 summarizes the chemical components of B-DBT found from B-DG and B-HQ by MSCC.

Table 6.6 Chemical components of B-DBT that were found in B-DG and B-HQ through MSCC

Peak clusters		
B-DBT (A)	B-DG (B)	B-HQ (C)
A1	B1 (all)	C1 (all)
A2	B2 (all)	C2 (all)
A3	B3 (parts)	-
A4	B4 (parts)	C3 (parts)
A5	B5 (all)	C4 (parts) and C5 (most)
A6	-	C6 (all)
A7	B6 (all)	C7 (all)
A8	B7 (no)	C8 (all)
A9	-	C9 (all)

Remarks: The labels “no”, “parts”, “most” and “all” mean, respectively, no, partial, most and all chemical components in the boiled herb can be found in B-DBT . “-“ represents no peak was found in the corresponding chromatographic position of the herb

.From Table 6.6, it can be seen that some components in B-DBT are clearly found in its component herbs. For example, the peak cluster A6 (or chemical components A6 eluted in this region) of B-DBT was identified by using MSCC and its spectral feature is almost the same as that of peak cluster C6 of B-HQ only. Yet, no one peak cluster of B-DG matched A6. Therefore, it is concluded that the component A6 of B-DBT comes solely from B-HQ. However, some peak clusters cannot be determined so clearly on which herb(s) they come from owing to the serious overlapping of the peaks. For example, the spectral feature of peak cluster A5 found to be the same as that of B5 of B-DG as well as partially similar to C4 and very similar to C5 of B-HQ (Table 6.6). Therefore, in this case, we can only say that the chemical components corresponding to A5 of B-DBT come from chemicals of B-DG and also from B-HQ with chemicals present in the corresponding elution time regions. More works need to be done to get more detailed information of this kind for B-DBT, B-HQ and B-DG.

6.5 Conclusion

This study clearly shows that it is possible to study the chemical compositions of the CM formulation DBT and its components herbs, HQ and DG, based on the three different data analysis methodologies, “marker approach”, “multi-component approach” and “pattern approach”. Besides, some chemometric techniques combined with these three approaches were applied to characterize the chemical components of DBT, HQ and DG. They included Multicomponent spectral correlative chromatography (MSCC) and Similarity index (SI). These techniques are found to be efficient and effective to mine

more chemical information from the HPLC chromatographic fingerprints of DBT, HQ and DG extracts.

In the "marker approach", astragaloside IV, calycosin, formononetin, ferulic acid, n-butylidenephthalides and *Z*-ligustilide were used to characterize the chemical composition of B-DBT, B-HQ and B-DG extracts. However, astragaloside IV was not detected at its λ max (203nm) under the chromatographic so that was used in this study so that finally it was not used for qualitative determination. As for the other four standards, they were not found in the 50 mg/ml of B-DBT, B-HQ and B-DG through matching the retention times and UV spectra of these four standards. Therefore characterization of the chemical composition of the three boiled samples in this case is not suitable to be carried out by using "marker approach" proposed. But at least, it shows how the "marker approach" looked for characterization of chemical composition of CM and CM formulation could not used for qualitative determination.

"Pattern approach" combined with a chemometric technique called SI was applied to study and compare the chemical composition of DBT, HQ and DG under two different extraction methods, boiled and soaked. The SI values between the HPLC chromatograms of B-DBT and U-DBT, B-DG and U-DG, and B-HQ and U-HQ are 86%, 93.6% and 85.6%, respectively. It was found that these two types of extracts did give slightly difference in their chemical compositions as indicated by their chromatograms. This also implied that DBT extracts obtained from boiled and soaked may give different biological activities but more works are needed to be done to confirm this.

Finally, it is interesting to know which chemical constituents of B-DBT coming from those of the components herbs HQ and DG. With the help of "multi-component approach" and MSCC, this work was successfully carried out. Nine peak clusters in the chromatogram of B-DBT was identified. One peak cluster of B-DBT was confirmed coming from DG, three from HQ and five from both DG and HQ. This strategy could hopefully be and successfully applied to other CM formulations to find out which ones of them chemical components coming from those of their components herbs with higher confidence.

Chapter 7

Summary

Chromatographic techniques combined with chemometric data processing methods and /or bioassay have been found desirable in chemical and biochemical studies. In this investigation, chromatographic techniques were applied to analyze the chemical constituents of Radix Ginseng, Radix Panacis Quinquefolii, Ganoderma amboinense, RDLP and Danggui Buxue Tang (DBT). In addition, the complex data obtained from Ganoderma amboinense and DBT were processed extensively by using chemometric data processing methods to extract more chemical information. An *in-vitro* bioassay was also utilized for studying the pharmacological activity of chemical composition of RDLP. A brief summary of these studies is given in the following paragraphs.

The techniques applied in this research programme were briefly introduced in Chapter 2. High performance liquid chromatography (HPLC) is one of the most popular chromatographic techniques applied for analyzing the complex chemical constituents in Chinese medicine (CM). It is commonly coupled with various kinds of detectors such as diode array detector (DAD), mass spectroscopy (MS) and evaporative light scattering detector (ELSD). In our work, HPLC-DAD, HPLC-DAD-ELSD and preparative HPLC-DAD were applied for analyzing the selected CMs and CM formulations. Two-way data acquired from HPLC-DAD can be further analyzed with chemometric techniques. From various applications of chemometric techniques, data processing is important for the analysis of CM. Here, two chemometric data processing methods, evolving window orthogonal projections (EWOP) and multicomponet spectral correlative chromatographic (MSCC) methods, were employed and described in the same chapter. Moreover, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide) cell proliferation assay (MTT assay) was employed for studying the cytotoxicity of fractions in CM.

In Chapter 3, a fast experimental procedure for the analysis of commercial products of Radix Ginseng and Radix Panacis Quinquefolii has been developed for introducing the basic concept of quality control of natural products to tertiary students. This procedure was developed including sample extraction and chromatographic condition of HPLC-DAD-ELSD. In this investigation, homogeneization was employed for the sample extraction, the chromatographic condition was optimized with consideration of the six common ginsenosides (Rg1, Rf, Rb1, Rc, Rb2 and Rd), and the operation condition of ELSD was the recommended condition suggested by the manufacturer. This laboratory was designed for one triple period of lesson and an overnight. During the lesson, the sample extracts and a serial concentration of ginsenoside mixture were prepared, and the operation of the instrument was introduced. The lesson was also flexible to be adjusted by the users in tertiary institute.

Ganoderma amboinense is a rare type of *Ganoderma lucidum*. In Chapter 4, the six nucleosides (uracil, uridine, inosine, guanosine, adenine and adenosine) in the fruit body, cap and stipe of Ganoderma amboinense were studied with combination of HPLC-DAD and EWOP methods. The stipe and fruit body of Ganoderma amboinense were identified to have uracil, uridine, guanosine and adenosine, while in its cap only uridine and guanosine were found. The concentrations of the identified nucleosides were accurately determined through EWOP method though their contents were too low to be interfered by the inconsistence instrumental variation. The combined techniques were applied successful to analysis the concentrations of the nucleosides in Ganoderma amboinense samples.

Preparative chromatography is the most widely used purification technique in the pharmaceutical and biotechnological industries. Scale-up from an analytical preparative separation is the first step for all preparative separations. In Chapter 5, the separation condition of the CM formulation RDLP was developed by a trial-and-error approach using analytical HPLC. Then, linear scale-up of the optimal condition was successful transferred to preparative HPLC by us. In order to find the potential bioactive fractions from the water extract of RDLP, bioassay-guided fractionation approach was applied by using preparative HPLC and MTT assay. Among four fractions collected, F-1B performed the strongest antiproliferation activity on both hepatoma cancer cell lines. Also, the other three fractions, F-1, F-2 and F-1A, gave different extent of antiproliferation activity which might result from the chemical components perform other biological functions based on the principle of CM formulation in the RDLP water extract. Moreover, the RDLP water extract was safe for liver and lung normal cell lines.

Finally, another CM formulation, DBT, and its component herbs, Radix Astragali (HQ) and Radix Angelicae Sinensis (DG) were studied by the combination of HPLC-DAD and MSCC. In Chapter 6, “marker approach”, “multi-component approach”, and “pattern approach” were applied to characterize DBT from its component herbs. Based on “marker approach” with the use of experimental data only, five herbal markers of HQ and DG were not identified in DBT. Therefore, two-way data of DBT, HQ and DG acquired from HPLC-DAD were processed in detail by MSCC. This approach was successful to identify peak clusters of DBT belonged to HQ and / or DG. Additionally, in “pattern approach”, the similarity indeices between the chromatograms obtained from boiled and soaked DBT, HQ and DG were found to have different chemical compositions obtained

by these treatments. Such additional information might imply different bioactivities performed, and useful for investigating the potential active ingredient.

In a conclusion, chemical studies of complex chemical systems like CMs were improved significantly by combination of the chromatographic technique, chemometric methods and biological screening. By using the combined approach, more useful information was extracted from the complex system. Commercial products of Radix Ginseng and Radix Panacis Quinquefolii, Ganoderma amboinense, RDLP and Danggui Buxue Tang (DBT) were the CMs and CM formulations analyzed in this way in this research programme.

Appendices

Appendix 3.1 Determination of the nitrogen gas flow rate and the draft temperature for ELSD operation (Alltech, 2003)

For running a binary mobile phaseof mixture of solvent A and B,

$$\textit{Draft temperature } (T_d) = (T_d)_A \times r_A + (T_d)_B \times r_B \quad [A3.1\text{-}1]$$

$$\textit{Gas flow rate } (R_{N2}) = (R_{N2})_A \times r_A + (R_{N2})_B \times r_B \quad [A3.1\text{-}2]$$

Where r_A and r_B are the ratio of solvents A and B in the binary mobile respectively.

Table A.3.1-1 Draft temperature (T_d) and gas flow rate (R_{N2})to different solvent used in ELSD (Alltech, 2003)

Solvent (1ml/min)	T_d (^oC)	R_{N2} (L/min)
Acetone	30	0.6
Acetonitrile	70	1.7
Chloroform	40	1.5
Heptane	50	1.5
Hexane	40	1.6
Isopropyl alcohol	55	1.7
Methanol	60	1.6
Methylene chloride	50	1.6
Tetrahydrofuran (stabilized)	60	1.7
Tetrahydrofuran (unstabilized)	40	1.6
Water	115	3.2
Methanol : Water (90:10)	75	2.0
Acetonitrile : Water (75:25)	80	2.0

Appendix 3.2 HPLC-DAD chromatograms of the six ginsenoside standards separated by the elution systems C2, C3, C4, C5, C6 and C7 with their corresponding chromatographic conditions

(a) Elution system C2

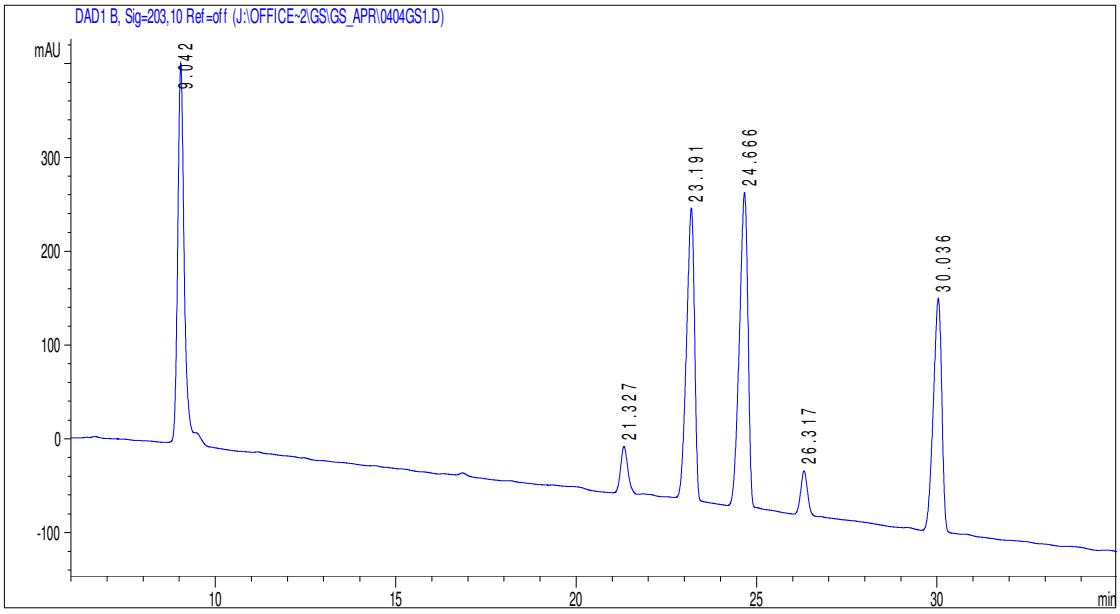


Table A.3.2-1 Gradient of mobile phase composition of Elution system C2 at *0.7 ml/min*

Elution time (min)	ACN (%)	Formic acid (0.001%) (%)
0	25	75
3	26	74
30	40	60
35	40	60

(b) Elution system C3

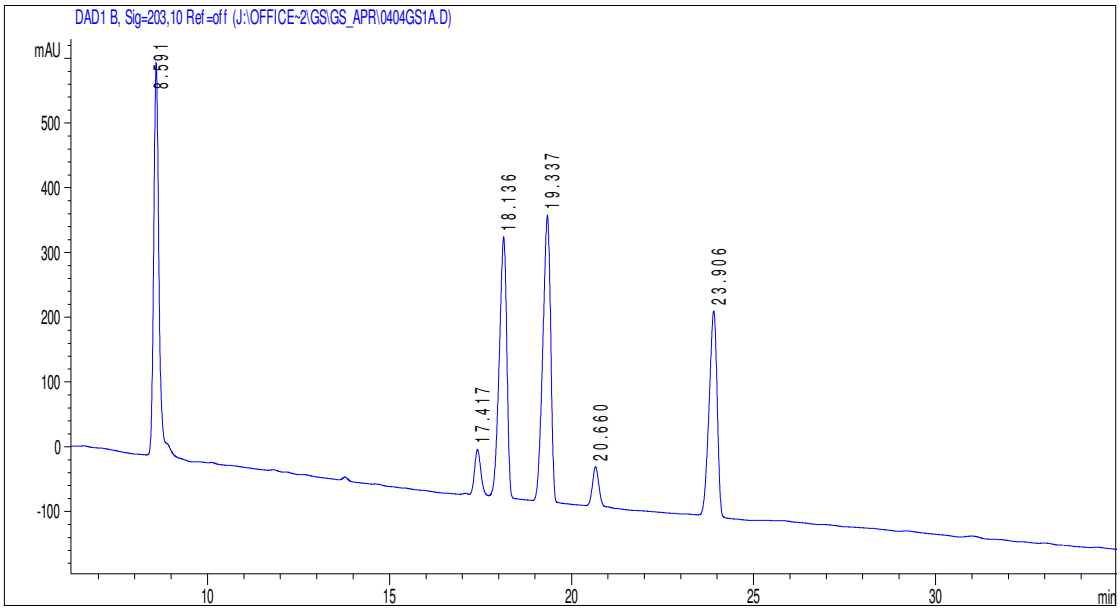


Table A.3.2-3 Gradient of mobile phase composition of Elution system C3 at *0.7 ml/min*

Elution time (min)	ACN (%)	Formic acid (0.001%) (%)
0	25	75
10	33	67
30	45	55
35	45	55

(c) Elution system C4

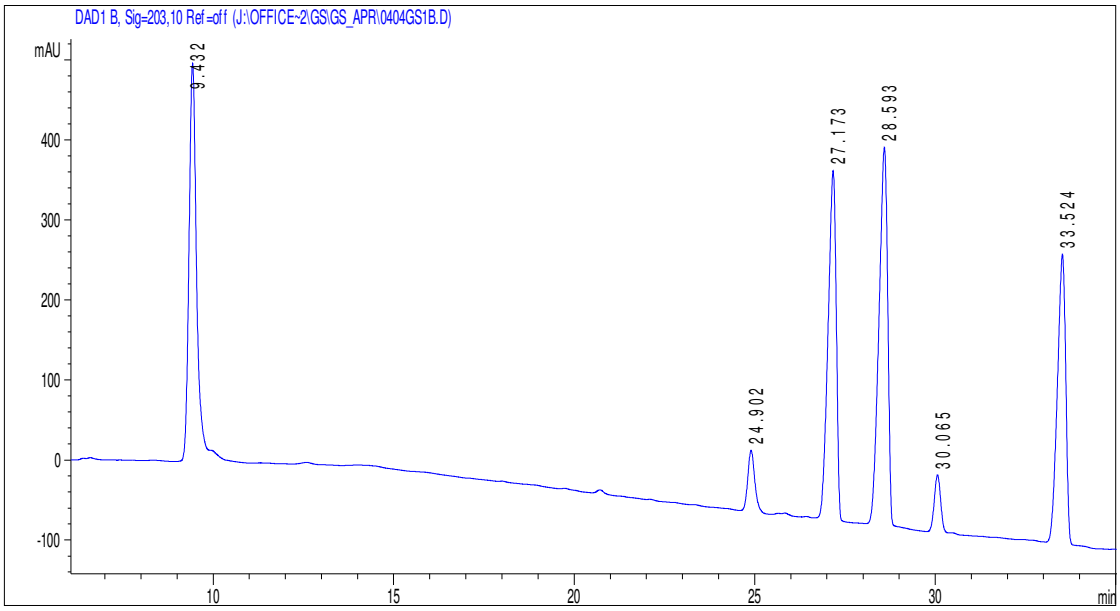


Table A.3.2-4 Gradient of mobile phase composition of Elution system C4 at *0.7 ml/min*

Elution time (min)	ACN (%)	Formic acid (0.001%) (%)
0	25	75
8	26	74
20	33	67
35	45	55

(d) Elution system C5 (Method GS_G5)

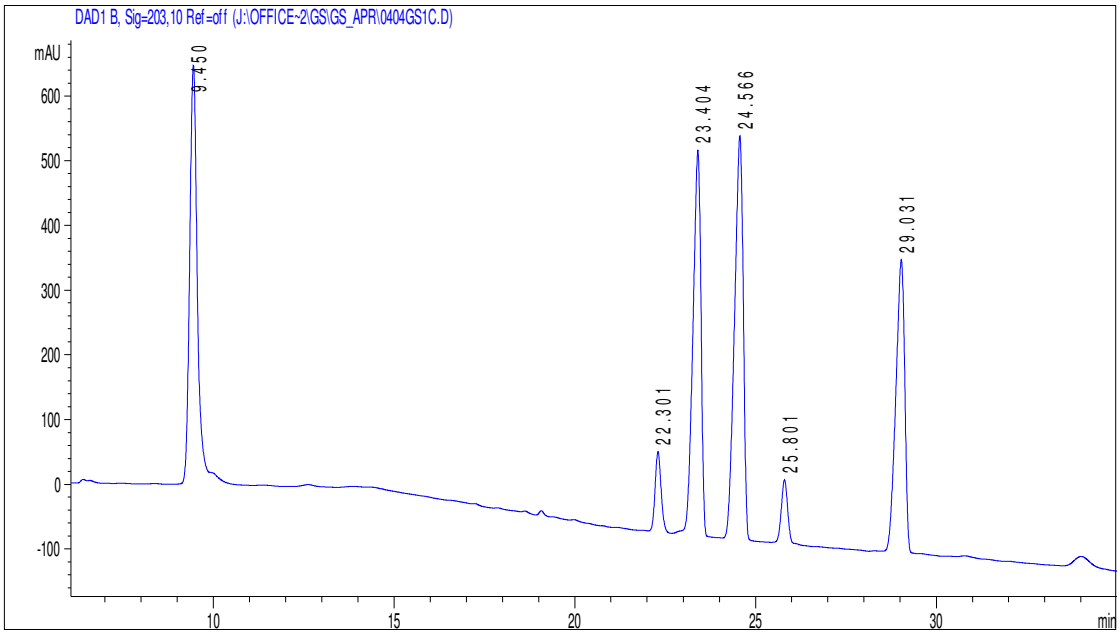


Table A.3.2-5 Gradient of mobile phase composition of Elution system C5 at *0.7 ml/min*

Elution time (min)	ACN (%)	Formic acid (0.001%) (%)
0	25	75
8	26	74
15	33	67
35	45	55

(e) Elution system C6

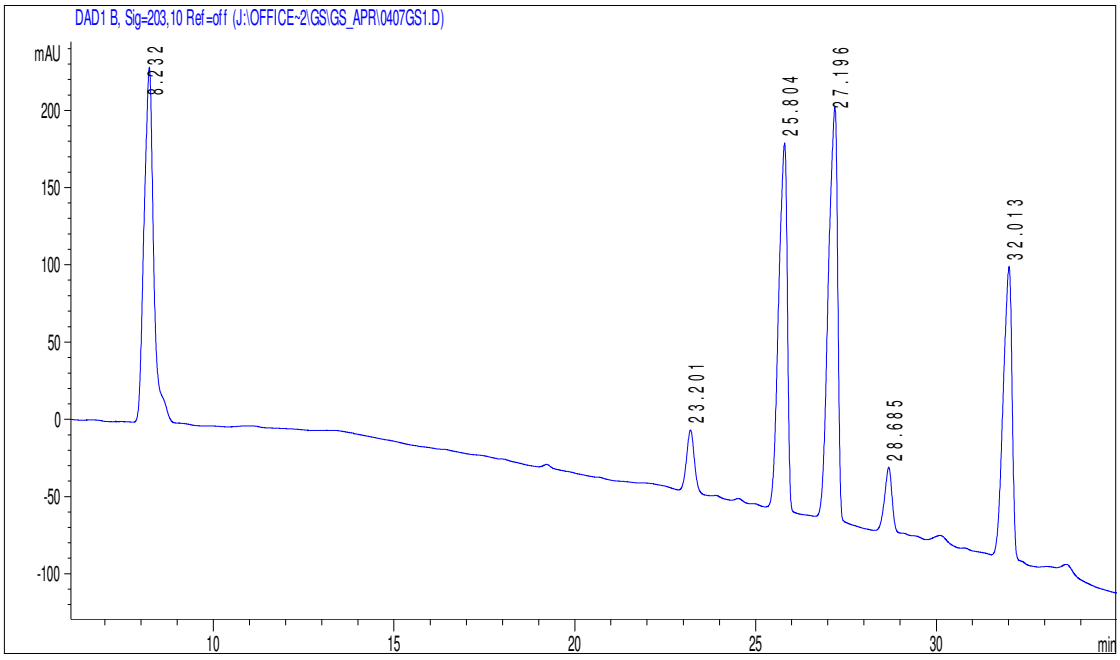


Table A.3.2-6 Gradient of mobile phase composition of Elution system C6 at *0.8 ml/min*

Elution time (min)	ACN (%)	Formic acid (0.001%) (%)
0	25	75
8	26	74
20	33	67
35	45	55

(f) Elution system C7

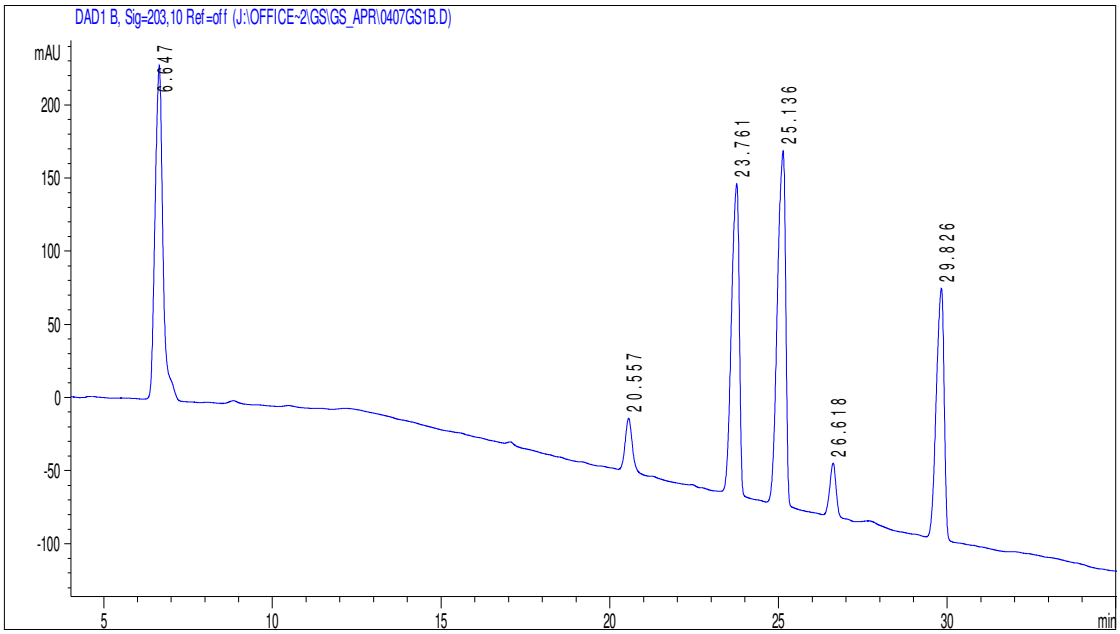
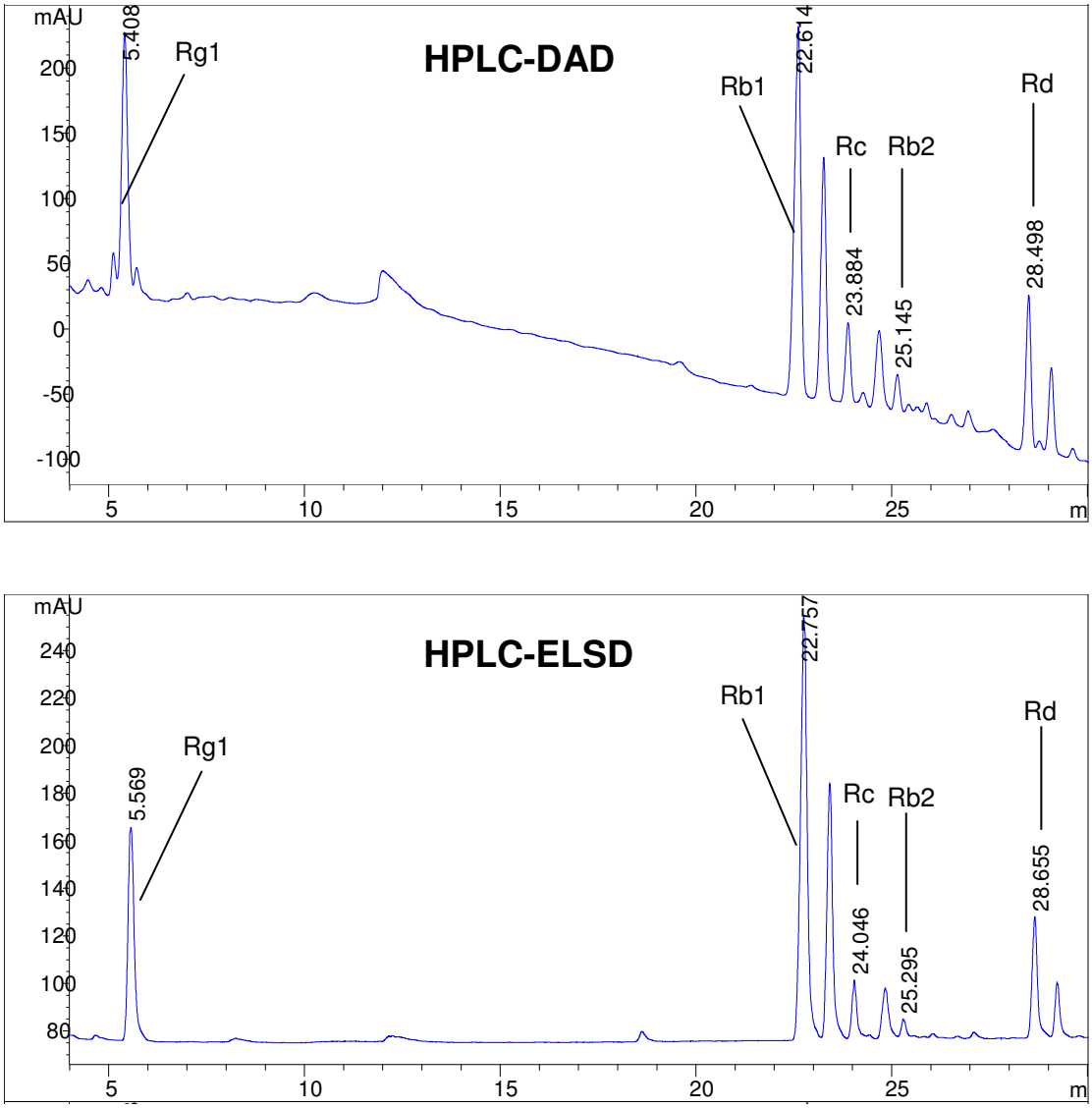


Table A.3.2-7 Gradient of mobile phase composition of Elution system C7 at *1.0 ml/min*

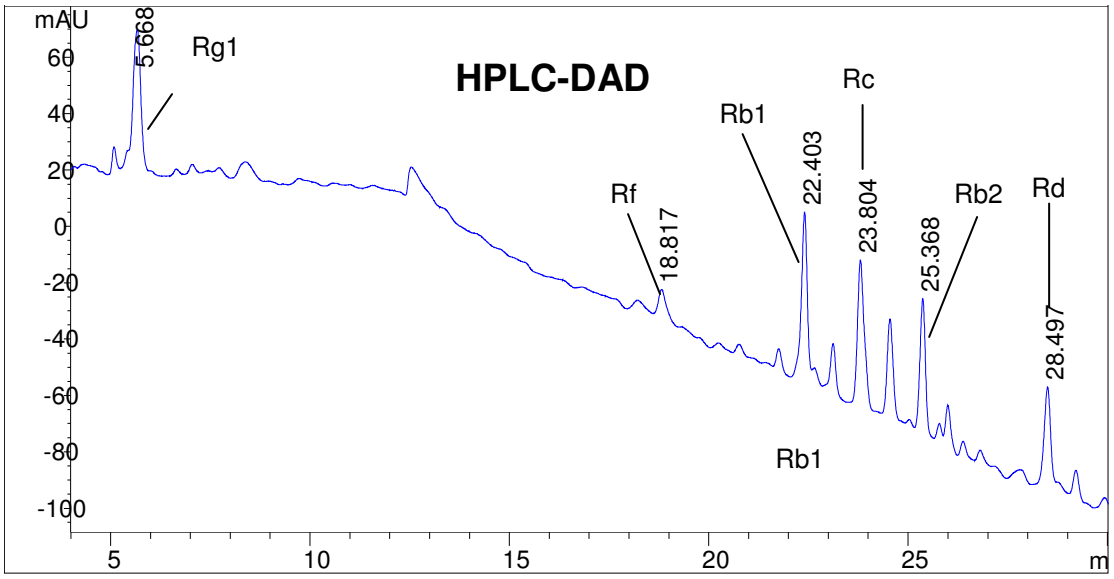
Elution time (min)	ACN (%)	Formic acid (0.001%) (%)
0	25	75
8	26	74
20	33	67
35	45	55

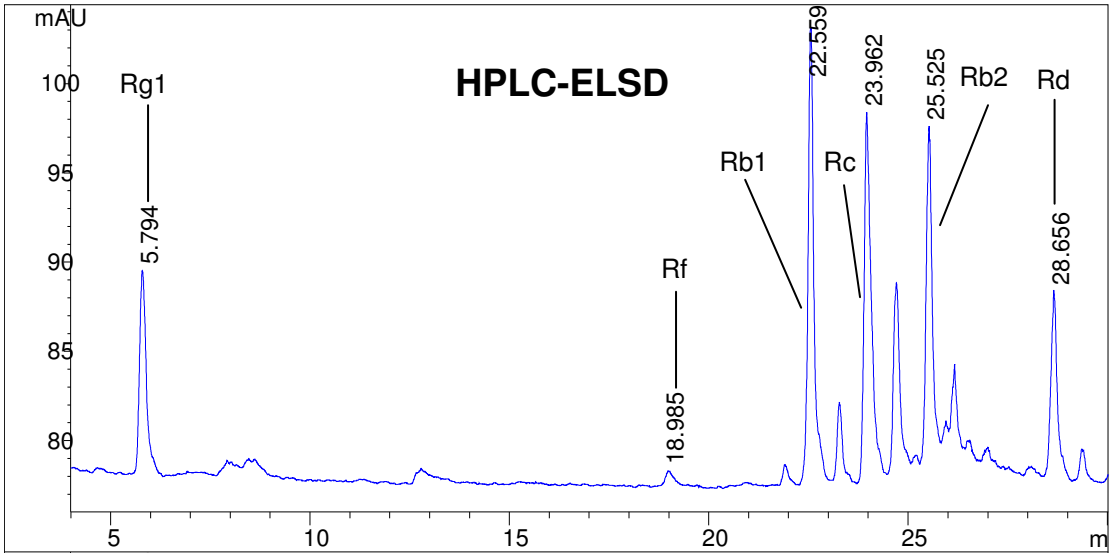
Appendix 3.4 HPLC-ELSD and HPLC-DAD chromatograms of AGS capsule and OGS tablet

(a) AGS capsule



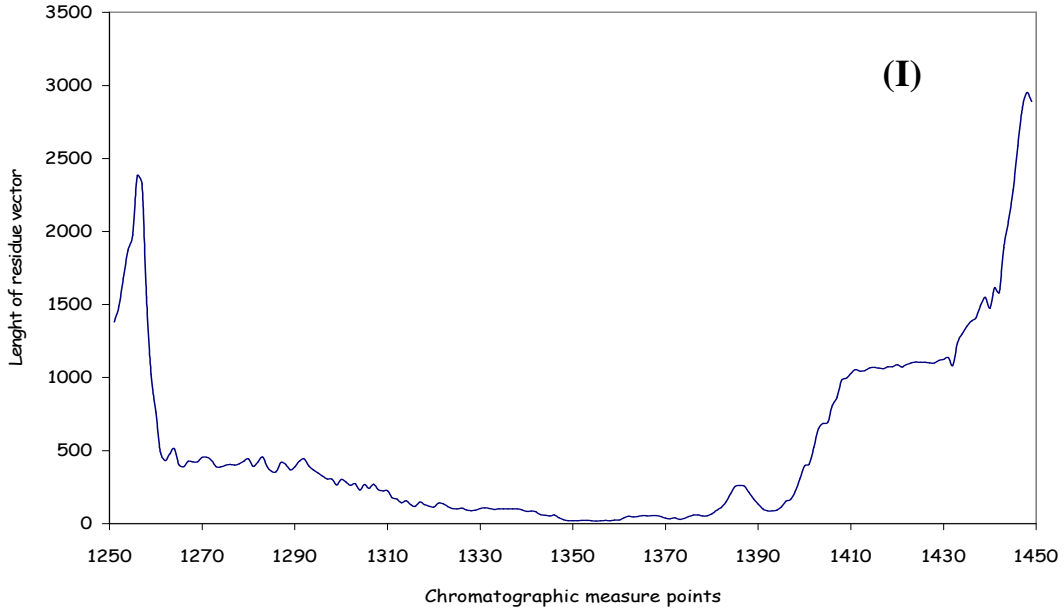
(b) OGS tablet

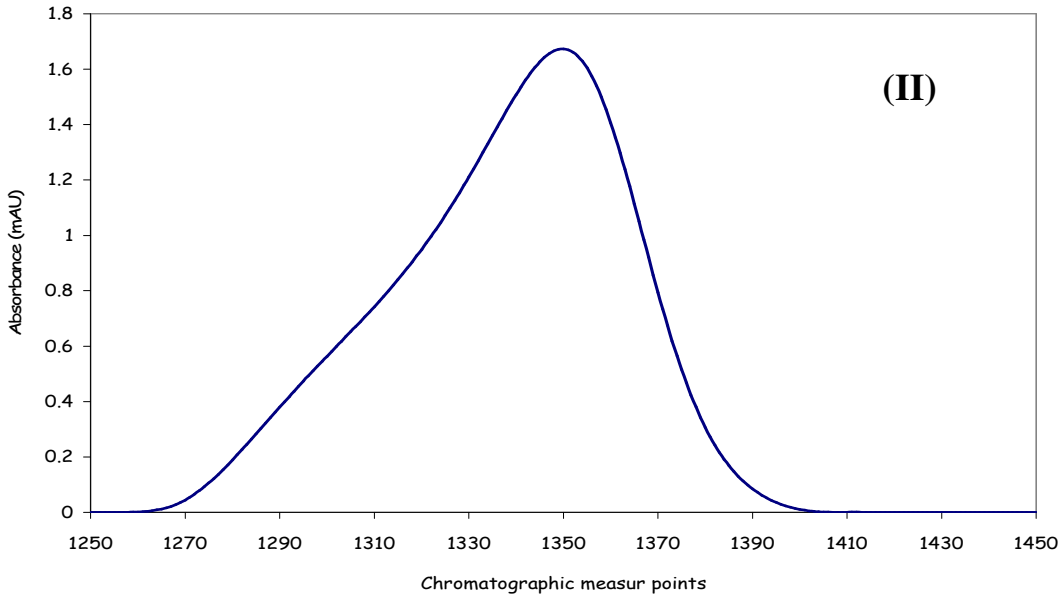

HPLC-DAD
mAU
60
40
20
0
-20
-40
-60
-80
-100
5.668
Rg1
Rf
18.817
Rb1
22.403
Rc
23.804
25.368
Rb2
Rd
28.497
Rb1
5
10
15
20
25
m


HPLC-ELSD
mAU
100
95
90
85
80
Rg1
5.794
Rf
18.985
Rb1
Rc
22.559
23.962
25.525
Rb2
Rd
28.656
5
10
15
20
25
m

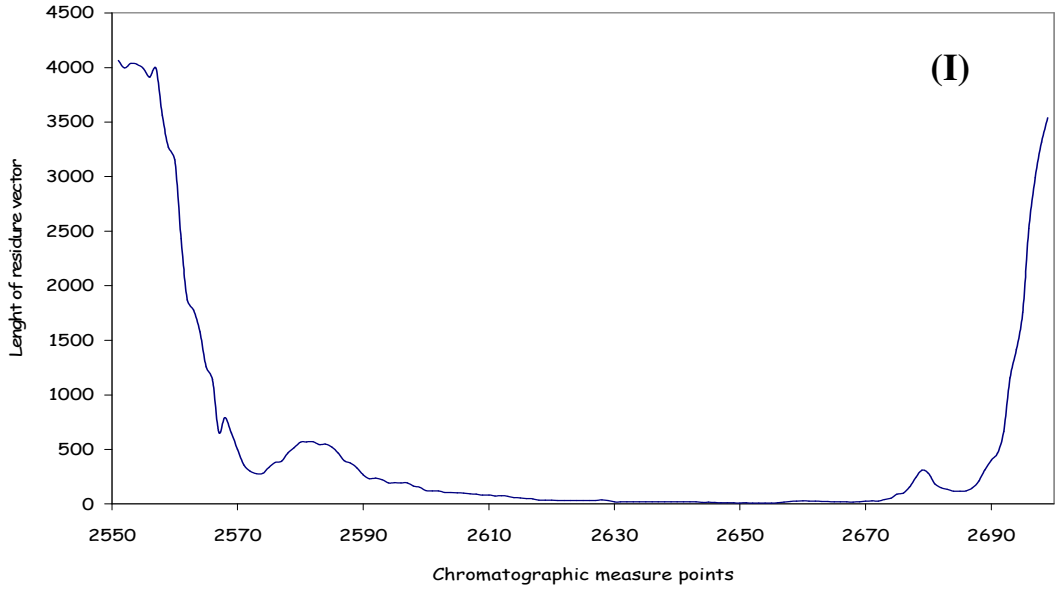
Appendix 4.1 Zero-concentration graph (ZCG) (I) and chromatographic profiles (II) of the nucleosides from the cap of Ganoderma amboinense

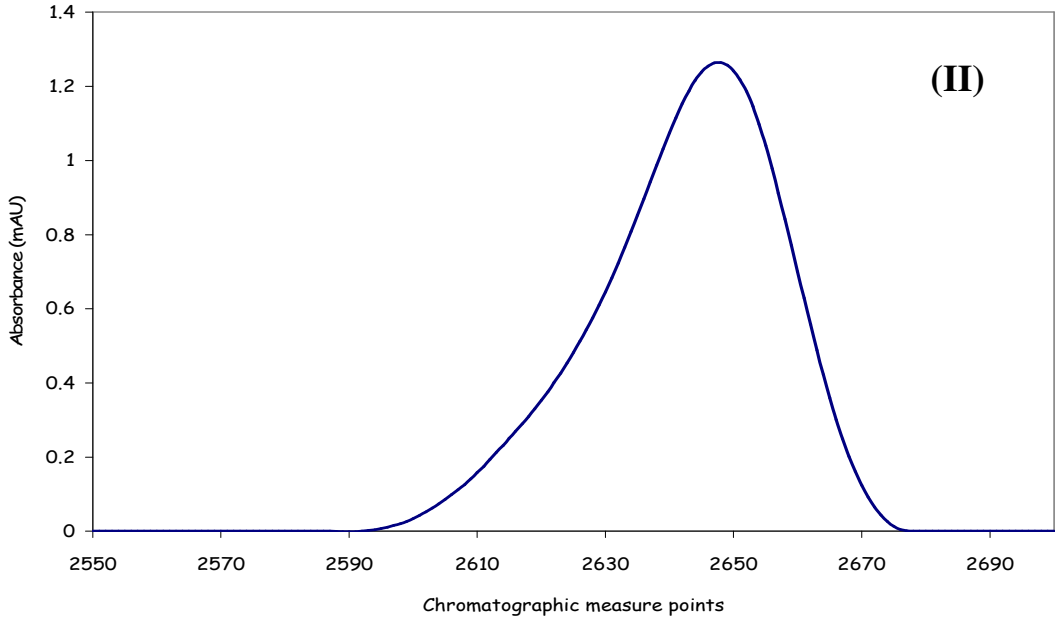
(a) Uridine





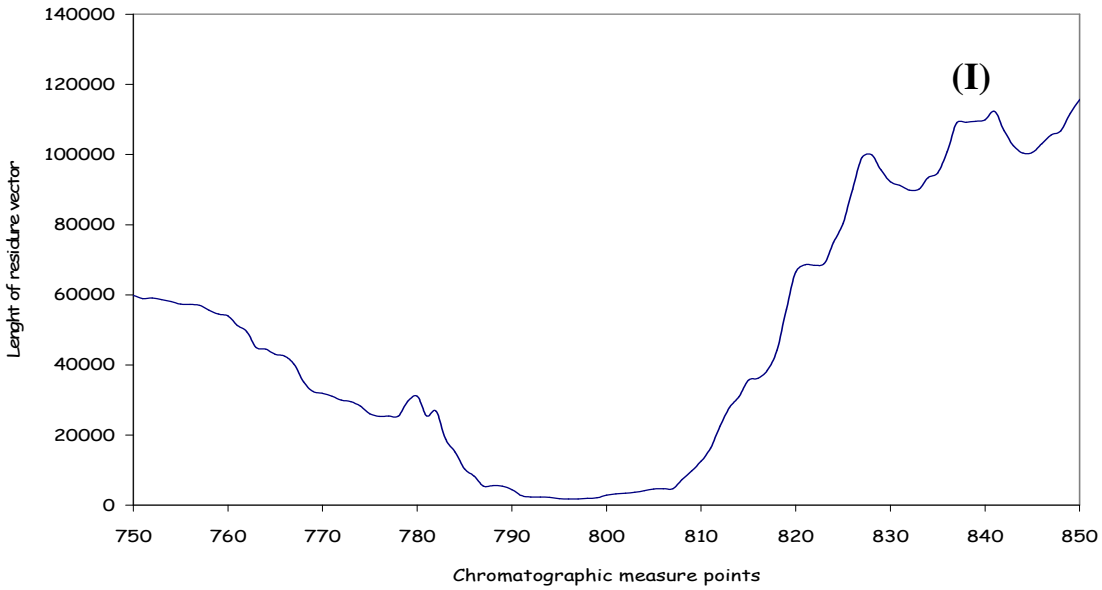
(b) Guanosine

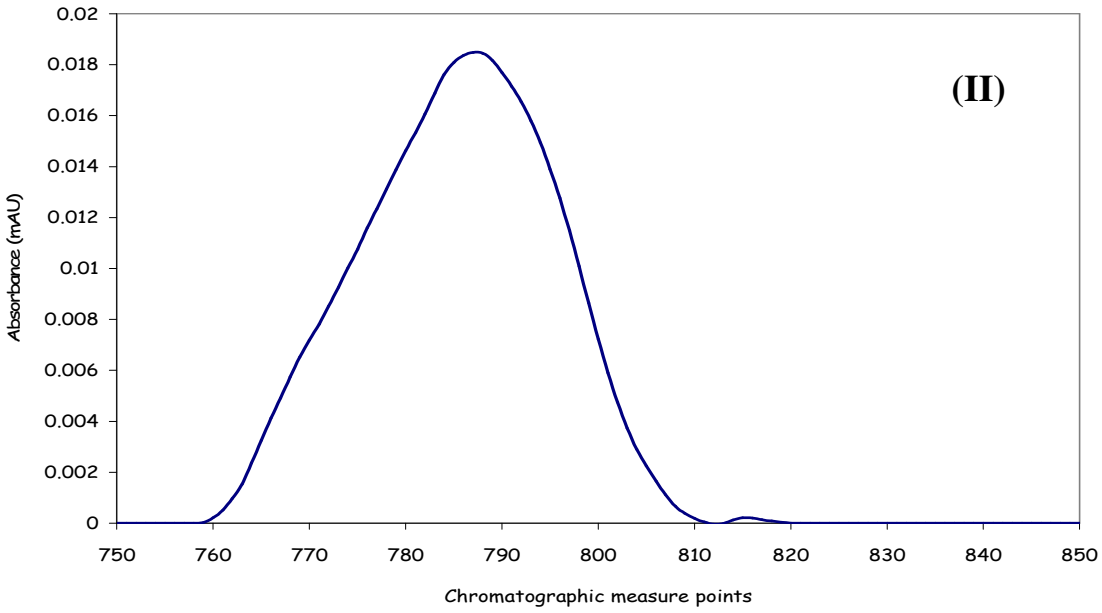

(I)
Lenght of residure vector
4500
4000
3500
3000
2500
2000
1500
1000
500
0
2550
2570
2590
2610
2630
2650
2670
2690
Chromatographic measure points


(II)
Absorbance (mAU)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2550
2570
2590
2610
2630
2650
2670
2690
Chromatographic measure points

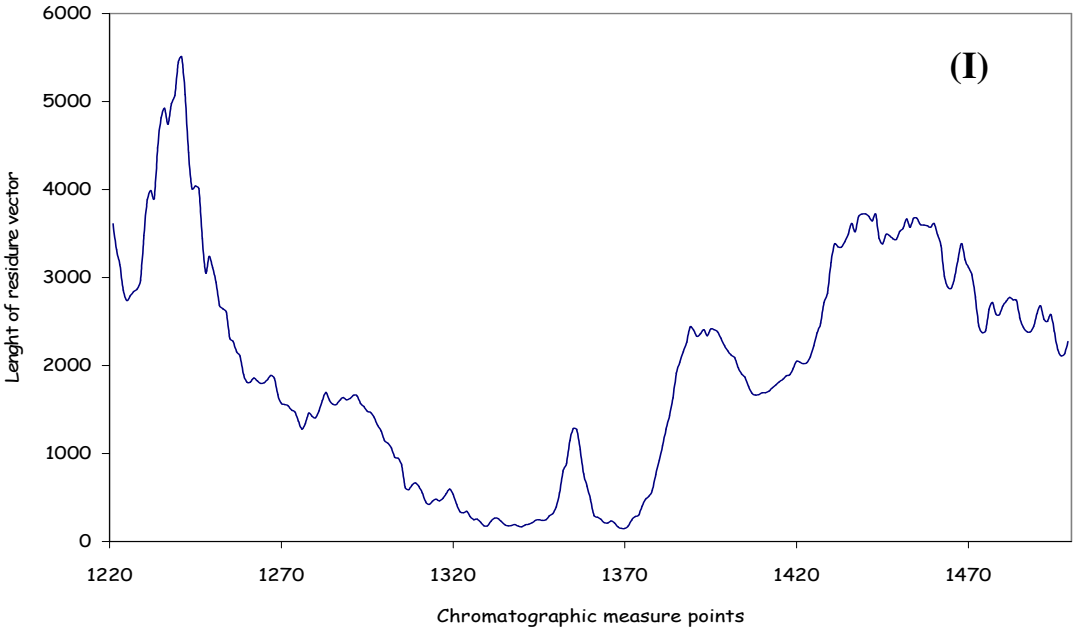
Appendix 4.2 Zero-concentration graph (ZCG) (I) and chromatographic profiles (II) of the nucleosides from the stipe of Ganoderma amboinense

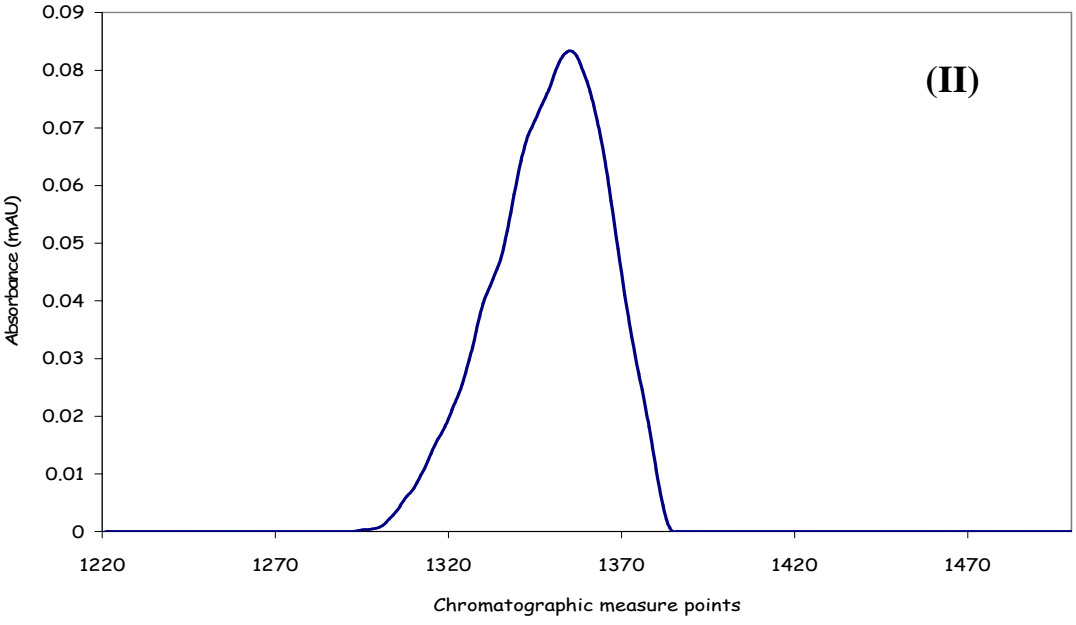
(a) Uracil



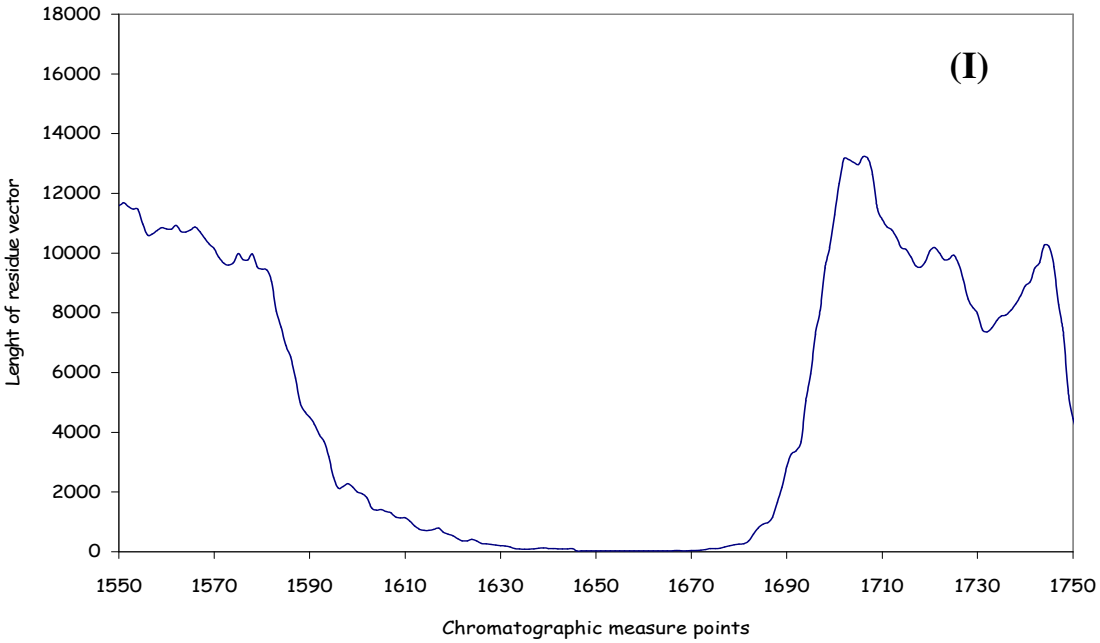


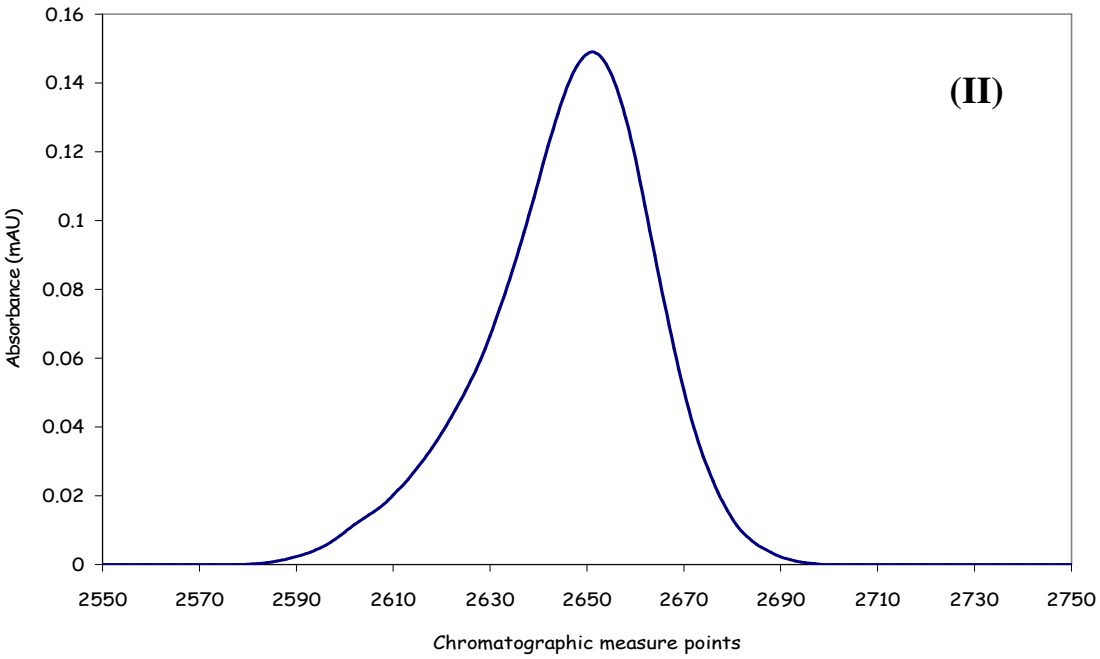
(b) Uridine


(I)
Lenght of residure vector
6000
5000
4000
3000
2000
1000
0
1220
1270
1320
1370
1420
1470
Chromatographic measure points


(II)
Absorbance (mAU)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1220
1270
1320
1370
1420
1470
Chromatographic measure points

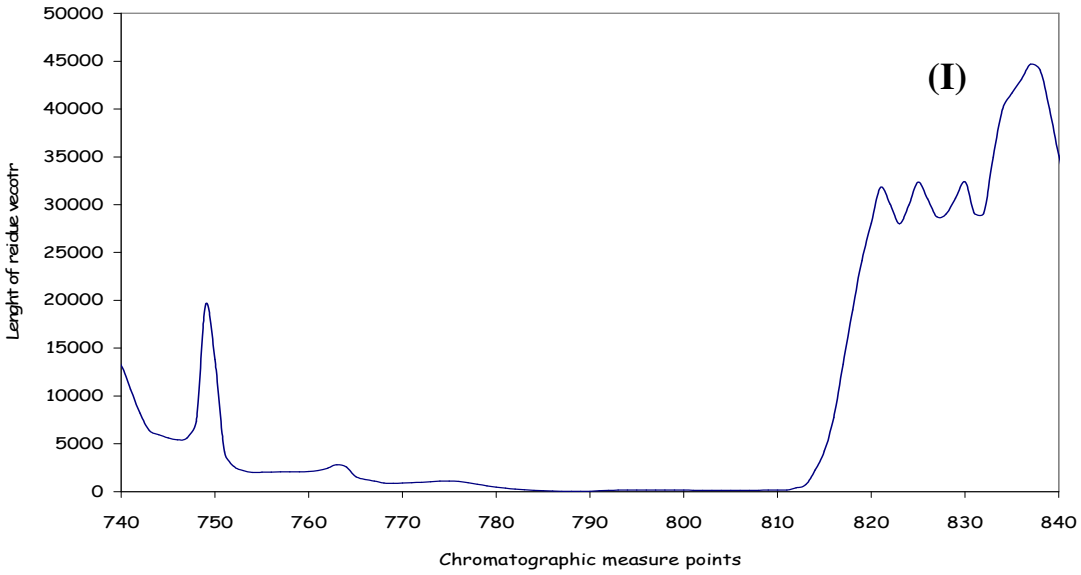
(c) Guanosine

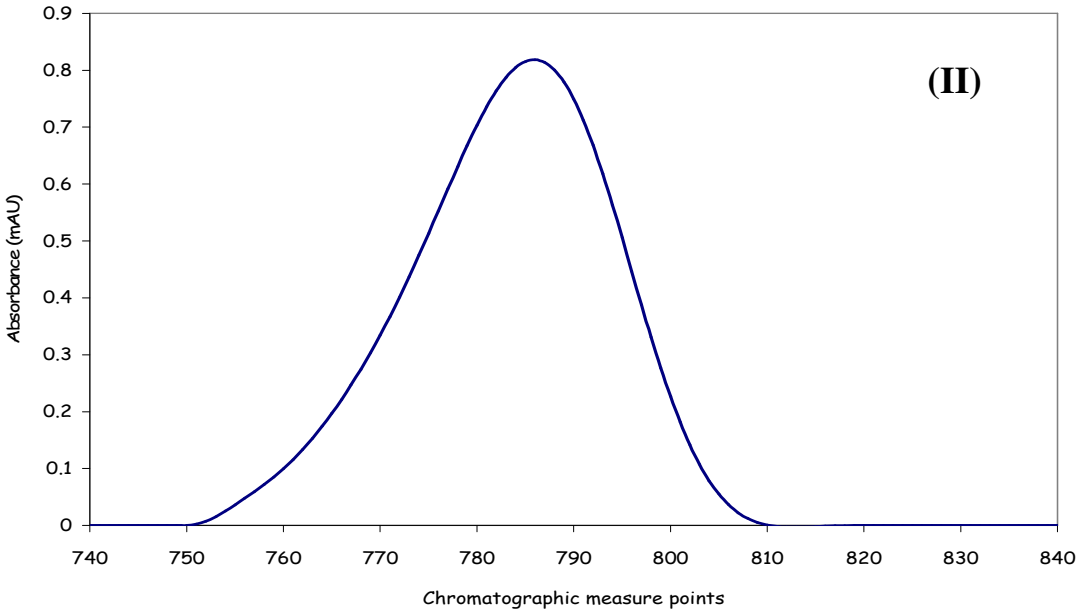

(I)
Lenght of residue vector
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
1550
1570
1590
1610
1630
1650
1670
1690
1710
1730
1750
Chromatographic measure points


(II)
Absorbance (mAU)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
2550
2570
2590
2610
2630
2650
2670
2690
2710
2730
2750
Chromatographic measure points

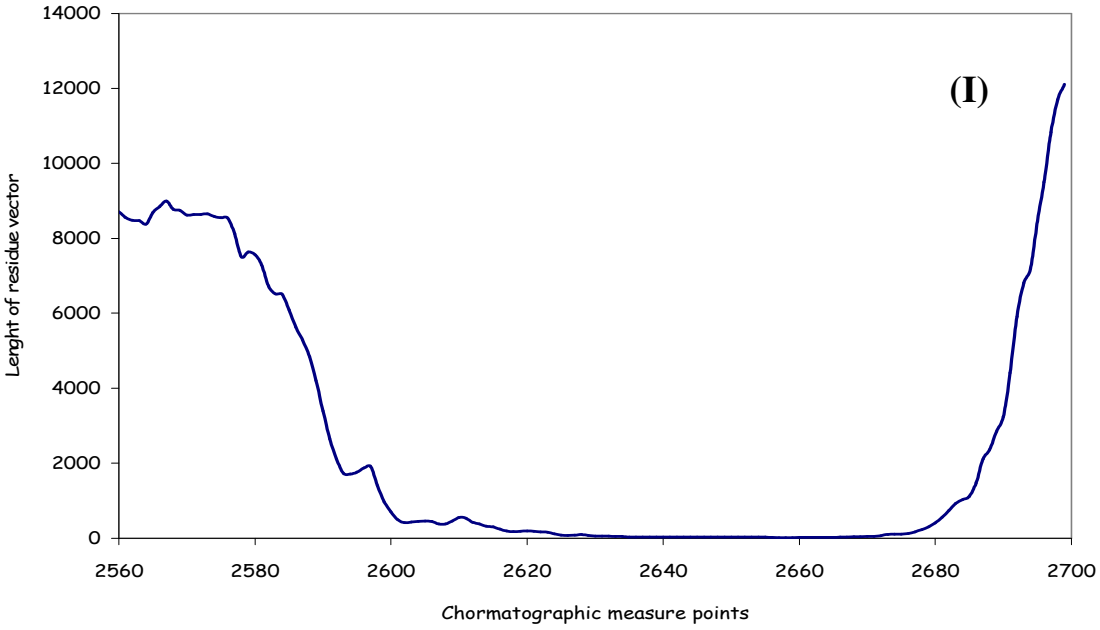
Appendix 4.3 Zero-concentration graph (ZCG) (I) and chromatographic profiles (II) of the nucleosides from the <u>fruit body</u> of Ganoderma amboinense

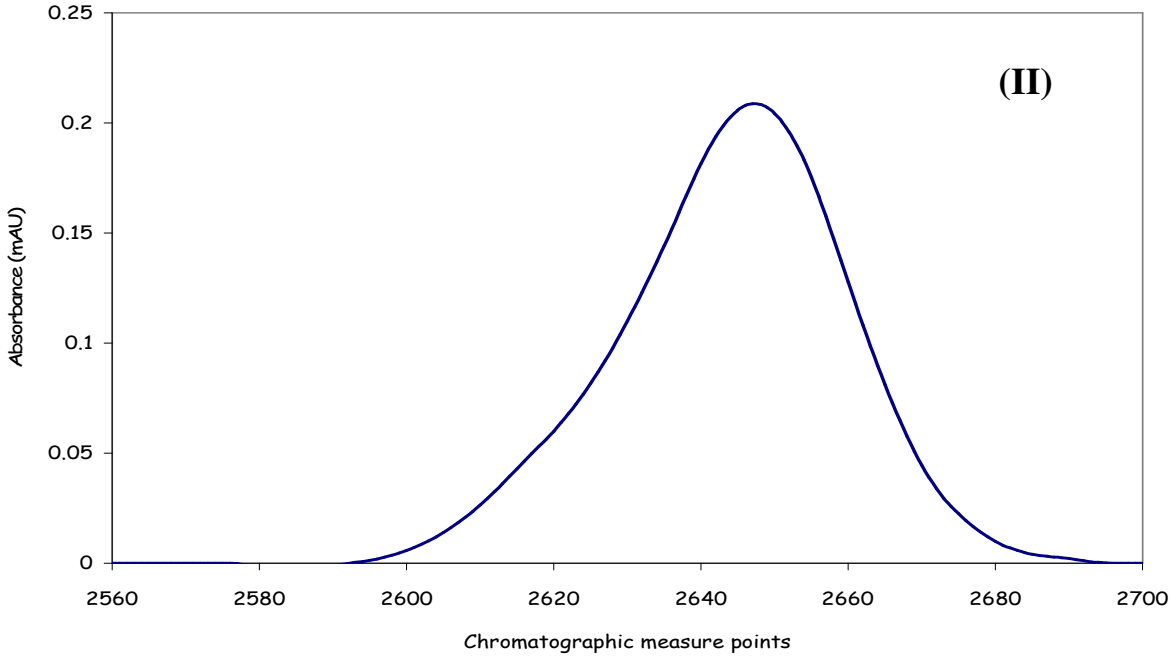
(a) Uracil



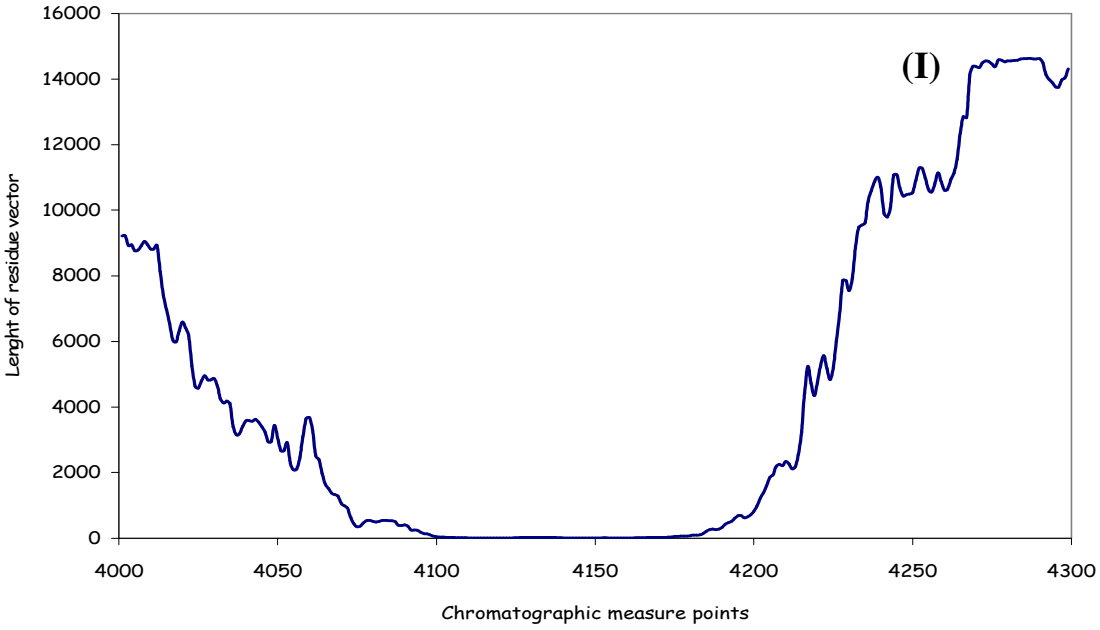


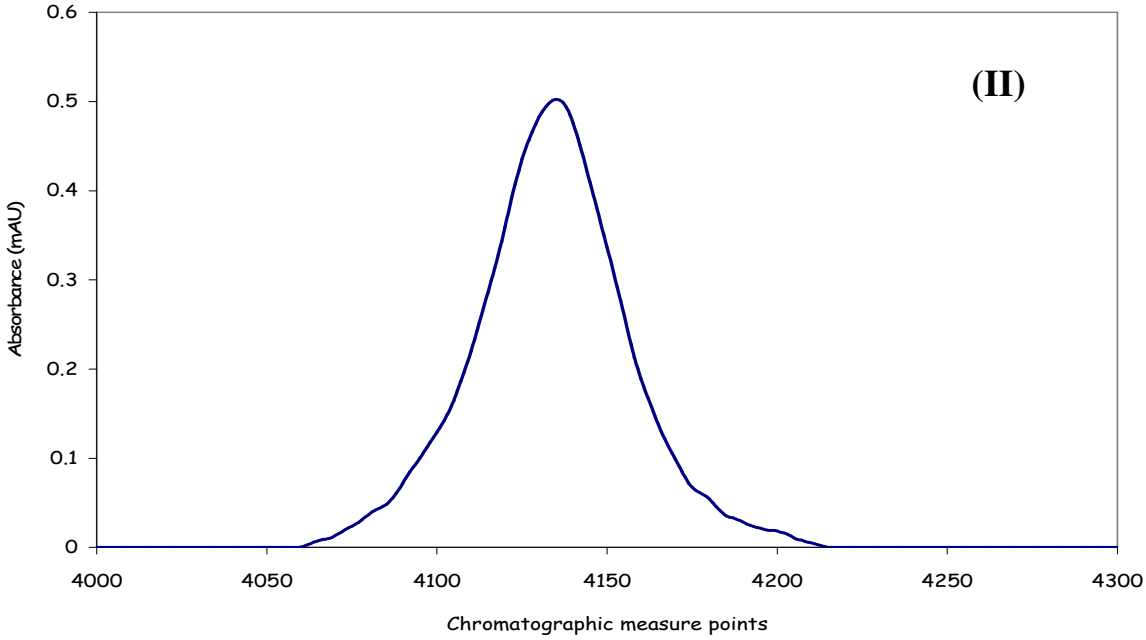
(b) Guanosine


(I)
Lenght of residue vector
14000
12000
10000
8000
6000
4000
2000
0
2560
2580
2600
2620
2640
2660
2680
2700
Chormatographic measure points


(II)
Absorbance (mAU)
0.25
0.2
0.15
0.1
0.05
0
2560
2580
2600
2620
2640
2660
2680
2700
Chromatographic measure points

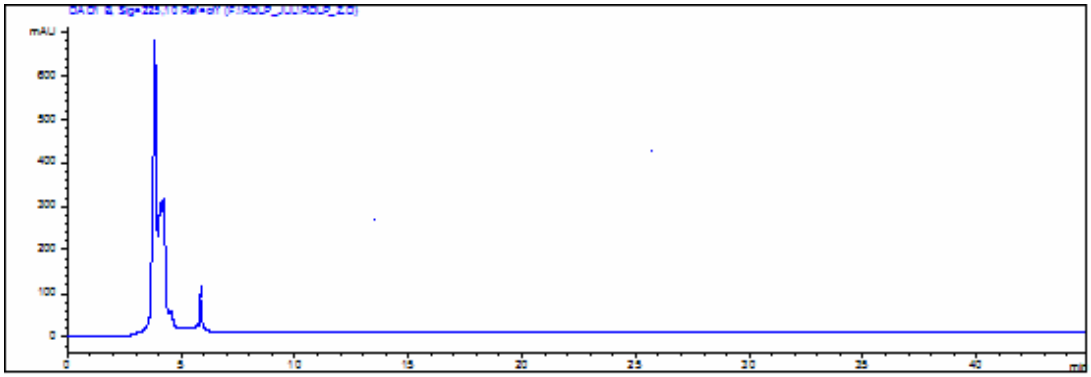
(c) Adenosine


(I)
Lenght of residue vector
16000
14000
12000
10000
8000
6000
4000
2000
0
4000
4050
4100
4150
4200
4250
4300
Chromatographic measure points

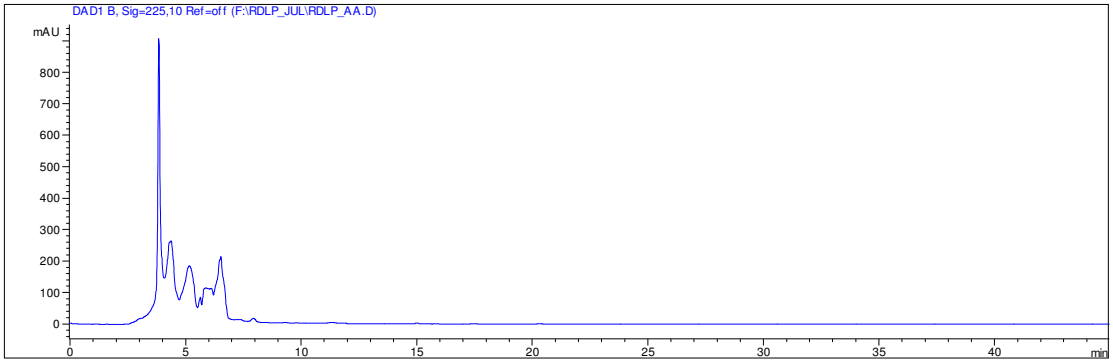

(II)
Absorbance (mAU)
0.6
0.5
0.4
0.3
0.2
0.1
0
4000
4050
4100
4150
4200
4250
4300
Chromatographic measure points

Appendix 5.1 HPLC chromatograms of the RDLP water obtained by different solvent systems

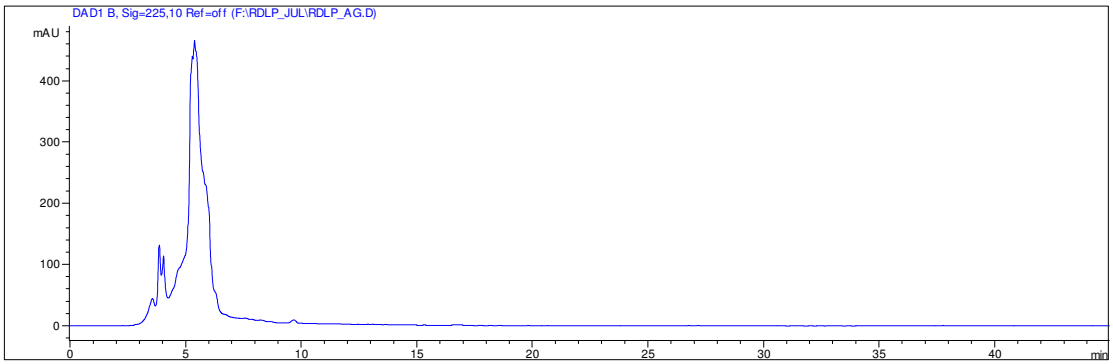
(a) Acetonitrile (ACN) : H2O = 20 : 80



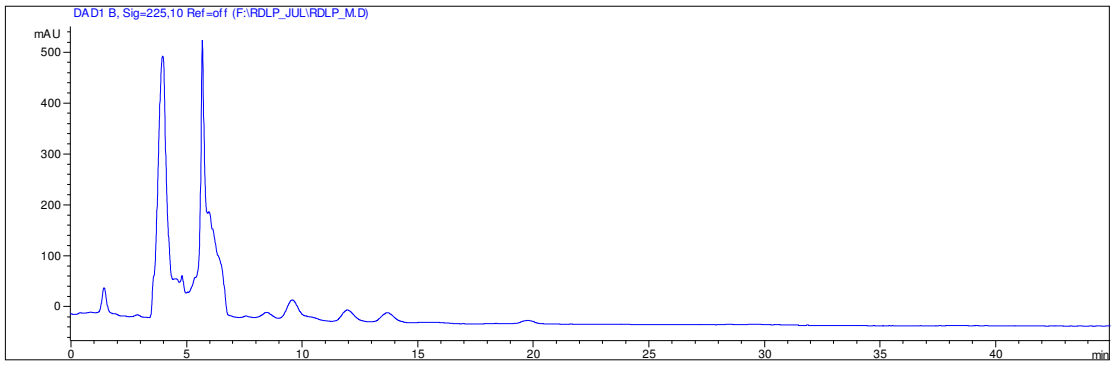
(b) ACN : H2O = 50:50



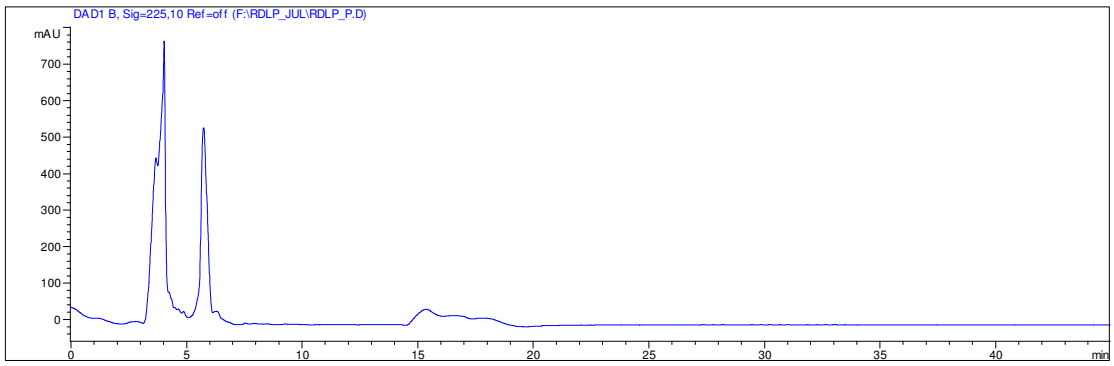
(c) ACN : H2O = 80:20



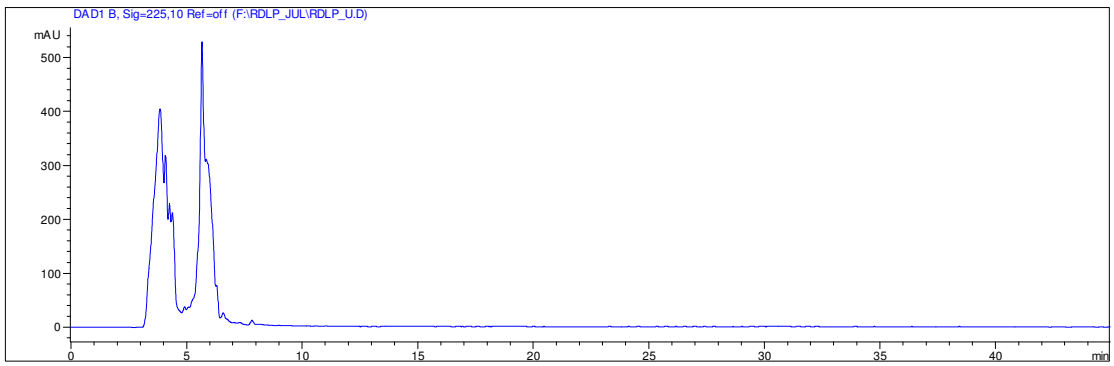
(d) Ethanol (EtOH) : H2O = 20 : 80



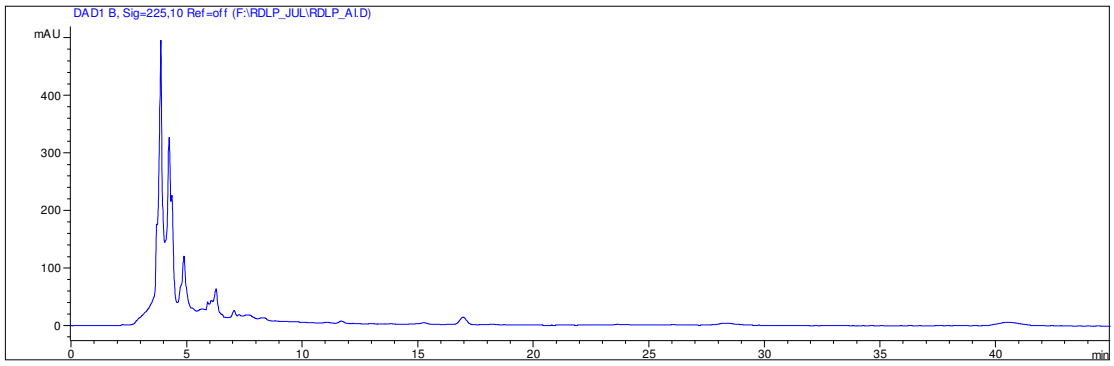
(e) EtOH : H2O = 50 :50



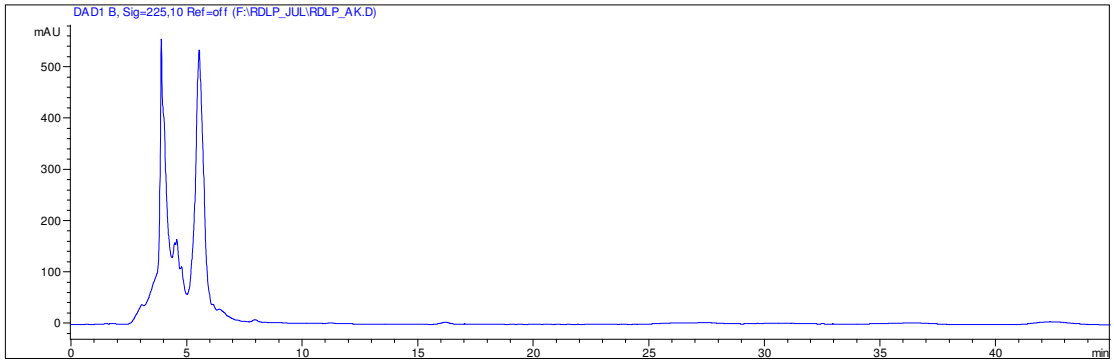
(f) EtOH : H2O = 80 :20



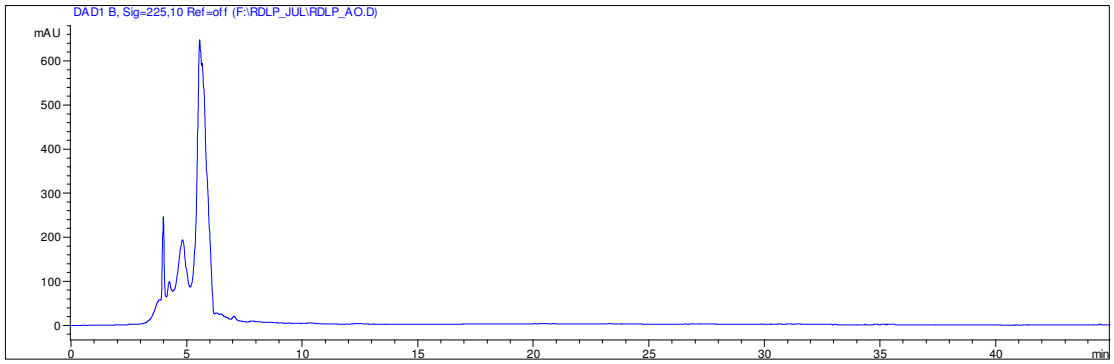
(g) Isopropanol : H2O = 20 :80



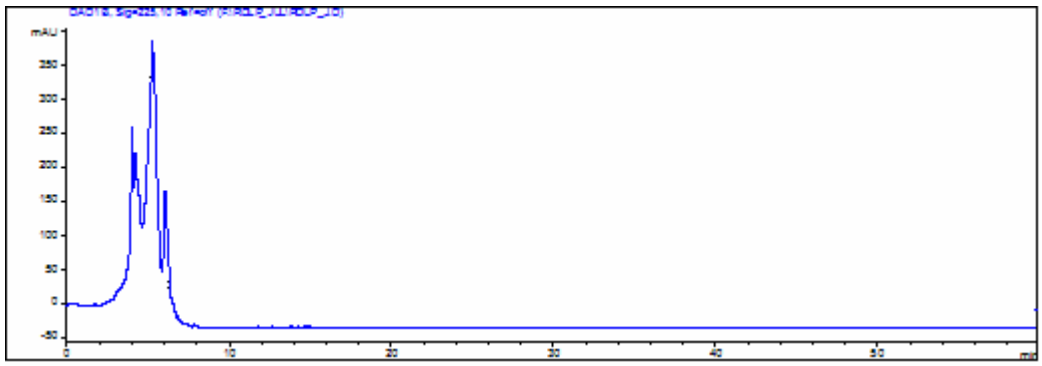
(h) Isopropanol : H2O = 50 :50



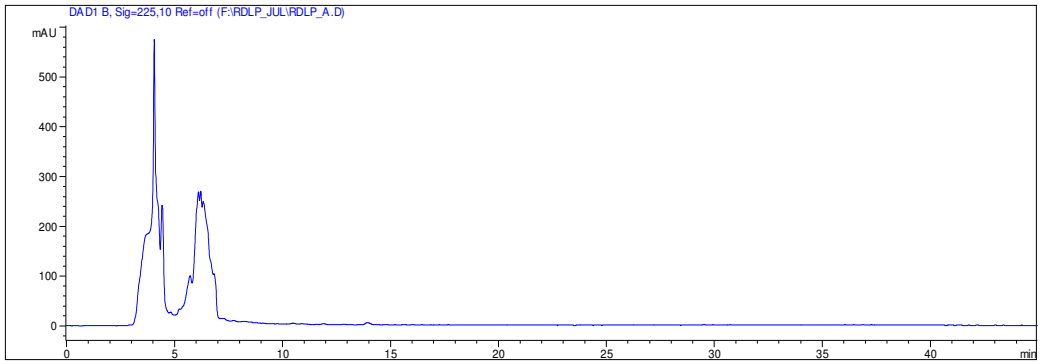
(i) Isopropanol : H2O = 80 :20



(j) MeOH : H2O = 20 : 80

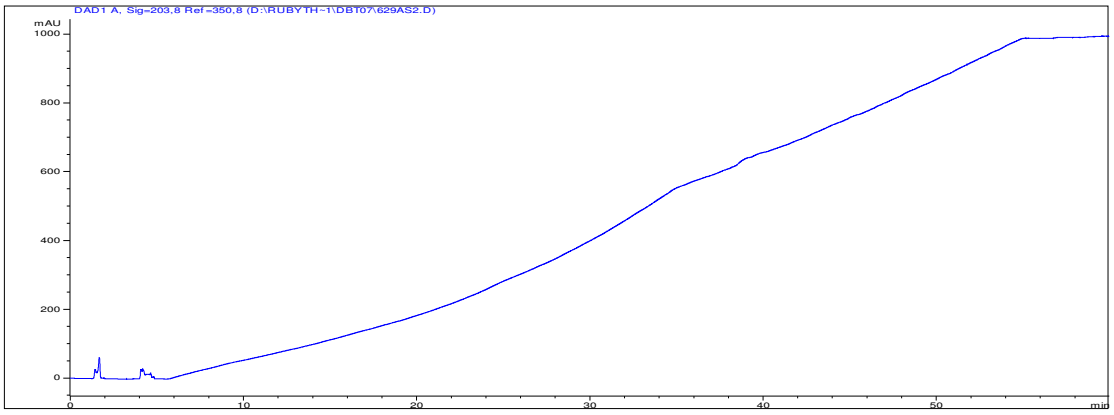


(k) MeOH : H2O = 80 : 20

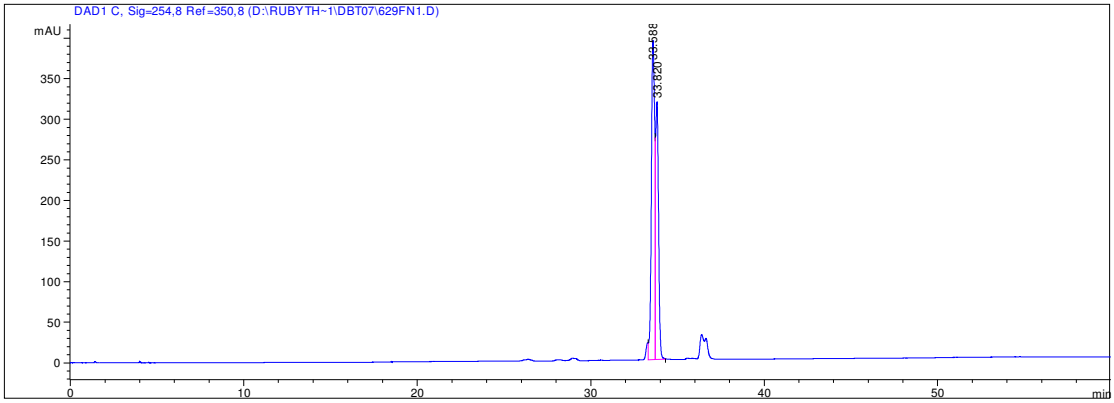


Appendix 6.1 HPLC chromatograms of the five standards

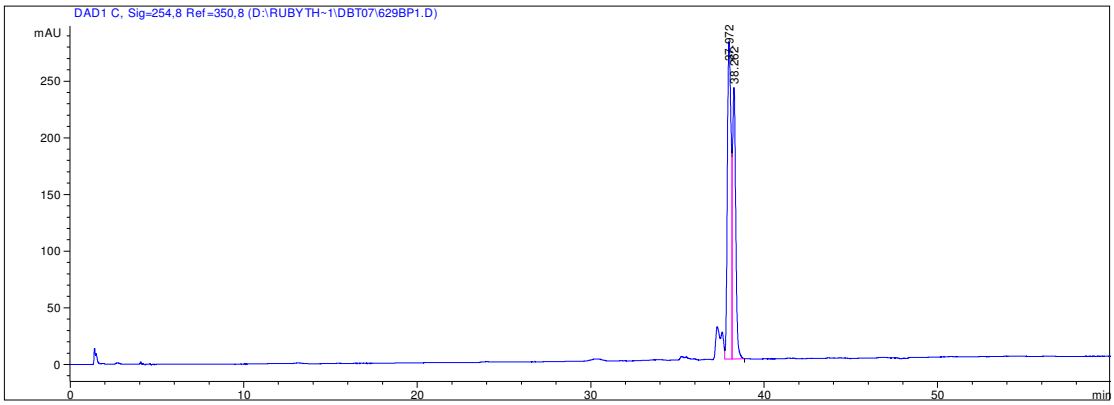
(a) HPLC chromatogram Astragaloside IV at 203nm



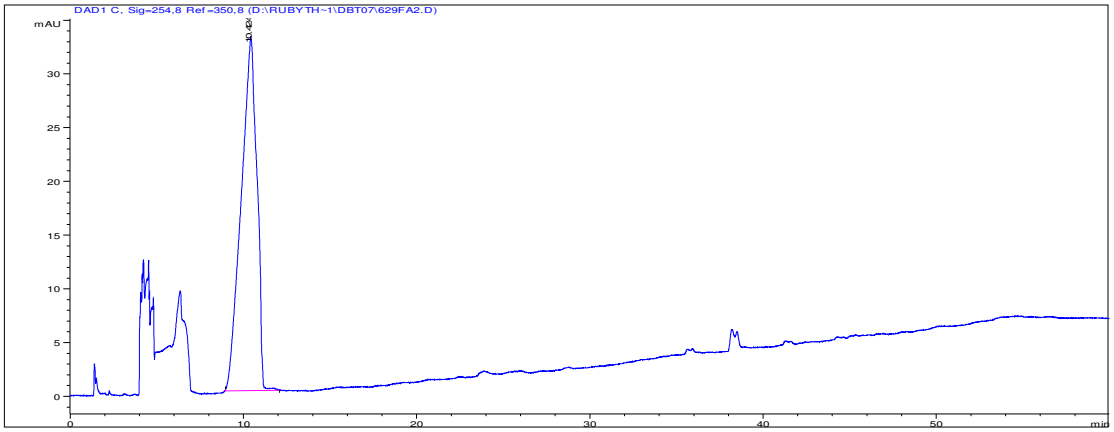
(b) HPLC chromatogram of Formononetin at 254nm



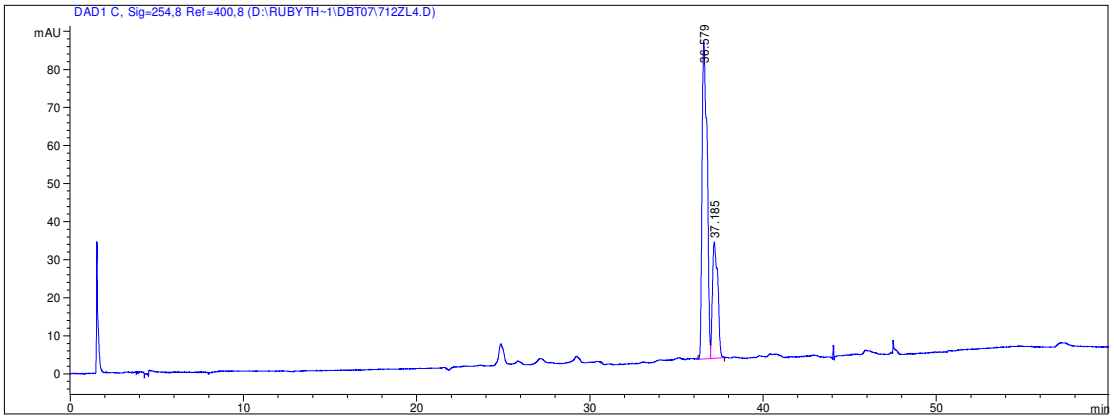
(c) HPLC chromatogram of n-butylidenephthalide at 254nm



(d) HPLC chromatogram of Ferulic acid at 254nm

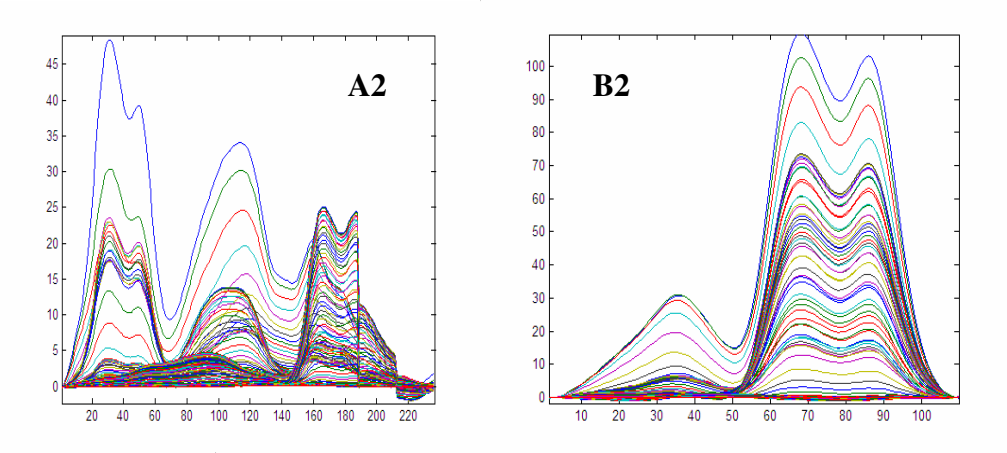


(e) HPLC chromatogram of Z-ligustilide at 254nm

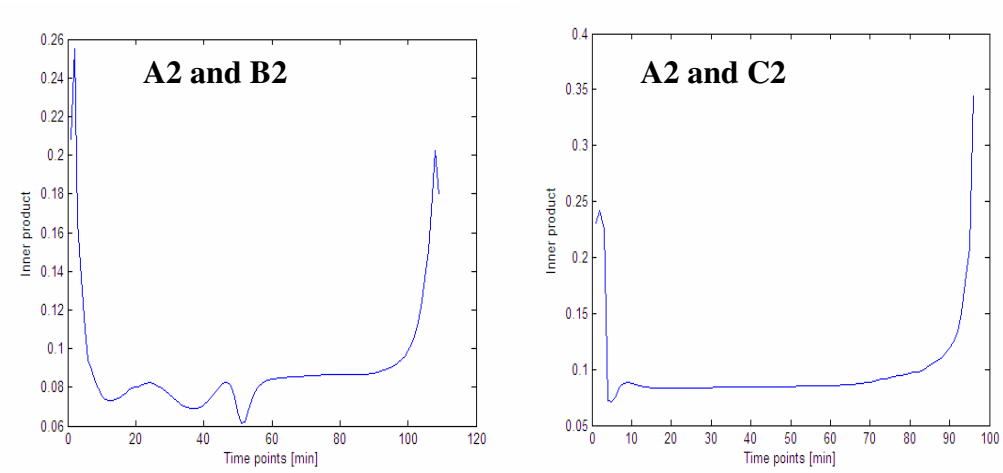


Appendix 6.2 Data sets (spectrochromatogram and MSCC results) of peak clusters of B-DBT, B-DG and B-HQ and

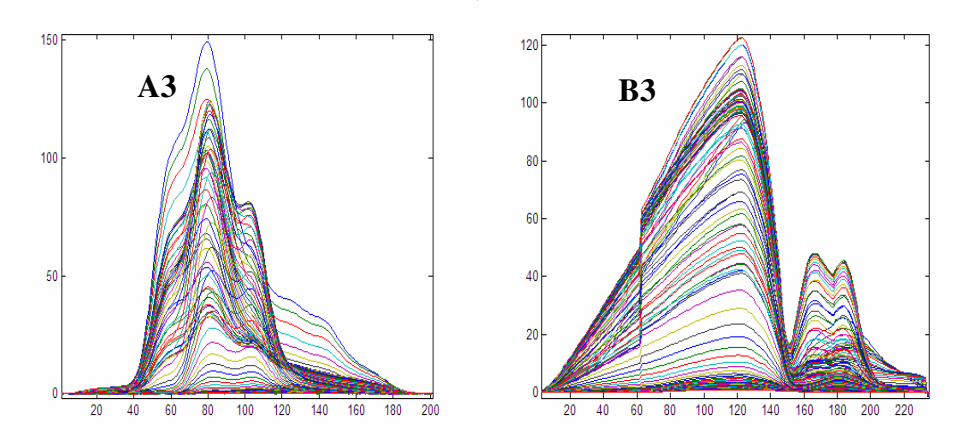
(a) Data sets A2, B2 and C2



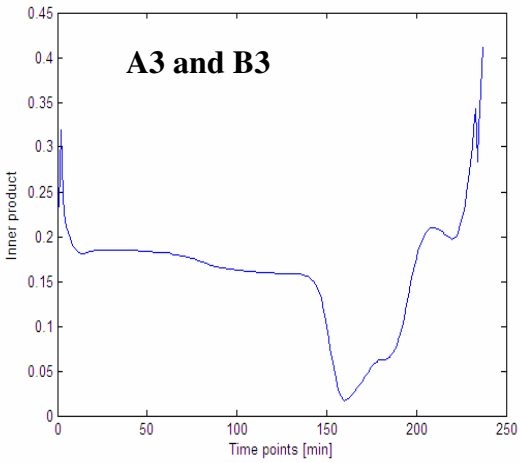
MSCC results of A2 and B2 (left) and A2 and C2 (right)



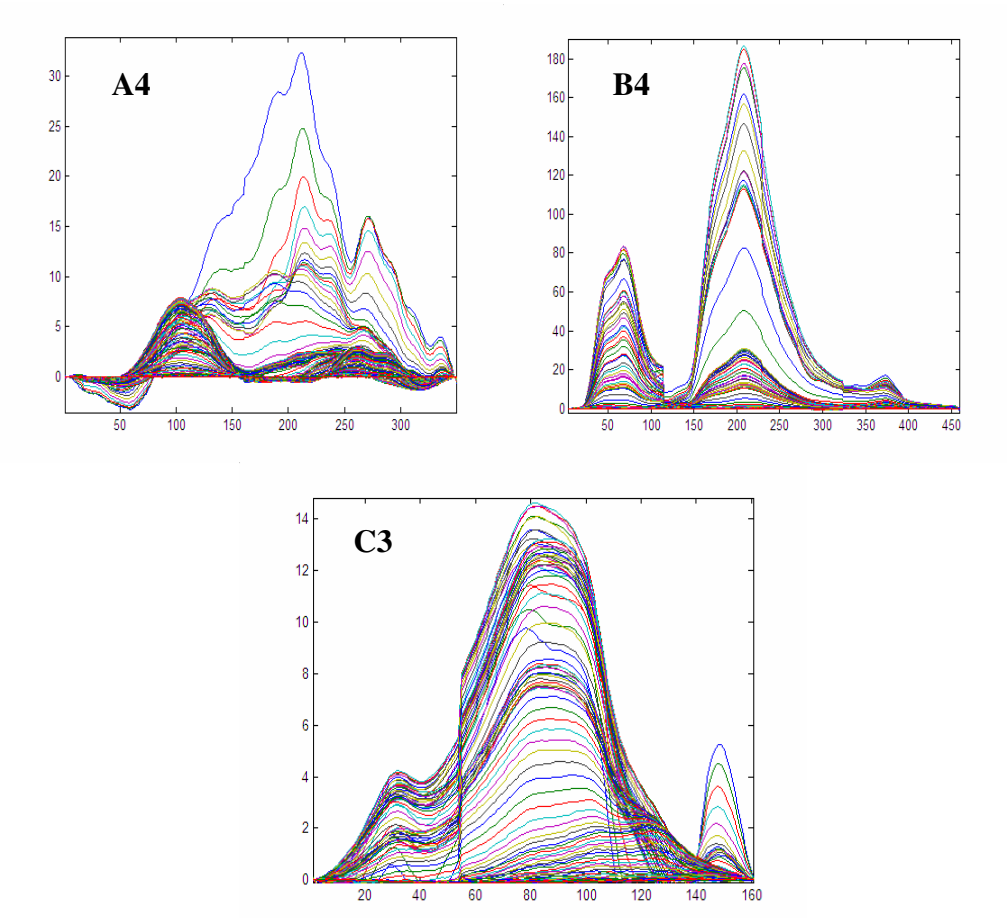
(b) Data sets A3 and B3



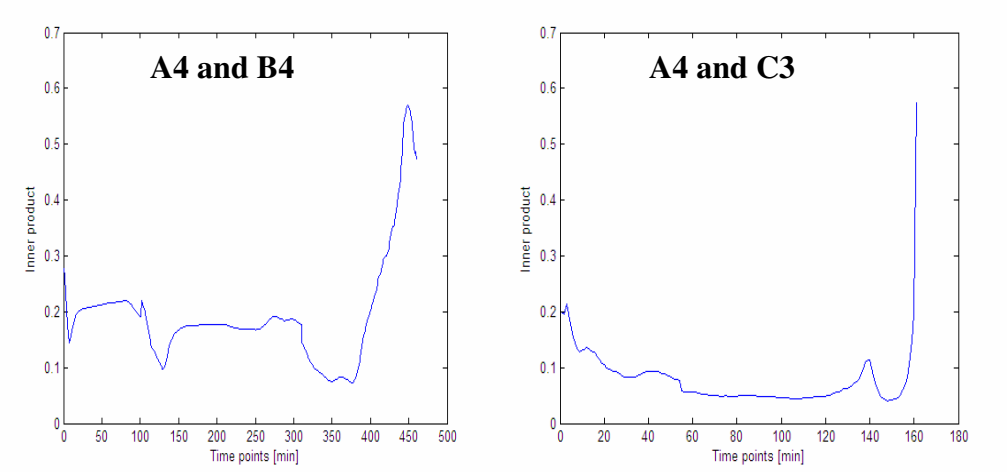
MSCC results of A3 and B3



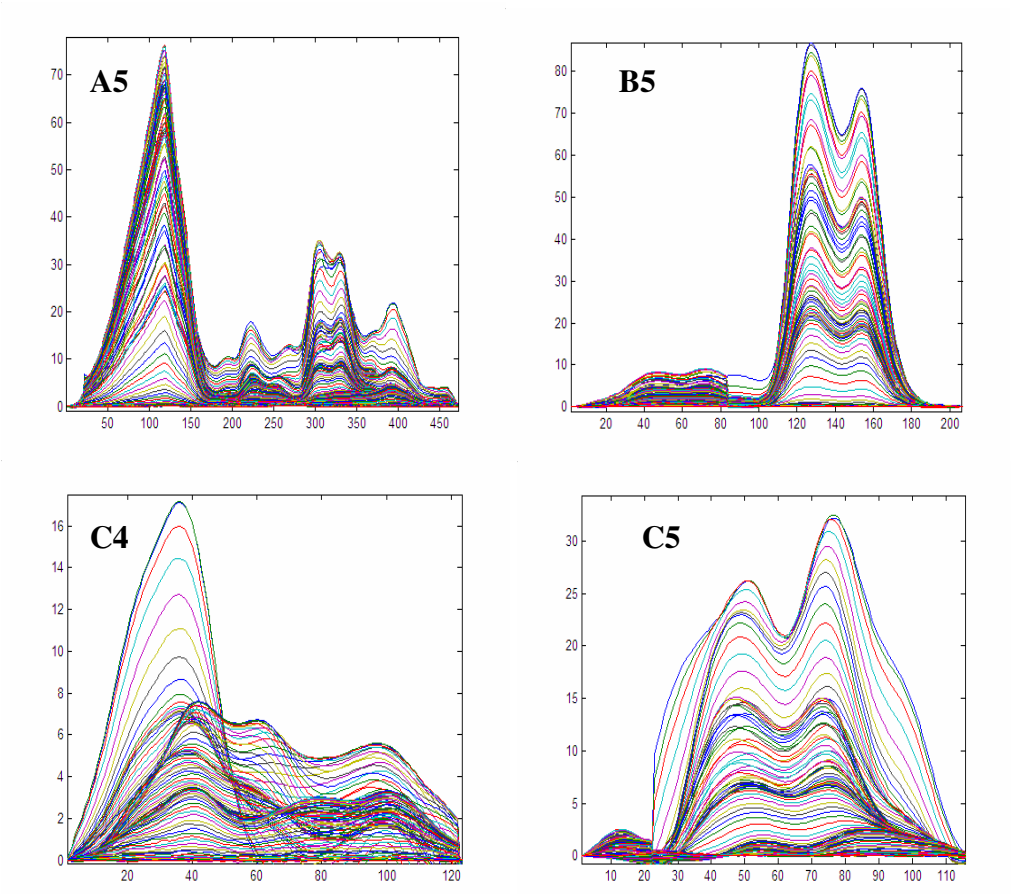
(c) Data sets A4, B4 and C3



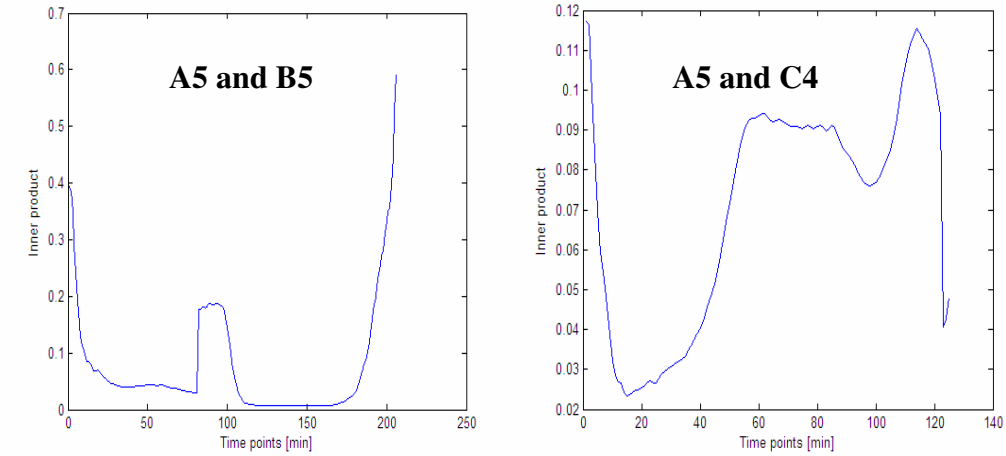
MSCC results of A4 and B4 (left) and A4 and C3 (right)

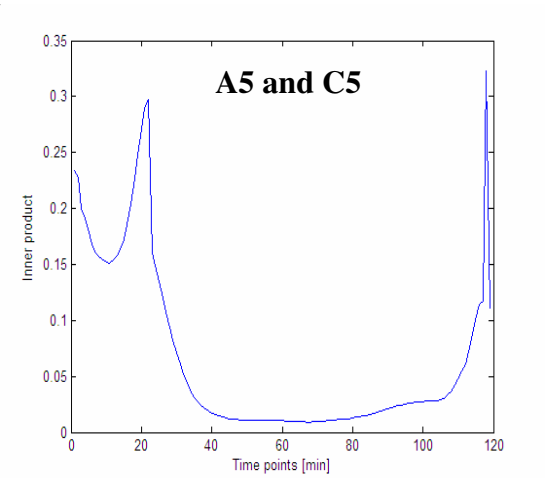


(d) Data sets A5, B5, C4 and C5

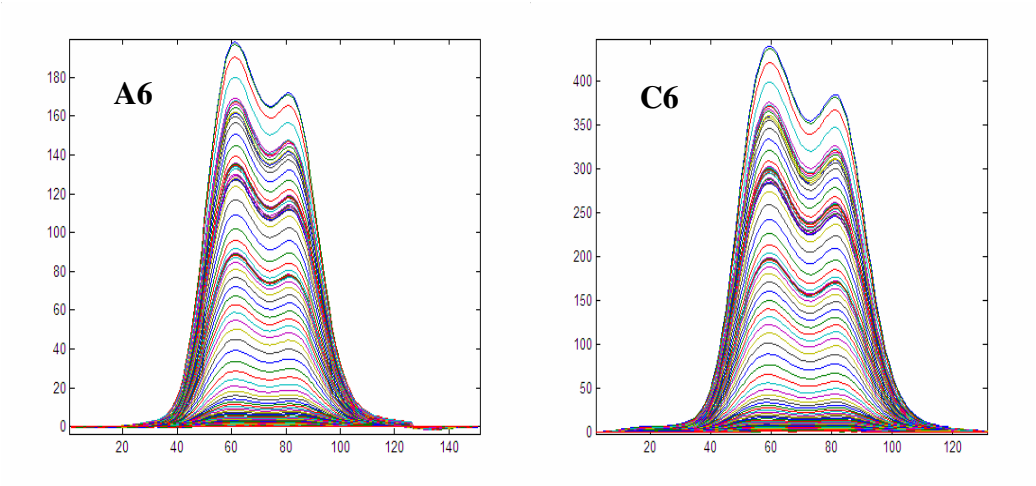


MSCC results of A5 and B5 (left) and A5 and C4 (right) and A5 and C5 (down)

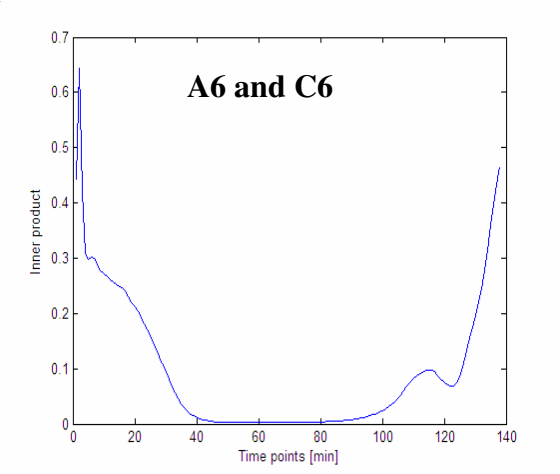




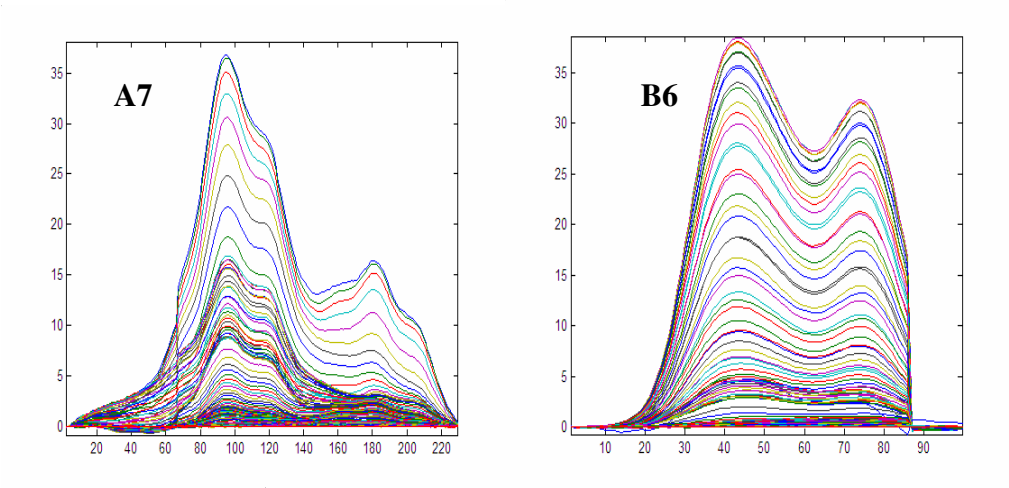
(e) Data sets A6 and C6



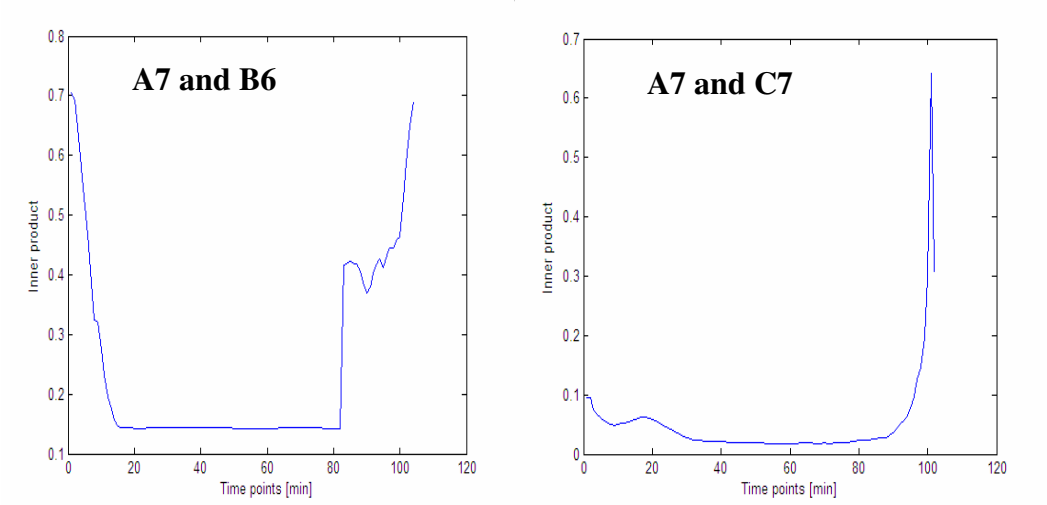
MSCC results of A6 and C6



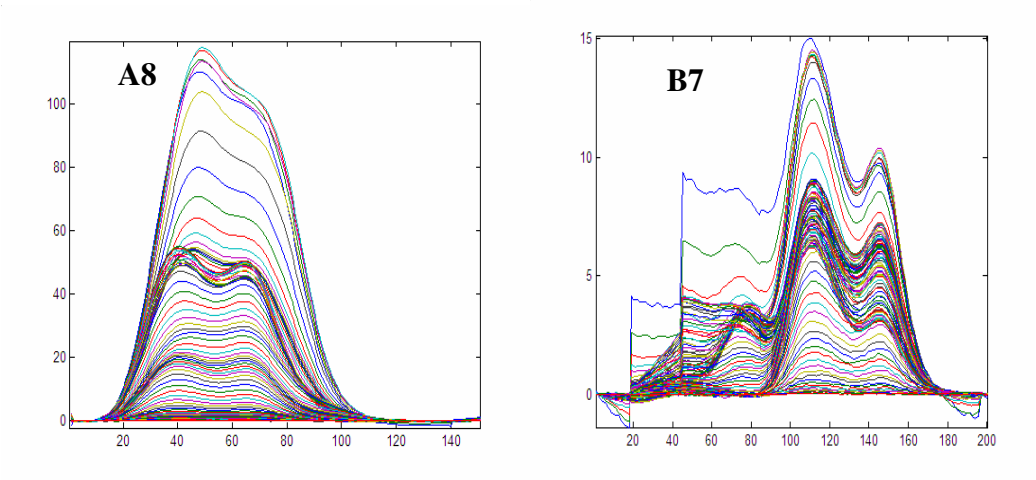
(f) Data sets A7, B6 and C7



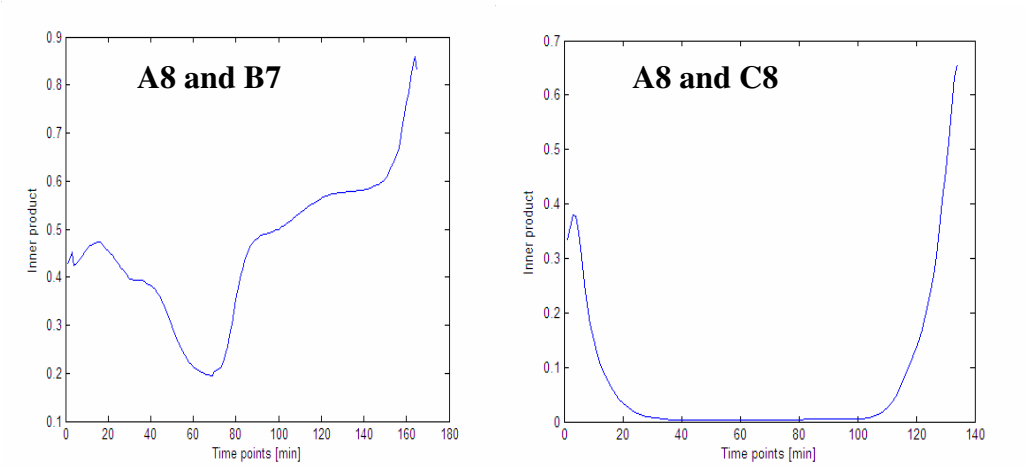
MSCC results of A7 and B6 (left) and A7 and C7 (right)



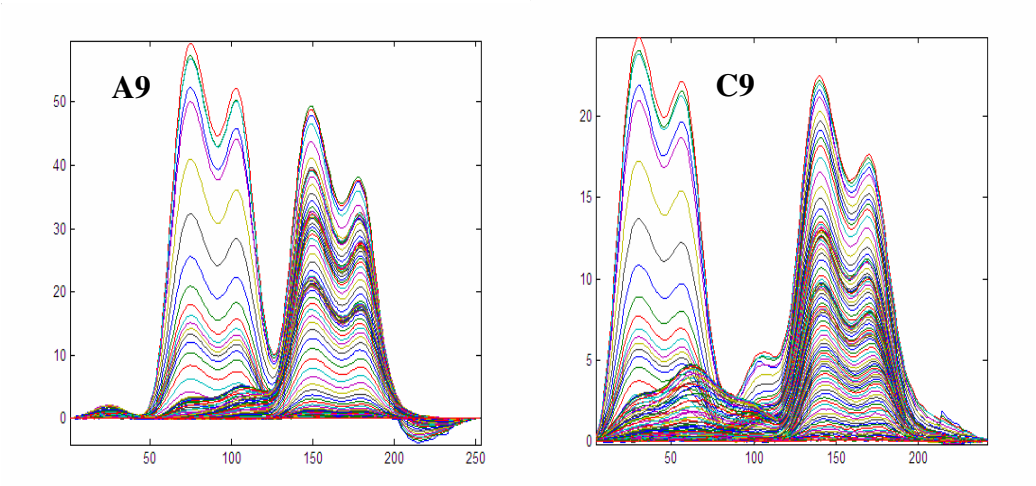
(g) Data sets A8, B7 and C8



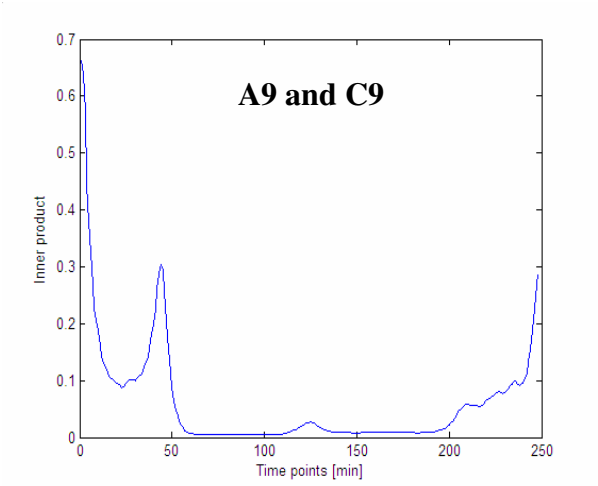
MSCC results of A8 and B7 (left) and A8 and C8 (right)



(h) Data sets A9 and C9



MSCC results of A9 and C9



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