Quantitative Evaluation of Herbal Medicines by Liquid Chromatography with Three-channel Electrochemical Detection

Liangmian Chen

TABLE OF CONTENTS

ABBREVIATIONS 1
INTRODUCTION 3

Chapter 1 The novel LC-3ECD method for the determination of various redox compounds 8
1.1 Development of a novel LC-3ECD system for the determination of complex compound mixture 8
1.1.1 Principle and assumed process for the design of an LC-3ECD system 8
1.1.2 Practical assembly of the novel LC-3ECD system 10
1.2 The LC-3ECD method for the determination of 9 redox compounds from *Flos Chrysanthemi* 13
1.2.1 Detection channel arragements and analytical process in LC-3ECD system for determining 9 redox compounds from *Flos Chrysanthemi* 13
1.2.2 Investigation and optimization of LC-3ECD system conditions for determining 9 redox compounds from *Flos Chrysanthemi* 14
1.2.3 Validation of LC-3ECD by standard substances for the determination of 9 compounds from *Flos Chrysanthemi* 18
1.3 The LC-3ECD method for the determination of 11 redox compounds from SHL preparations 20
1.3.1 Analytes and LC-3ECD chromatographic conditions for SHL preparations 20
1.3.2 Hydrodynamic voltammograms of 11 analytes 21
1.3.3 Validation of LC-3ECD by standard substances for the determination of 11 compounds from SHL preparations 22

Chapter 2 System repeatability estimation in quantitative HPLC-UV and LC-3ECD for herbal medicines by ISO 11843-7 27
2.1 Scope and principle of ISO11843-7 for the system repeatability estimation 28
2.2 System repeatability estimation of quantitative HPLC-UV by ISO 11843-7 29
2.2.1 HPLC-UV for determining baicalin from *Scutellaria Radix* 29
2.2.2 Precision profile of baicalin in quantitative HPLC-UV by ISO 11843-7 29
2.2.3 System repeatability test of HPLC-UV 31

2.2.4 Determination of baicalin in JP *Scutellaria Radix* 32
2.3 System repeatability estimation of quantitative LC-3ECD by ISO 11843-7 32

Chapter 3 Quantitative evaluation of *Flos Chrysanthemi* and SHL preparations by LC-3ECD 35
3.1 Quantitative evaluation of *Flos Chrysanthemi* by LC-3ECD 35
3.1.1 Determination of caffeoylquinic acids and flavonoids in the real samples of *Flos Chrysanthemi* 35
3.1.2 Quantitative comparison of caffeoylquinic acids and flavonoids in *Flos Chrysanthemi* 38
3.1.3 PCA for the classification of *Flos Chrysanthemi* 43
3.2 Quantitative evaluation of SHL preparations by LC-3ECD 46

GENERAL DISCUSSION AND CONCLUSIONS 53
EXPERIMENTAL SECTION 55
ACKNOWLEDGEMENTS 59
REFERENCES 60

ABBREVIATIONS

AAS	atomic absorption spectrometry
AFS	atomic fluorescence spectrometry
BC	baicalin
BCE	baicalein
BG	butyl gallate
BJ	‘Boju’
BP 2014	British Pharmacopoeia 2014 edition
CCHA	cryptochlorogenic acid
CE	capillary electrophoresis
CFA	caffeic acid
CHA	chlorogenic acid
CJ	‘Chuju’
ChP 2010	Chinese Pharmacopoeia 2010 edition
DAD	diode array detection
ECD	electrochemical detection
EG	ethyl gallate
ELSD	evaporative light scattering detection
FL	fluorescence detection
EP 6	Europe Pharmacopoeia 6th edition
FS	*Fructus Forsythiae* plus *Radix Scutellariae*
FTA	forsythoside A
FUMI	function of mutual information
GC	gas chromatography
GJ	‘Gongju’
H_2O	water
H_3PO_4	phosphoric acid
HJ	‘Hangju’
HPLC	high performance liquid chromatography
ICHA A	isochlorogenic acid A
ICHA B	isochlorogenic acid B
ICHA C	isochlorogenic acid C
IS	internal standard
ISO 11843-7	International Organization for Standardization 11843 part 7
JP 16	Japanese Pharmacopoeia 16th edition
KF	kaempferol
LC	liquid chromatography
LC-3ECD	LC with three-channel isocratic elution ECD

LF	*Flos Lonicerae* plus *Fructus Forsythiae*
LOD	limit of detection
LS	*Flos Lonicerae* plus *Radix Scutellariae*
LT	luteolin
LTG	luteolin 7-O-glucoside
MeCN	acetonitrile
MeOH	methanol
MP	mobile phase
MS	mass spectrometry
NCHA	neochlorogenic acid
ND	not detected
PAD	protocatechuic aldehyde
PCA	principle component analysis
PG	propyl gallate
pre-C	pre-column
r	correlation coefficient
RRLC	rapid resolution liquid chromatography
R_s	resolution
RSD	relative standard deviation
S-BJ	sulfur-fumigated ‘Boju’
SD	standard deviation
S-HJ	sulfur-fumigated ‘Hangju’
SHL	Shuang-Huang-Lian
STL	scutellarin
SV	switching valve
S/N	signal-to-noise ratio
TCM	traditional Chinese medicine
UPLC	ultra performance liquid chromatography
USP 37	United State Pharmacopoeia 37th edition
UV	ultraviolet detection
WGD	wogonoside

INTRODUCTION

Herbal medicines such as traditional Chinese and Kampo medicines, have attracted considerable attention worldwide in recent years for their effective treatment with minimum side effects in many diseases [1–2]. Owing to the fact that the medicinal herbs and their preparations generally exert their therapeutic effects through the synergic action of the multiple active components [3], the simultaneous determination of various bioactive components becomes the preferable method for the quality evaluation of herbal medicines.

Liquid chromatography (LC) is the most frequently used separation and analytical method to deal with complex chemical mixtures. The general detection methods on LC contain ultraviolet detection (UV) and/or diode array detection (DAD), fluorescence detection (FL), electrochemical detection (ECD), mass spectrometry (MS), evaporative light scattering detection (ELSD), *etc.* UV is the most general method for the determination of organic compounds. In contrast, an ECD would be a useful analytical method due to its high sensitivity and selectivity for the redox compounds such as phenolic acids and flavonoids. These compounds have the structure of phenolic hydroxy groups, which can be oxidized in an electrochemical redox reaction [4-6]. Compared with UV/DAD, a very commonly used detection method, which is possible to detect various organic compounds with less selectivity, ECD has higher selectivity for the redox compounds. Thus, these redox compounds could be more easily quantified without interference of other non-redox compounds in herbal medicines. Moreover, in the case of UV and/or DAD, because chromatographic peak height/area of the analyte has a linear relationship to a molar absorption coefficient (ε), standard substance of analyte is required to perform semi- and quantitative determination. In the case of ECD, chromatographic peak current height has a linear relationship to a number of electrons (*e.g.* n = 1, 2, or 4) in an electrochemical reaction of an analyte. Although most ECD methods use standard substances for the determination, using chromatographic peak current height, semi-quantitative determination, at least in some cases, can be performed without standard substances. Furthermore, literature survey shows that the sensitivity of ECD is remarkably higher than that of UV/DAD [7]. Thus, the low content redox compounds but with strong biological activities in herbal medicines which are hard to be detected by UV/DAD can be quantified by an ECD method. Therefore, an LC-ECD would be a very valuable method for the quantitative evaluation of herbal medicines.

Flos Chrysanthemi, the dried capitulum of *Chrysanthemum morifolium* Ramat. (Compositae), called ‘Ju Hua’ in China, is a well-known herbal medicine, tea material, and food resource in many Asian countries such as China, Japan, South Korea, and Thailand [8]. In China, there are many cultivars of *Flos Chrysanthemi* available. Among these cultivars, four major varieties of *C. morifolium* cv. ‘Hangju’ (HJ), ‘Boju’ (BJ), ‘Chuju’ (CJ), and ‘Gongju’ (GJ) have been recorded into Chinese Pharmacopoeia 2010 edition (ChP 2010) as standard varieties of *Flos Chrysanthemi* for their therapeutic effects of scattering cold, cleaning heat and toxin properties, and brightening eyes [9]. *Flos Chrysanthemi* was abundant

of caffeoylquinic acids and flavonoids, which were considered to be the main bioactive components. Modern pharmacological studies revealed that these components from *Flos Chrysanthemi* possess extensive biological activities, such as anti-oxidation [10, 11], anti-mutagenesis [12], cardiovascular protection [13, 14], anti-cancer [15], anti-human immune deficiency viruses [16], *etc*. Therefore, it is significant to quantitatively analyze these bioactive components by a feasible analytical method. Several methods have been reported to analyze the chemical profiles of *Flos Chrysanthemi*, such as capillary electrophoresis (CE) with ECD [17], high performance liquid chromatography (HPLC) with DAD [18-20], LC-MS [21, 22], gas chromatography (GC) with MS [23, 24], LC-DAD-MS [25, 26], *etc*. The caffeoylquinic acids and flavonoids have the structure of phenolic hydroxy groups, which can be oxidized in an electrochemical redox reaction. Therefore, these compounds from *Flos Chrysanthemi* can be analyzed by an LC-ECD method.

Most medicinal herbs need to undergo a post-harvesting processing to convert the raw material into a form readily useable for prescriptions. In China, there are many kinds of post-harvesting processing methods for herbal medicines, such as sun-drying, stir-frying, steaming, *etc* [27]. The main traditional post-harvesting processing methods for *Flos Chrysanthemi* are shade-drying, sun-drying, and hot-air drying. In addition, a sulfur fumigation method is often used by herbal farmers and producers for its benefits of decreasing drying time, preserving color, preventing insects and molds, easy operation, and low-cost. However, sulfur fumigation can cause the quality of the herbs to vary in terms of chemical profiles, pharmacokinetics, bioactivities, and even toxicities [28]. A literature survey showed that the chemical variation of *Flos Chrysanthemi* caused by sulfur fumigation has been qualitatively evaluated by LC-MS [21] and GC-MS [23]. For further study, it is urgent to establish an analytical method for the quantitative comparison of bioactive components between the non-fumigated *Flos Chrysanthemi* and their sulfur-fumigated products.

Shuang-Huang-Lian (SHL) is a composite formula of traditional Chinese medicine (TCM) comprised of three herbs: *Flos Lonicerae*, *Radix Scutellariae*, and *Fructus Forsythiae*, which is commonly used to treat upper respiratory illnesses caused by viruses or bacterial infections, such as tonsillitis, pharyngitis, pneumonia, acute enteritis, and viral dysentery [9]. Due to its high efficacy for viral diseases, it has become one of the top selling TCM products in China with a great variety of dosage forms, such as oral liquid, lyophilized powder for injection, capsule, granule, tablet, and suppository. Previous studies on the three medicinal crude herbs have revealed the presence of caffeoylquinic acids, flavonoids, and phenylethanoid glycosides. These components have been considered to be the main bioactive components in SHL preparations [29]. Caffeoylquinic acids such as chlorogenic acid (CHA), neochlorogenic acid (NCHA), cryptochlorogenic acid (CCHA), isochlorogenic acid A (ICHA A), isochlorogenic acid B (ICHA B), isochlorogenic acid C (ICHA C), and caffeic acid (CFA) have been reported to possess antioxidant [30], antibacterial [31], and antiviral activities [32], tyrosinase-inhibitory and antiproliferation effects [33], and so on. Flavonoids such as baicalin (BC), scutellarin (STL), wogonoside (WGD), and baicalein (BCE) have been found to exhibit

anti-oxidant and free radical-scavenging [34, 35], anti-inflammatory [36, 37], and anti-tumor activities [38, 39], vasodilative effects [40], anxiolytic-like effects [41], *etc*. Forsythoside A (FTA), one of the representative components of phenylethanoid glycosides, showed strong antioxidant [42, 43], antibacterial [42-44], and anti-inflammatory activities [45], cyclic adenosine monophosphate phosphor-diesterase inhibitory effects [46], neuroprotective effects [47], *etc*. The simultaneous analysis of these bioactive components was imperative for the quality evaluation of SHL preparations. Literature survey revealed that several analytical methods have been employed to analyze the bioactive components from SHL preparations including CE-ECD [48], CE-UV [49], rapid resolution liquid chromatography (RRLC) with DAD [50], ultra performance liquid chromatography (UPLC) with MS [51], HPLC-ELSD [52], HPLC-UV/DAD [53-55], HPLC-MS [56], HPLC-ECD [57], HPLC-DAD-ECD [58], *etc*. Most of these analytical methods involved with gradient elution LC system. LC is commonly used for the multi-components analyses due to its powerful separation abilities by gradient elution.

An LC-ECD is a valuable analytical method due to its high sensitivity and selectivity for redox compounds. However, a single channel isocratic elution LC-ECD was unsatisfactory for the analyses of multi-components as these components usually had remarkably different hydrophobic properties, which were hard to be fully separated by a single channel isocratic elution LC-ECD, simultaneously. On the other hand, in a gradient LC-ECD, the sensitivity of gradient LC-ECD was consequently inferior to that of isocratic LC-ECD [7, 59]. In an ECD, the structure of the electrical double layer on a working electrode in an electrochemical flow cell was largely affected by solvent molecules, supporting electrolytes, the applied potential, *etc*. The different mobile phase composition in a gradient LC-ECD would change the solvent molecules on the surface of the working electrode and influence the stability of the electrical double layer. Then, the chromatographic baseline fluctuation and drift would be increased. That is why the sensitivity of gradient LC-ECD is generally lower than that of isocratic LC-ECD. A two-channel isocratic elution ECD system for determining isoflavones [59] and a three-channel isocratic elution ECD system for determining phenolic acids and tanshinones [7] were established with shorter analytical time than single isocratic LC-ECD and higher sensitivity than gradient LC-ECD, previously. However, the separation of caffeoylquinic acids and flavonoids worsened by using the two-channel isocratic ECD system [59] due to the remarkably different hydrophobicity of these compounds. On the other hand, by using the previous three-channel isocratic elution ECD system [7], the very long analytical time and high system pressure presented, as well as the bad separation of caffeoylquinic acids and flavonoids, partly, owing to some design defects of the system. Therefore, these two LC-ECD systems were not suitable for the simultaneous analyses of caffeoylquinic acids and flavonoids.

In chapter 1 of this study, a novel LC with three-channel isocratic elution ECD (LC-3ECD) system has been developed using new design of channel connections and the technique of alternate rotations of switching valves to simultaneously analyze the different polarity

compounds with good separation, high sensitivity and efficiency. This LC-3ECD system consists of three isocratic elution flow ways, two switching valves, four columns (one pre-column and three separation columns), and three detection channels. Through alternately rotating switching valves to change the elution flow ways, the different polarity compounds in the complex compound mixture were alternatively eluted into different separation columns from the pre-column by the different mobile phases. Then, the similar polarity compounds were fully separated on the same separation column and detected in the same detection channel by an isocratic elution. This novel LC-3ECD system could obtain the good separations of various polarity compounds within satisfactory analytical time, without loss of the sensitivity. To verify its effectiveness and feasibility, this LC-3ECD was validated by using 9 standard compounds mixture concerned with *Flos Chrysanthemi* and 11 standard compounds mixture concerned with SHL preparations, respectively.

In a quantitative HPLC method, the system repeatability is of great importance to get an accurate, credible, and applicable quantitative result. To obtain highly reproducible results to quantitatively evaluate the herbal medicines, the precision of the analytical method must be estimated by the measurement of standard deviation (SD) and/or relative SD (RSD) as criteria for system repeatability. Well-known statistical ways to precisely estimate the measurement SD and/or RSD, require five or six runs of chromatographic experiments to be performed in the HPLC analysis [60, 61]. In pharmacopoeias such as Japanese, United State, Europe, British, and Chinese, it is described in the section of “System suitability” in “Liquid chromatography”, in principal, that the total number of replicate injections should be six [9, 62-65]. In order to quantify various compounds in herbal medicines, it would take a lot of time, effort, and chemicals to estimate the system repeatability by a statistical way of repetitive measurement. However, a new methodology called function of mutual information (FUMI) theory, which has been published in International Organization for Standardization 11843 part 7 (ISO 11843-7), titled as “Methodology based on stochastic properties of instrumental noise”, in 2012, could be used to estimate the precision in a simple way. By this new methodology, the measurement SD and RSD can be obtained from the stochastic aspects of the noise and signal data involved in a chromatogram with no requirement of repetitive chromatographic measurement of the real samples. And the measurement RSDs of stochastic ways by ISO 11843-7 have 95% confidence intervals corresponding to those for about 40 measurement (±20% of the true RSD) [66].

In chapter 2 of this study, the applicability of this new methodology of ISO 11843-7 was evaluated for the system repeatability estimation in quantitative HPLC system. Firstly, the HPLC-UV, which is commonly used for the quantitative evaluation of herbal medicines, was taken as an example to verify the applicability of ISO 11843-7. The system repeatability of HPLC-UV for determining baicalin from *Scutellaria Radix* was estimated by ISO 11843-7. The precision profile was established, in which, the stochastically observed RSDs by ISO 11843-7 were compared with the statistically observed RSDs by repetitive measurement. The results show that the stochastically observed RSDs were within the 95% confidence interval

of the statistically observed RSDs. It indicated that ISO 11843-7 is applicable to estimate the system repeatability of HPLC-UV without repetitive measurement. After the verification of its applicability in HPLC-UV, ISO 11843-7 was applied to estimate the system repeatability of the novel LC-3ECD for determining caffeoylquinic acids and flavonoids from *Chrysanthemi Flos*. The successful application indicates that ISO 11843-7 is a very helpful methodology for the system repeatability estimation of LC-3ECD, as well as for that of HPLC-UV, during the quantitative evaluation of herbal medicines. It is simple, credible, and applicable, saving not only the chemicals and energy, but also much experimental time.

Since the novel LC-3ECD system has been established by the new design of channel connection in chapter 1 and its good system repeatability has been reconfirmed by ISO 11843-7 in chapter 2, it is urgent to verify the applicability of the present LC-3ECD for the quantitative evaluation of herbal medicines. In chapter 3 of this study, the present LC-3ECD was applied to determine various bioactive components in *Flos Chrysanthemi* and SHL preparations. 7 caffeoylquinic acids of CHA, NCHA, CCHA, ICHA A, ICHA B, ICHA C, and CFA and 2 flavonoids of Luteolin 7-*O*-glucoside (LTG), and luteolin (LT) from *Flos Chrysanthemi* were simultaneously determined by the present LC-3ECD with high sensitivity and desirable analytical time. The present LC-3ECD coupled with principle component analysis (PCA) was applied to compare the ‘sameness’ and ‘differences’ in *Flos Chrysanthemi* and their sulfur-fumigated products. The quality of *Flos Chrysanthemi* and their sulfur-fumigated ones has been quantitatively evaluated and compared. Furthermore, 6 caffeoylquinic acids of CHA, NCHA, CCHA, ICHA B, ICHA C and CFA, 4 flavonoids of STL, BC, WGD, and BCE, and 1 phenylethanoid glycoside of FTA were simultaneously determined within 70 min by the present LC-3ECD. Then, the quality of 14 batches of SHL oral liquid and 12 batches of SHL lyophilized powder for injection produced by different manufacturers in China was evaluated by simultaneous determination of these 11 bioactive redox components.

Chapter 1 The novel LC-3ECD method for the determination of various redox compounds

In order to analyze the various compounds with remarkably different hydrophobic properties by an LC-ECD method with good separation, high efficiency, and without loss of sensitivity, the development of multi-channel isocratic elution ECD system was necessary and urgent for the quantitative evaluation of herbal medicines, as there were so many bioactive components existing in herbal medicines. In the previous study, a three-channel isocratic elution ECD system was established for determining phenolic acids and tanshinones [7]. However, the application of this system for herbal medicines was very limited owing to some design defects of the system. Firstly, the mobile phase once flowed through a column by the reverse direction during the analysis procedure. It would influence the separation effects of the analytes. Secondly, some analytes were eluted through three columns for separation by the mobile phase. It would increase the analytical time and reduce the analysis efficiency. Thirdly, the system pressure was really high when three columns were in the same flow way. It would weaken the system suitability. Therefore, the analysis capability could be markedly improved by the more feasible design of the system.

In this chapter, a novel LC with three-channel isocratic elution ECD (LC-3ECD) system have been developed by the new design of channel connections and the technique of alternate rotations of the switching valves to determine the different polarity compounds, simultaneously, with high sensitivity and efficiency. The effectiveness and feasibility of the present LC-3ECD method have been verified by using the complex compound mixtures concerned with the herbal medicines of *Flos Chrysanthemi* and Shuang-Huang-Lian (SHL) preparations.

1.1 Development of a novel LC-3ECD system for the determination of complex compound mixture

1.1.1 Principle and assumed process for the design of an LC-3ECD system

Generally, a test solution of a certain herbal medicine, such as *Flos Chrysanthemi*, is a complex compound mixture containing various components with different properties. Hereon, in order to help to explain the retention properties of the compounds on the chromatographic column, the compounds in the mixture were divided into three groups, the high polarity compounds (x_1, x_2, x_3, x_i …) as Group Ⅰ, the middle polarity compounds (y_1, y_2, y_3, y_i …) as Group Ⅱ, and the low polarity compounds (z_1, z_2, z_3, z_i …) as Group Ⅲ as shown in Fig. 1-1. When these compounds are simultaneously analyzed by a common LC-ECD method, the following contradiction and/or problems exist:

An isocratic elution LC-ECD couldn’t meet the analytical needs of these compounds. Most of the time, the reversed-phase columns were used in HPLC to separate the various components for herbal medicines. When the high polarity compounds had good separation in

the chromatographic column with a low ratio of organic phase as the mobile phase, the low polarity compounds were hard to be eluted out from the column. While using a high ratio of organic phase as the mobile phase to elute the low polarity compounds, the high and middle polarity compounds were hard to be separated in the column, simultaneously. On the other hand, a gradient elution LC-ECD may elute all compounds out of the column, successively, but the chromatographic baseline noise was enhanced and the sensitivity of these compounds would be reduced remarkably owing to the change of the mobile phase composition [7, 59]. In addition, even using a gradient elution, it may be difficult to obtain good separation of all these compounds from a single separation column.

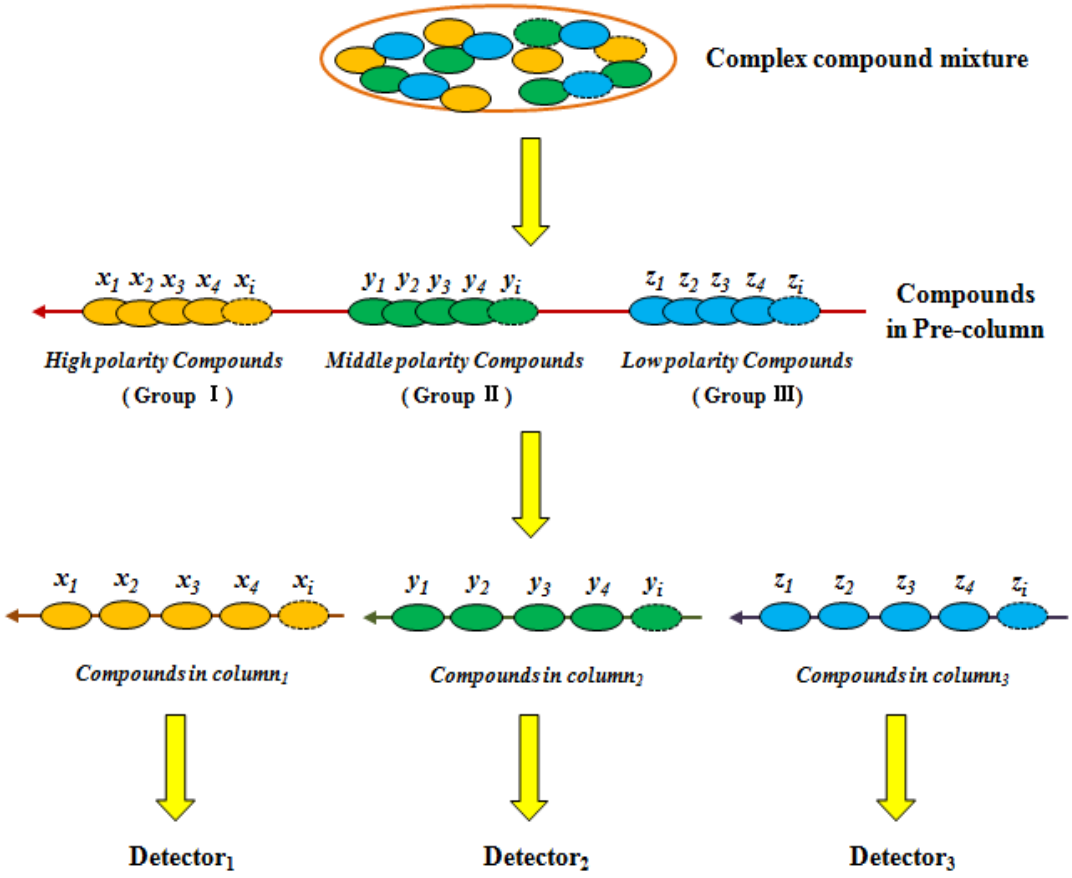


Fig. 1-1 Assumed flow diagram for the design of LC-3ECD system.

Therefore, in order to analyze these different polarity compounds simultaneously by a LC-ECD method with high sensitivity and efficiency, and avoid the problems mentioned above, an LC-3ECD system with four chromatographic columns (reversed-phase columns) was assumed and tried as shown in Fig. 1-1. A pre-column was designed for the pre-separation of these different polarity compounds. On the pre-column, it was really hard to separate each compound in the complex compound mixture, especially among the compounds with similar polarities. However, the full separation among the high polarity compounds (x_1,

x_2, x_3, x_i …) in Group Ⅰ, the middle polarity compounds (y_1, y_2, y_3, y_i …) in Group Ⅱ, and the low polarity compounds (z_1, z_2, z_3, z_i …) in Group Ⅲ on the pre-column could be obtained by the elution of different mobile phase, probably. It ensures that each group of compounds could be eluted into different columns from the pre-column in the different time, separately. Then the high polarity compounds (x_1, x_2, x_3, x_i …) in Group Ⅰ were separated on $column_1$ and detected in $detector_1$ using a low ratio of organic phase as the mobile phase. The middle polarity compounds (y_1, y_2, y_3, y_i …) in Group Ⅱ were separated on $column_2$ and detected in $detector_2$ using a middle ratio of organic phase as the mobile phase. The low polarity compounds (z_1, z_2, z_3, z_i …) in Group Ⅲ were separated on $column_3$ and detected in $detector_3$ using a high ratio of organic phase as the mobile phase. Meanwhile, each mobile phase should always flow through the same detector to keep the isocratic elution in each detection channel. Based on the above assumptions and design flow, the different polarity compounds may be analyzed simultaneously in short analytical time without loss of sensitivity.

In order to verify the above assumptions and achieve the stated goals, the technique of column-switching and the feasible channel connections should be well designed and applied.

1.1.2 Practical assembly of the novel LC-3ECD system

According to the above design principle and assumed process, several equipment accessories were used to assemble the LC-3ECD system by the feasible connecting method as shown in Fig. 1-2. These equipment accessories include a vacuum degasser (DG-2080-53, Jasco, Tokyo, Japan), three pumps (301M, Flom, Tokyo, Japan), a sample injector fitted with a 5 μl injection loop (7725i, Rheodyne, Cotati, CA, USA), two automatic six ports-switching valves (Model 405, Flom, Tokyo, Japan), four chromatographic columns, a column oven (CTO-10ASvp, Shimadzu, Kyoto, Japan), three electrochemical detectors (LC-4C, BAS, Tokyo, Japan), and a recorder (TR-V1000, Keyence, Osaka, Japan). Each electrochemical cell (Radial flow cell, BAS) was constructed from a glassy carbon working electrode, an Ag/AgCl reference electrode, and a stainless steel auxiliary electrode.

As shown in Fig. 1-2A, when the LC-3ECD system was in the initial state, the flow way on switching valve $_1$ (SV_1) and SV_2 were at the initial positions. The mobile phase $_1$ (MP_1) with a low ratio of organic phase flowed through pre-column (Pre-C), column $_1$ (C_1), and detection channel $_1$ (D_1) in the first channel (CN_1) under a certain flow rate. All compounds in the compound mixture were eluted into Pre-C by MP_1 after sample injection. The high polarity compounds (x_1, x_2, x_3, x_i …) in Group Ⅰ (Fig. 1-1) were eluted into C_1 by MP_1 in CN_1 within certain time after sample injection, whereas other compounds were retained in Pre-C. Then, the flow way on SV_1 was changed by rotating the SV_1 automatically, on which, the rotation time has been set up beforehand, as shown in Fig. 1-2B, whereas SV_2 was still at the initial position. The MP_2 with a middle ratio of organic phase flowed through Pre-C, C_2, and D_2 in the second channel (CN_2) under a certain flow rate. The on-column concentration (frontal concentration) of the middle polarity compounds (y_1, y_2, y_3, y_i …) in Group Ⅱ (Fig. 1-1) occurred on Pre-C by MP_2, and then they were eluted into C_2 from Pre-C in CN_2 within

certain time after sample injection, whereas other compounds were still retained in Pre-C. Then, SV_2 was changed by the automatic rotation of the SV_2, on which, the rotation time has also been set up beforehand, as shown in Fig. 1-2C, whereas the position of SV_1 was the same as that shown in Fig. 1-2B. The MP_3 with a high ratio of organic phase flowed through Pre-C, C_3, and D_3 in the third channel (CN_3) under a certain flow rate. The on-column concentration of the low polarity compounds (z_1, z_2, z_3, z_i …) in Group Ⅲ (Fig. 1-1) retained in Pre-C occurred by MP_3, and they were eluted into C_3 in CN_3. In summary, MP_1, MP_2, and MP_3 alternately flowed through the Pre-C to elute the high polarity compounds (x_1, x_2, x_3, x_i …) in Group Ⅰ, the middle polarity compounds (y_1, y_2, y_3, y_i …) in Group Ⅱ, and the low polarity compounds (z_1, z_2, z_3, z_i …) in Group Ⅲ into C_1, C_2, and C_3, respectively. At that time, on Pre-C, the step-wise on-column concentration was induced by MP_2 and MP_3. Then, the high polarity compounds (x_1, x_2, x_3, x_i …) in Group Ⅰ were fully separated on C_1 and detected in CN_1. The middle polarity compounds (y_1, y_2, y_3, y_i …) in Group Ⅱ were fully separated on C_2 and detected in CN_2. The low polarity compounds (z_1, z_2, z_3, z_i …) in Group Ⅲ were fully separated on C_3 and detected in CN_3. Meanwhile, MP_1, MP_2, and MP_3 always flowed through D_1, D_2, and D_3, respectively, to keep the isocratic elution in each detection channel in the whole analytical process.

Compared with the previous LC-3ECD [7], the present LC-3ECD was more predominant. Each mobile phase could alternatively flow through the pre-column in the same direction. The different polarity compounds in the compound mixture could be alternatively eluted into different separation columns from the pre-column by the different mobile phases. Each mobile phase alternatively flowed through two columns at most. These situations couldn't be achieved by the previous LC-3ECD.

For the purpose of simultaneous determination of various compounds in a certain herbal medicine, the feasible time for rotating the two switching valves should be investigated and the chromatographic conditions including the mobile phase, flow rates, chromatographic columns, oven temperature, and applied potentials should be optimized by experiments to obtain good separation, high sensitivity and efficiency.

To verify the effectiveness and feasibility of the present LC-3ECD system, the compound mixtures concerned with *Flos Chrysanthemi* and Shuang-Huang-Lian preparations will be analyzed by the present LC-3ECD.

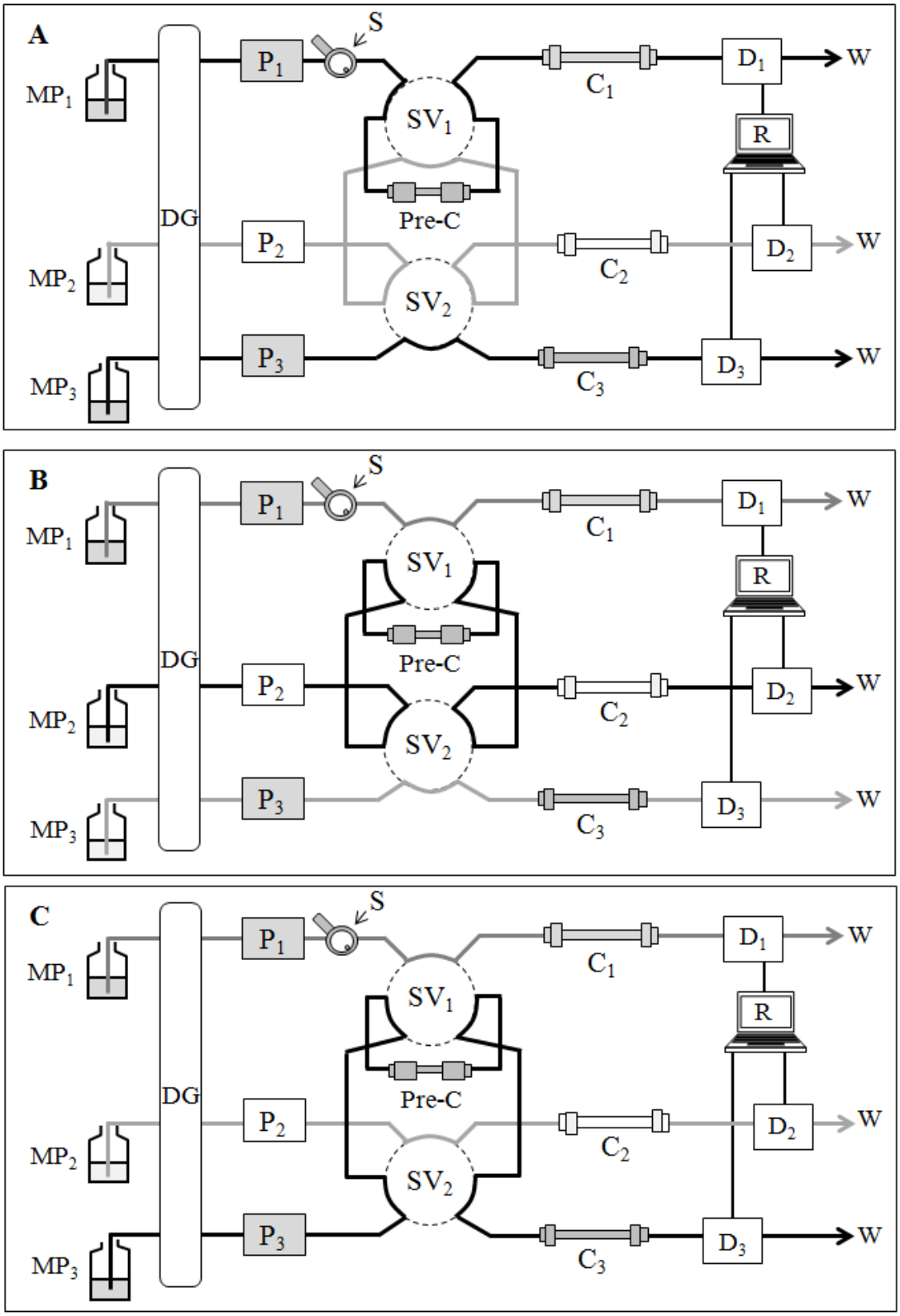


Fig. 1-2 Block diagram of the LC-3ECD system. $MP_{1\text{-}3}$, mobile phases; DG, vacuum degasser; $P_{1\text{-}3}$, pumps; S, sample injector; $SV_{1\text{-}2}$, switching valves; Pre-C and $C_{1\text{-}3}$, chromatographic columns; $D_{1\text{-}3}$, detection channels: detectors and electrochemical flow cells; R, recorder; W, waste.

1.2 The LC-3ECD method for the determination of 9 redox compounds from *Flos Chrysanthemi*

1.2.1 Detection channel arragements and analytical process in LC-3ECD system for determining 9 redox compounds from *Flos Chrysanthemi*

There are various bioactive components in *Flos Chrysanthemi*, which mainly contain caffeoylquinic acids and flavonoids. In this study, 7 caffeoylquinic acids of chlorogenic acid (CHA), neochlorogenic acid (NCHA), cryptochlorogenic acid (CCHA), isochlorogenic acid A (ICHA A), isochlorogenic acid B (ICHA B), isochlorogenic acid C (ICHA C), and caffeic acid (CFA) and 2 flavonoids of luteolin 7-*O*-glucoside (LTG), and luteolin (LT) were selected as analytes for determination by LC-3ECD. The structures of these 9 compounds were shown in Fig. 1-3. In addition, an internal standard (IS) method was used to obtain accurate measurement in the analytical process. Considering the factors of stability, dissolubility, the response value of peak current, and the resolution (R_s), the compounds of protocatechuic aldehyde (PAD), ethyl gallate (EG), and butyl gallate (BG) were selected as IS_1, IS_2, and IS_3 in CN_1, CN_2, and CN_3, respectively. The structures of these 3 ISs were shown in Fig. 1-4.

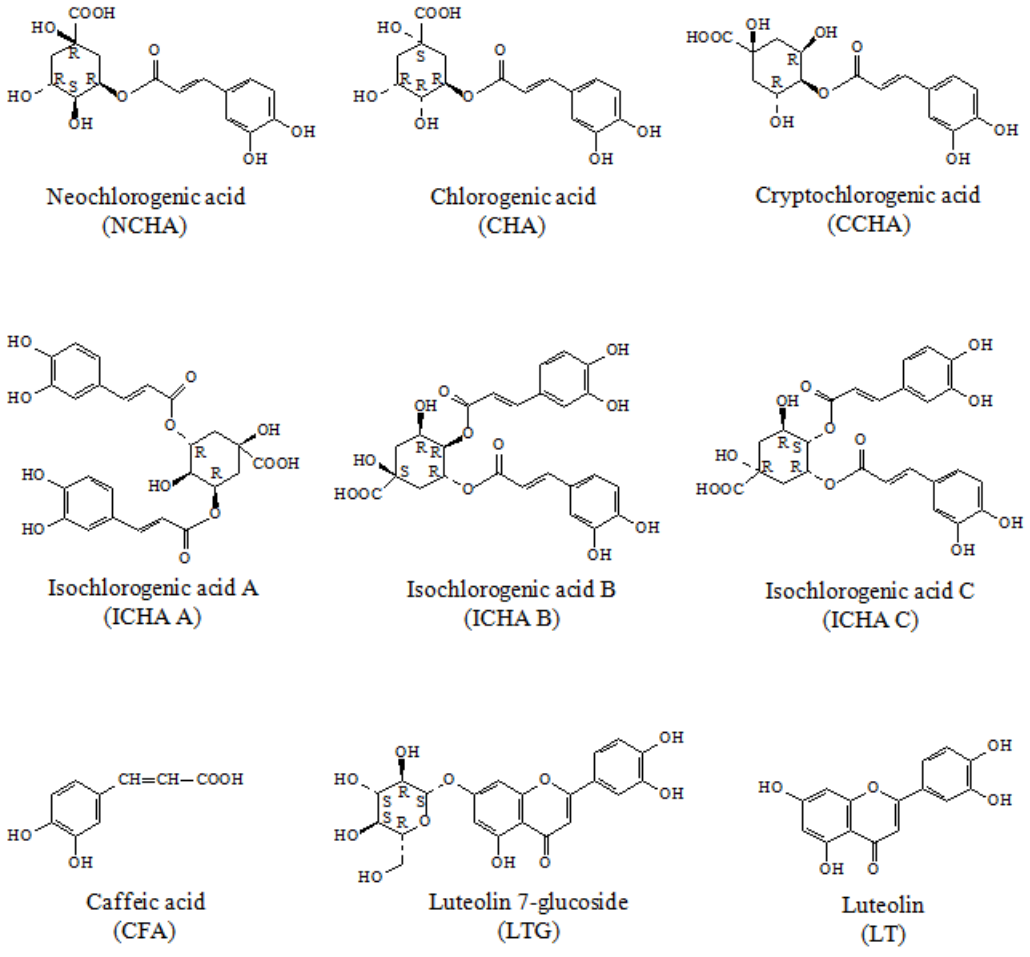
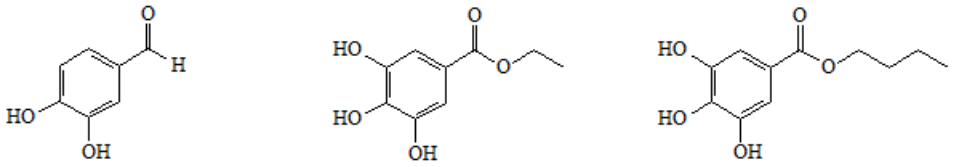


Fig. 1-3 Structures of 9 compounds determined from *Flos Chrysanthemi* by LC-3ECD.



Protocatechuic aldehyde (PAD) | Ethyl gallate (EG) | Butyl gallate (BG)

Fig. 1-4 Structures of 3 ISs for the determination of 9 compounds from *Flos Chrysanthemi* by LC-3ECD.

According to the polarities of 9 analytes and their elution orders from the Pre-C by mobile phase, the analytes of NCHA, CHA, CCHA, and CFA were arranged to CN_1 for detection. The compounds of LTG, ICHA B, and ICHA A were arranged to CN_2 for detection. And the compounds of ICHA C, and LT were arranged to CN_3 for detection. The whole analytical process of these analytes by the present LC-3ECD was as follows.

In the initial state of the LC-3ECD system, the flow way on SV_1 and SV_2 were at the initial positions as shown in Fig. 1-2A. MP_1 (acetonitrile-water-phosphoric acid (MeCN-H_2O-H_3PO_4 = 8.4:91.6:0.5, v/v/v)) flowed through the Pre-C (Capcell pak C18 UG-120, 35×1.0 mm, i.d., 3 μm), C_1 (Intertsustain C18, 250×1.0 mm, i.d., 3 μm), and D_1 in CN_1 at a 30 μl/min flow rate. Within 9 minutes after injecting the sample, NCHA, PAD, CHA, CCHA, and CFA were eluted from Pre-C to the C_1 by MP_1, whereas ICHA A, ICHA B, LTG, ICHA C, LT, EG, and BG were retained in the Pre-C. At 9 minutes after injecting the sample, the flow way on SV_1 was automatically changed as shown in Fig. 1-2B, whereas SV_2 was still at the initial position. MP_2 (MeCN-H_2O-H_3PO_4 (17.2:82.8:0.5, v/v/v)) flowed through Pre-C, C_2 (Develosil C30 XG-M-3, 250×1.0 mm, i.d., 3 μm), and D_2 in CN_2 at a 40 μl/min flow rate. Between 9 and 17 minutes after injecting the sample, LTG, EG, ICHA B, and ICHA A were eluted from Pre-C to the C_2 by MP_2, whereas ICHA C, BG and LT were still retained in the Pre-C. At 17 min after injecting the sample, SV_2 was automatically changed to be the flow way shown in Fig. 1-2C, whereas the position of SV_1 was the same as that shown in Fig. 1-2B. MP_3 (MeCN-H_2O-H_3PO_4 (22:78:0.5, v/v/v)) flowed through Pre-C, C_3 (Capcell pak C18 UG-120, 150×1.0 mm, i.d., 3 μm), and D_3 in CN_3 at a 45 μl/min flow rate. ICHA C, BG and LT retained in Pre-C were eluted from Pre-C to the C_3 by MP_3. Then, three groups of compounds were fully separated on C_1, C_2, and C_3 and detected in CN_1, CN_2, and CN_3, respectively. MP_1, MP_2, and MP_3 always flowed through D_1, D_2, and D_3, respectively, to keep the isocratic elution in each detection channel in the whole analytical process. Column temperature was set at 32 ℃. The applied potential was set at +0.6, +0.7, and +0.7 V *vs.* Ag/AgCl in D_1, D_2, and D_3, respectively.

1.2.2 Investigation and optimization of LC-3ECD system conditions for determining 9 redox compounds from *Flos Chrysanthemi*

Compared with a single channel LC system, in which one chromatographic column was

generally used, the present LC-3ECD system was much more complicated, which was composed of three channels containing one Pre-C and three separation columns (C_1, C_2, and C_3). In present LC-3ECD, the column-switching by changing the switching valves could make the different analytes flow into different separation columns from the Pre-C because the Pre-C was series-connected to the separation columns. However, the same analyte may be eluted into two separation columns from the Pre-C before and after changing the switching valves, and detected in two channels, simultaneously, owing to the unsuitable time for changing the switching valves. This situation would lead to the ineffective determination results. Furthmore, the chromatographic conditions in three channels were also the main factors for the development of the present LC-3ECD, such as column types, mobile phase composition, flow rates, applied potentials, *etc*. Therefore, these determinants should be investigated and optimized.

Firstly, the time for changing the two switching valves was investigated to make each analyte be eluted into a single channel, entirely. The compounds of NCHA, PAD, CHA, CCHA, and CFA eluted from pre-C by MP_1 were detected in D_1 within 9 min after sample injection using the present LC-3ECD but without C_1, whereas other compounds were retained in the Pre-C. Thus, the rotation of SV_1 was performed at 9 min. After changing the SV_1, MP_2 flowed through the Pre-C. The on-column concentration (frontal concentration) of LTG, EG, ICHA B, and ICHA A occurred on Pre-C by the elution of MP_2, and then they were eluted out pre-C by MP_2 and detected in D_2 between 9 min and 17.0 min after sample injection using the present LC-3ECD but without C_2, whereas ICHA C, BG and LT were still retained in the Pre-C. Thus, the rotation of SV_2 was performed at 17 min. After changing the SV_2, MP_3 flowed through the Pre-C. The on-column concentration of ICHA C, BG and LT occurred on Pre-C by the elution of MP_3, and then they were eluted into C_3 by MP_3 and detected in D_3 using the present LC-3ECD. Therefore, when the switching times of SV_1 and SV_2 were set at 9 min and 17 min after sample injection, respectively, three groups of these compounds can be eluted into CN_1, CN_2, and CN_3 by MP_1, MP_2, and MP_3, respectively. During these periods, on Pre-C, the step-wise on-column concentration was induced by MP_2 and MP_3.

Secondly, the chromatographic conditions including column types, mobile phase composition, flow rates, and column temperature were optimized to achieve good separation of each compound within a short period of the analytical time. The column of Capcell pak C18 UG-120 (35×1.0 mm, i.d., 3 μm) was selected as the Pre-C, on which all compounds could be easily eluted to the respective detection channel by respective mobile phase to save analytical time. Meanwhile, three groups of the compounds, which would be eluted into the different detection channels, were completely separated on the Pre-C. Three columns of Intertsustain C18 (250×1.0 mm, i.d., 3 μm), Develosil C30 XG-M-3 (250×1.0 mm, i.d., 3 μm) and Capcell pak C18 UG-120 (150×1.0 mm, i.d., 3 μm) as C_1, C_2 and C_3, respectively, were found to be the best selections for the separation of the analytes. The separation effects of the analytes varied greatly among the different column types. It depends on not only the column length and inner diameter, but also the bonded group, particle size, surface area, pore size, pore volume, carbon contents, *etc*. All column types used in this study were investigated and

these columns' characteristic parameters were found from the column manufacturers, as shown in Table 1-1. For the mobile phase composition, using methanol (MeOH) as an organic phase led to the poor separation of the compounds and high pressure of the system. By replacing MeOH with MeCN, lower system pressure and better separation were achieved. The pH value of the mobile phase is also an important factor in LC-ECD, which affects not only the peak shape, but also the redox reaction occurring in the electrochemical cell [7]. The H_3PO_4 content in each mobile phase was investigated. The peak shapes of caffeoylquinic acids became satisfactory while the H_3PO_4 content was up to 0.5% (v/v). The flow rates of MP_1, MP_2, and MP_3 were set to 30, 40, and 45 μl/min, respectively, thereby avoiding high system pressure in each detection channel. Based on the good separation of each compound, increasing column temperature reduced the column pressure and shortened the analytical time. The investigation showed that the appropriate choice for the column temperature was 32 ℃.

Thirdly, an optimal applied potential in each detection channel was investigated for the detection of 9 analytes, ranging from +0.4 to +1.0 V *vs.* Ag/AgCl. Nine analytes of caffeoylquinic acids and flavonoids have the same structure of *ortho*-dihydroxybenzene, which could be converted to *ortho*-quinone easily by losing two electrons in an electrochemical oxidation reaction [4, 5]. The results showed that 9 analytes were oxidized at potentials of more positive than +0.5 V *vs.* Ag/AgCl. The peak current heights of NCHA, CHA, CCHA, and CFA stayed at a plateau as the potentials applied in D_1 were in the range of +0.6 V to +1.0 V *vs.* Ag/AgCl. In D_2 and D_3, the peak current heights of LTG, ICHA B, ICHA A, ICHA C, and LT reached a plateau at potentials of more than +0.7 V *vs.* Ag/AgCl. Considering the highly sensitive determination without loss of selectivity and reproducibility, +0.6 V *vs.* Ag/AgCl applied in D_1, and +0.7 V *vs.* Ag/AgCl applied in D_2 and D_3 were adopted, respectively, in this study.

Table 1-1 Characteristic parameter of analytical columns for LC-3ECD studied

Particle packed columns	Base	Bonded group	Particle size	Pore size	Carbon contents	Surface area	Pore volume	End-capping
Capcell pak C18 UG-120	Spherical silica gel	Octadecyl group	3 μm	12 nm	15%	300 m^2/g	1.00 ml/g	Complete
Inertsustain C18	Spherical silica gel	Octadecyl group	3 μm	10 nm	14%	350 m^2/g	0.85 ml/g	Complete
Develosil C30 XG-M-3	Spherical silica gel	Triacontyl group	3 μm	14 nm	19.5%	300 m^2/g	1.10 ml/g	Complete
Develosil C30 UG3	Spherical silica gel	Triacontyl group	3 μm	14 nm	18%	300 m^2/g	1.05 ml/g	Complete

Monolith column	Base	Bonded group	Macropore		Mesopore		Carbon contents	Surface area	Pore volume	End-capping
			Size	Total porosity	Size	Total porosity				
MonoBis C18	Monolithic silica rod	Octadecyl group	1.4 μm	65%	11 nm	10%	19%	360 m^2/g	3.0 ml/g	Complete

1.2.3 Validation of LC-3ECD by standard substances for the determination of 9 compounds from *Flos Chrysanthemi*

Under the optimized LC-3ECD system conditions, the chromatogram of the mixed standard solution with the concentrations of 80 ng/ml for 9 analytes, 10 ng/ml for PAD (IS_1) and EG (IS_2), and 25 ng/ml for BG (IS_3) was obtained by the present LC-3ECD as shown in Fig. 1-5. The peaks of NCHA, PAD (IS_1), CHA, CCHA and CFA appeared at 24.7, 32.4, 54.2, 56.9, and 60.4 min in D_1, respectively. The peaks of EG (IS_2), LTG, ICHA B and ICHA A appeared at 26.4, 53.5, 60.2, and 70.2 min in D_2, respectively. The peaks of ICHA C, BG (IS_3), and LT appeared at 27.8, 44.4, and 47.2 min in D_3, respectively. The analytical time was within 80 min for the analytes. The R_s between CHA and CCHA was greater than 1.80. The R_s between CCHA and CFA was greater than 2.0. The tailing factors of all analytes were in the range of 0.95 to 1.05, so the peak height quantification made sense. These results exhibit good separation and desirable analytical time for determining these 9 compounds from *Flos Chrysanthemi*.

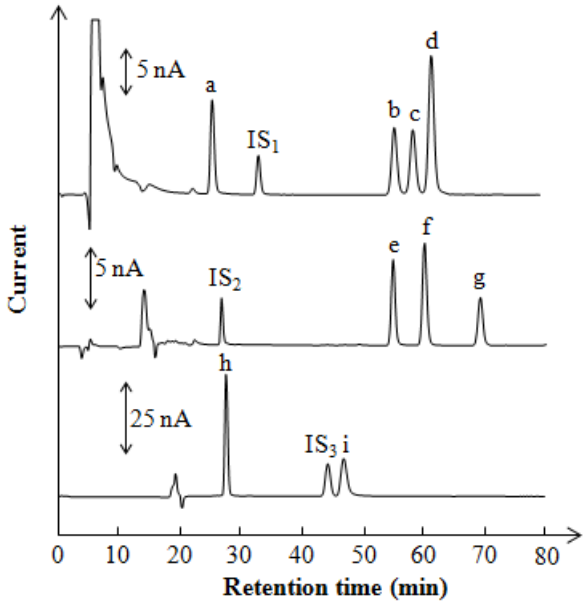


Fig. 1-5 Chromatogram of the standard mixture for the analyses of *Flos Chrysanthemi*. 80 ng/ml for NCHA (a), CHA (b); CCHA (c), CFA (d); LTG (e), ICHA B (f), ICHA A (g), ICHA C (h), and LT (i); 10 ng/ml for PAD (IS_1) and EG (IS_2); 25 ng/ml for BG (IS_3).

A series of mixed standard solutions of 9 concentration levels (2.5, 5.0, 7.5, 10, 25, 50, 75, 100, and 250 ng/ml for each analyte) with constant concentrations of 3 ISs (10, 10, and 25 ng/ml for PAD, EG, and BG, respectively) were freshly prepared, and then were analyzed by the present LC-3ECD to determine the linearity and linear range of the analytes. Linearity was assessed by means of linear regression with respect to the concentrations and peak height ratios of the analytes to ISs. The peak height ratios of the analytes to ISs at the different concentrations were subjected to regression analyses to establish regression equations and correlation coefficients (r). The results were shown in Table 1-2. The calibration curves were

linear over the ranges from 2.5 to 100 ng/ml for NCHA, CHA, CCHA, and CFA, and 2.5 to 250 ng/ml for LTG, ICHA B, ICHA A, ICHA C, and LT, respectively. All correlation coefficients (*r*) were greater than 0.999. It shows good linearity and wide linear range by the present LC-3ECD.

The limits of detection (LODs) were determined by diluting the mixed standard solution to calculate the signal-to-noise ratio (S/N) of 3:1. As shown in Table 1-2, the LODs of 9 analytes were ranging from 0.17 to 0.56 ng/ml. It shows the high sensitivity of each analyte by the present LC-3ECD.

Table 1-2 Linearity and LOD of 9 compounds for the analyses of *Flos Chrysanthemi* by LC-3ECD

Analyte	Regression equation [a]	*r*	Linear range (ng/ml)	LOD (ng/ml)
NCHA	$y = 30.4\,x + 4.35\times10^{-2}$	(0.999)[b]	2.5 - 100	0.29
CHA	$y = 22.1\,x + 1.40\times10^{-2}$	(0.999)[b]	2.5 - 100	0.40
CCHA	$y = 21.4\,x + 1.04\times10^{-2}$	(0.999)[b]	2.5 - 100	0.42
CFA	$y = 46.6\,x - 1.20\times10^{-3}$	(0.999)[b]	2.5 - 100	0.23
LTG	$y = 25.9\,x - 3.83\times10^{-2}$	(0.999)[c]	2.5 - 250	0.36
ICHA B	$y = 30.5\,x - 2.42\times10^{-2}$	(0.999)[c]	2.5 - 250	0.27
ICHA A	$y = 14.1\,x - 9.50\times10^{-3}$	(0.999)[c]	2.5 - 250	0.56
ICHA C	$y = 49.8\,x + 4.15\times10^{-2}$	(0.999)[c]	2.5 - 250	0.17
LT	$y = 16.9\,x - 4.88\times10^{-2}$	(0.999)[c]	2.5 - 250	0.52

[a] *x*, concentration of standard solution (ng/ml); *y*, mean peak height ratio of analyte to IS.
[b] $n = 8$ [c] $n = 9$

The precision of the present LC-3ECD for determining 9 compounds from *Flos Chrysanthemi* was assessed by six successive injections of the mixed standard solutions with a low concentration (6.0 ng/ml for 9 analytes, 10 ng/ml for IS_1 and EG IS_2, and 25 ng/ml for IS_3) and a high concentration (80 ng/ml for 9 analytes, 10 ng/ml for IS_1 and EG IS_2, and 25 ng/ml for IS_3). The RSDs (n = 6) of each analyte with low concentration and high concentration ranged from 0.83% to 2.4% and from 0.66% to 1.7%, respectively. It indicates the good precision of the present LC-3ECD.

In summary, the desirable separation, wide linear range and high sensitivity of these 9 compounds were obtained by the present LC-3ECD method. It also showed good precision of the present LC-3ECD. Therefore, the present LC-3ECD method for determining 9 compounds from *Flos Chrysanthemi* was validated by the standard mixtures, successfully.

1.3 The LC-3ECD method for the determination of 11 redox compounds from SHL preparations

1.3.1 Analytes and LC-3ECD chromatographic conditions for SHL preparations

The main bioactive components in SHL preparations were caffeoylquinic acids, flavonoids and phenylethanoid glycosides. In this study, 6 caffeoylquinic acids of NCHA, CHA, CCHA, CFA, ICHA B, ICHA C (Fig. 1-3), 4 flavonoids of scutellarin (STL), baicalin (BC), wogonoside (WGD), and baicalein (BCE), and a representative phenylethanoid glycoside of Forsythoside A (FTA) were determined from SHL preparations by LC-3ECD, simultaneously. The structures of 4 flavonoids (STL, BC, BCE, and WGD) and 1 phenylethanoid glycoside (FTA) were shown in Fig. 1-6.

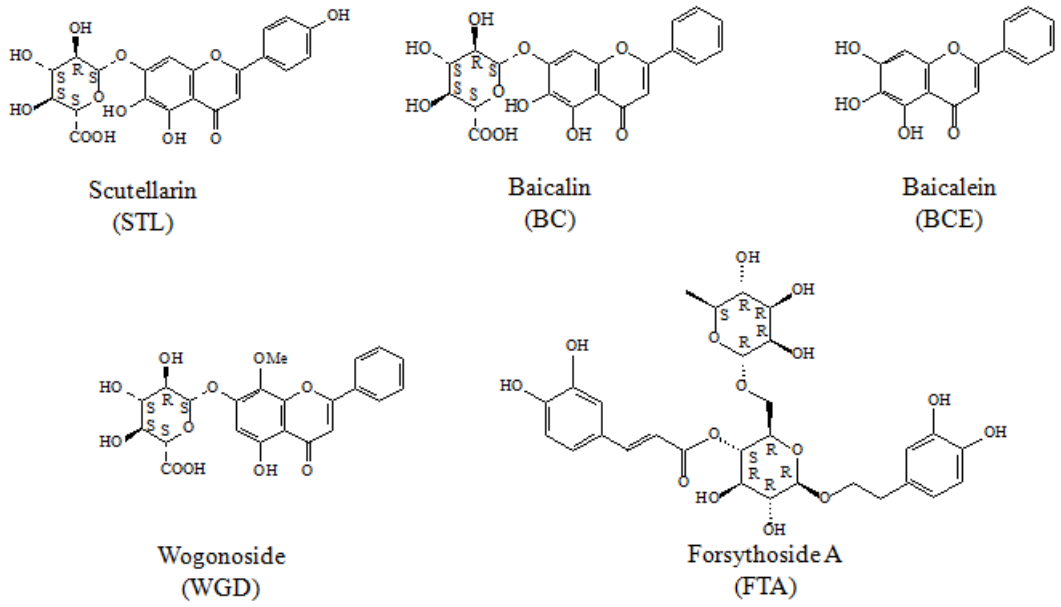


Fig 1-6 Structures of flavonoids and phenylethanoid glycosides determined from SHL preparations by LC-3ECD.

The LC-3ECD system for the analyses of SHL preparations was essentially the same as that for the analyses of *Flos Chrysanthemi* as shown in Fig. 1-2. However, the analytes in each detection channel and the chromatographic conditions including columns, flow rates, mobile phases, and switching time for the valves were obviously different. Eleven analytes were divided into three groups. NCHA, CHA, CCHA, and CFA were in Group Ⅰ. FTA, STL, and ICHA B were in Group Ⅱ. ICHA C, BC, WGD, and BCE were in Group Ⅲ. The analytes in Group Ⅰ, Group Ⅱ, and Group Ⅲ were arranged to be detected in CN_1, CN_2, and CN_3, respectively.

As shown in Fig. 1-2A, The MP_1 flowed through pre-C, C_1, and D_1 in CN_1. All analytes were eluted into pre-C by MP_1 after sample injection. The analytes in Group Ⅰ (NCHA, CHA, CCHA, and CFA) were eluted into C_1 by MP_1 in CN_1 within 8 min after sample injection,

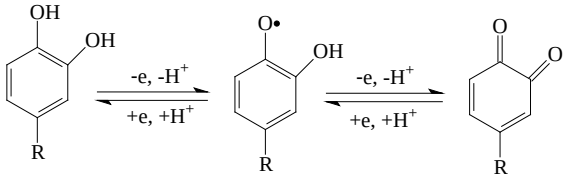
whereas other analytes were retained in pre-C. At 8 min after sample injection, SV_1 was automatically changed as shown in Fig. 1-2B, whereas SV_2 kept the same position as shown in Fig. 1-2A. The MP_2 flowed through pre-C, C_2, and D_2 in CN_2. The analytes in Group Ⅱ (FTA, STL, and ICHA B) were eluted into C_2 from pre-C by MP_2 in CN_2 between 8 min and 15 min after sample injection, whereas other analytes were still retained in pre-C. At 15 min after sample injection, SV_2 was automatically changed to be the flow way shown in Fig. 1-2C, whereas the position of SV_1 was the same as that shown in Fig. 1-2B. The MP_3 flowed through pre-C, C_3, and D_3 in CN_3. The analytes in Group Ⅲ (ICHA C, BC, WGD, and BCE) retained in pre-C were eluted into C_3 by MP_3 in CN_3. Then, three groups of analytes were fully separated on C_1, C_2, and C_3 and detected in CN_1, CN_2, and CN_3, respectively, within 70 min.

Columns of Capcell pak C18 UG-120 (35×1.0 mm, i.d., 3 μm), Intertsustain C18 (250×1.0 mm, i.d., 3 μm), Develosil C30 UG3 (250×1.0 mm, i.d., 3 μm), and MonoBis C18 (150×1.0 mm) were used as pre-C, C_1, C_2, and C_3, respectively. The mobile phases of MP_1, MP_2, and MP_3 were MeCN-H_2O-H_3PO_4 (8.6:91.4:0.5, v/v/v), MeCN-H_2O-H_3PO_4 (17.6:82.4:0.5, v/v/v), and MeCN-H_2O-H_3PO_4 (24:76:0.5, v/v/v), respectively. The flow rates of the mobile phase in CN_1, CN_2, and CN_3 were 30, 50, and 45 μl/min, respectively. The applied potentials in D_1, D_2, and D_3 were set at +0.6, +0.6, and +0.9 V *vs.* Ag/AgCl, respectively. The temperature of the column oven was kept at 32 ℃ and the sample injection volume was 5 μl.

1.3.2 Hydrodynamic voltammograms of 11 analytes

The appropriate selection of the applied potential was critical for accurate measurement as the lower potential would affect the peak current height and the higher potential would cause higher baseline noise [7, 59]. Therefore, the hydrodynamic voltammograms of these 11 analytes were investigated by a mixed standard solution with the concentrations of 0.04 μg/ml for NCHA, CHA, CCHA, FTA, STL, ICHA B, and ICHA C, 0.02 μg/ml for CFA and BCE, and 0.1 μg/ml for BC and WGD. The peak current heights of each analyte applied to different potentials were shown in Fig. 1-7. As shown in Fig. 1-7A, the peak current heights of NCHA, CHA, CCHA, and CFA in Group Ⅰ were raised with the increase of the applied potential. When the potential was more than +0.6 V *vs.* Ag/AgCl, the peak current heights stayed at a plateau. So the potential applied in the D_1 was set at +0.6 V *vs.* Ag/AgCl. The same results existed in the standard compounds of FTA, STL and ICHA B in Group Ⅱ (Fig. 1-7B) and the potential applied to D_2 was also set at +0.6 V *vs.* Ag/AgCl. In the D_3, the higher peak current height of WGD was obtained (Fig. 1-7C) when the potential was set at +0.90 V vs. Ag/AgCl. So the potential applied in the D_3 was set at +0.9 V *vs.* Ag/AgCl.

As shown in Fig. 1-3, the analytes of NCHA, CHA, CCHA, CFA, ICHA B, and ICHA C have the structures of *ortho*-dihydroxybenzene. The analytes of STL, BC, BCE, and FTA also have two phenolic hydroxy groups in the *ortho*-position as shown in Fig. 1-6. The mechanism of oxidation of these compounds in an electrochemical redox reaction was as follows [4-6]:



Thus, the oxidation reactions of these compounds proceeded easily and the low applied potentials were required. Oppositely, the compound of WGD, which has only one phenolic hydroxy group, was hard to be oxidized, and high potentials need to be applied. That is why the potential applied to D_3 was higher than that of D_1 and D_2.

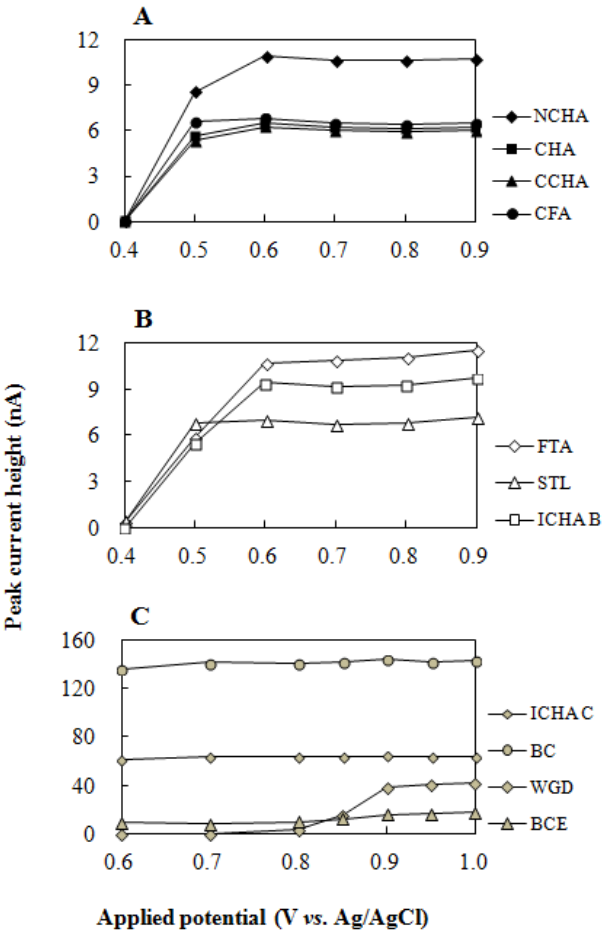


Fig. 1-7 Hydrodynamic voltammograms of 11 analytes in D_1 (A), D_2(B), and D_3 (C).

1.3.3 Validation of LC-3ECD by standard substances for the determination of 11 compounds from SHL preparations

The linearity of the present LC-3ECD was established by an IS method. The compounds of PAD, propyl gallate (PG), and kaempfeol (KF) were selected as IS_1, IS_2, and IS_3 in CN_1, CN_2, and CN_3, respectively, due to their good stabilities, R_s, and peak current values. The structures of these 3 ISs were shown in Fig. 1-8.

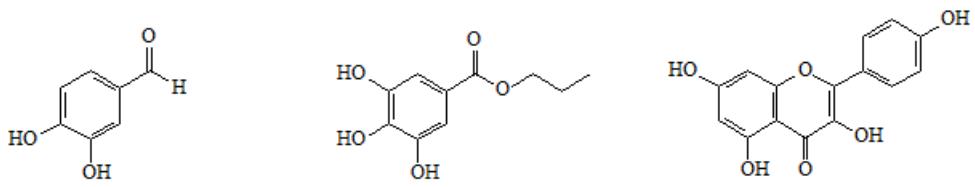


Fig. 1-8 Structures of 3 ISs for the determination of 11 compounds from SHL preparation by LC-3ECD.

The linearity and linear range were investigated by using mixed standard solutions of 11 concentration levels (0.002, 0.004, 0.006, 0.008, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10, and 0.20 μg/ml for NCHA, CHA, CCHA, CFA, FTA, STL, ICHA B, ICHA C, WGD, and BCE, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 2.0 μg/ml for BC) with constant concentrations of 3 ISs (0.05, 0.02, and 0.08 μg/ml for PAD, PG, and KF, respectively). The representative chromatogram of standard mixture was shown in Fig. 1-9. The good separations of each compound and satisfying analytical time were exhibited under the present system conditions by LC-3ECD.

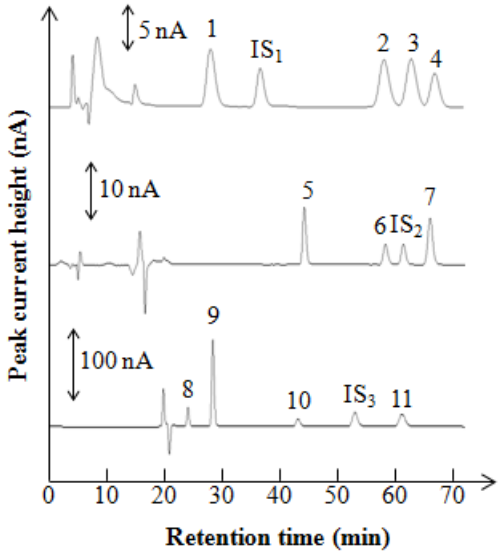


Fig 1-9 Chromatogram of standard mixture for the analyses of SHL preparations by LC-3ECD. Peaks: 1, NCHA; 2, CHA; 3, CCHA; 4, CFA; 5, FTA; 6, STL; 7, ICHA B; 8, ICHA C; 9, BC; 10, WGD; 11, BCE; IS_1, PAD; IS_2, PG; IS_3, KF.

Linearity was assessed by means of linear regression with respect to the concentrations and peak height ratios of the analytes to ISs. Calibration curves were prepared by plotting the peak height ratios against the corresponding concentrations. The results were shown in Table 1-3 and Fig. 1-10. A linearity correlation (r >0.999) was found between the peak height ratios and the concentrations of each analyte in the assayed range, individually. Good linearity and wide linear range of these analytes were obtained by LC-3ECD.

Table 1-3 Linearity and LOD of 11 compounds for the analyses of SHL preparations by LC-3ECD

Analyte	Regression equation [a]	*r*	Linear range (μg/ml)
NCHA	$y = 19.5\,x - 6.90\times10^{-3}$	(0.999)[b]	0.002 - 0.20
CHA	$y = 13.4\,x - 2.60\times10^{-3}$	(0.999)[b]	0.002 - 0.20
CCHA	$y = 11.3\,x - 2.50\times10^{-3}$	(0.999)[b]	0.002 - 0.20
CFA	$y = 25.4\,x - 1.13\times10^{-2}$	(0.999)[b]	0.002 - 0.20
FTA	$y = 37.8\,x - 2.46\times10^{-2}$	(0.999)[c]	0.004 - 0.20
STL	$y = 19.5\,x - 2.68\times10^{-2}$	(0.999)[c]	0.004 - 0.20
ICHC B	$y = 27.1\,x - 2.06\times10^{-2}$	(0.999)[c]	0.004 - 0.20
ICHA C	$y = 37.8\,x + 4.75\times10^{-2}$	(0.999)[c]	0.004 - 0.20
BC	$y = 17.5\,x - 4.22\times10^{-2}$	(0.999)[d]	0.040 - 1.00
WGD	$y = 10.7\,x + 2.80\times10^{-3}$	(0.999)[d]	0.004 - 0.10
BCE	$y = 23.4\,x + 2.26\times10^{-2}$	(0.999)[d]	0.004 - 0.10

[a] *x*, concentration of standard solution (μg/ml); *y*, mean peak height ratio of analyte to IS.
[b] $n = 11$; [c] $n = 10$; [d] $n = 9$.

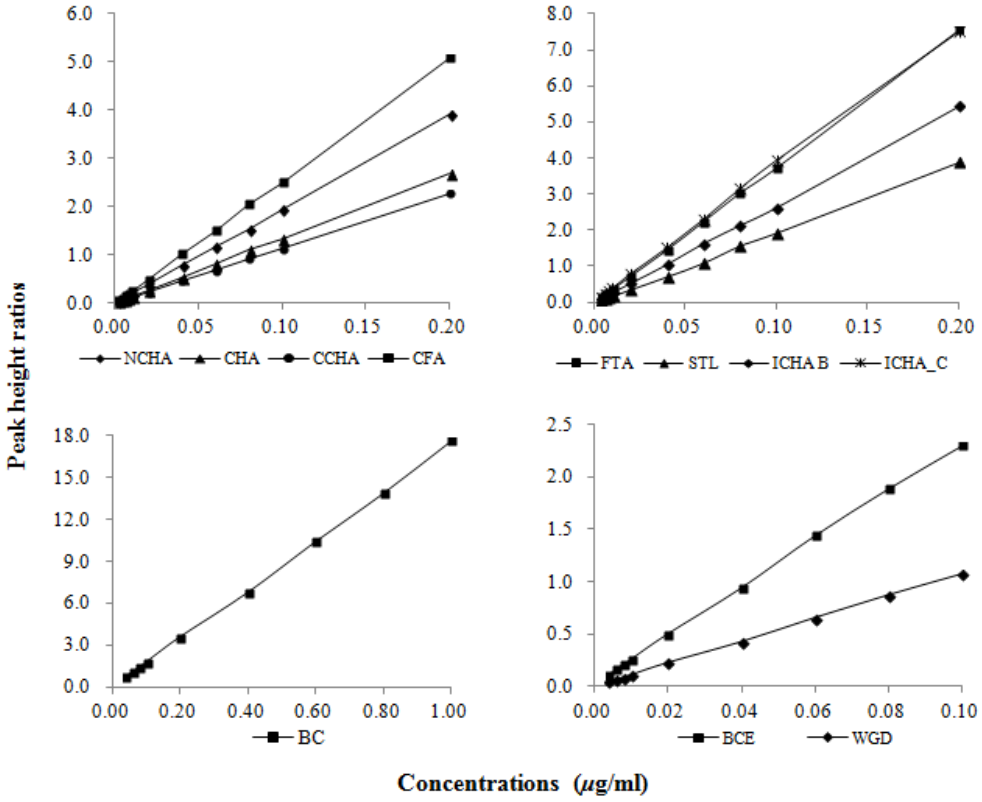


Fig. 1-10 Calibration curves of 11 compounds for the analyses of SHL preparations by LC-3ECD.

The precision of the present LC-3ECD was assessed by six successive injections of the mixed standard solution (0.06 μg/ml for NCHA, CHA, CCHA, and WGD, 0.04 μg/ml for FTA, ICHA B, ICHA C, and BCE, 0.40 μg/ml for BC, and 0.01 μg/ml for CFA and STL). The RSDs (n = 6) of the peak height ratios of each analyte in mixed standard solutions ranged from 0.63% to 2.5% (Table 1-4). It indicates the good precision of the present LC-3ECD.

Table 1-4 The RSD of the peak height ratios of each analyte for precision (n = 6)

Analyte	Con. (μg/ml)	$H_{standard}/H_{IS}$						RSD/%
		1	2	3	4	5	6	
NCHA	0.06	2.222	2.286	2.276	2.260	2.255	2.199	**1.5**
CHA	0.06	1.500	1.570	1.536	1.562	1.538	1.500	**1.9**
CCHA	0.06	1.305	1.372	1.342	1.368	1.345	1.320	**2.0**
CFA	0.01	0.500	0.532	0.519	0.535	0.524	0.513	**2.5**
FTA	0.04	2.890	2.941	2.956	2.896	2.879	2.914	**1.0**
STL	0.01	0.326	0.317	0.322	0.317	0.310	0.325	**1.9**
ICHA B	0.04	2.065	2.153	2.122	2.104	2.065	2.104	**1.6**
ICHA C	0.04	1.211	1.209	1.213	1.195	1.199	1.214	**0.66**
BC	0.40	6.799	6.875	6.858	6.741	6.747	6.822	**0.82**
WGD	0.06	0.691	0.690	0.688	0.680	0.684	0.690	**0.63**
BCE	0.04	1.034	1.041	1.019	1.019	1.012	1.039	**1.2**

The LODs of 11 analytes were determined by calculating the S/N of 3:1. The LODs of NCHA, CHA, CCHA, CFA, FTA, STL, ICHA B, ICHA C, BC, WGD, and BCE were 0.22, 0.33, 0.39, 0.17, 0.48, 0.90, 0.69, 0.46, 0.11, 0.53, and 0.67 ng/ml, respectively. The LODs of these compounds by other reported analytical methods were shown in Table 1-5. The LODs of analytes by the present LC-3ECD were two orders of magnitude lower than that of LC-DAD and approximate to that of LC-MS. Moreover, the sensitivity of the isocratic LC-ECD was remarkably higher than that of the gradient LC-ECD. In an ECD, the structure of the electrical double layer on a working electrode in an electrochemical flow cell was largely affected by solvent molecules and supporting electrolytes, as well as the applied potential [59]. The unstable electrical double layer in the gradient LC-ECD caused by the change of the mobile phase composition increased the baseline noise and reduced the sensitivity. In the present LC-3ECD, a stable electrical double layer was maintained by an isocratic mobile phase that flowed through each electrochemical flow cell and seemed to be the cause for the relatively stable baseline and high sensitivity.

In summary, the desirable separation, wide linear range, and high sensitivity of these 11 compounds, as well as the good precision, were obtained by the present LC-3ECD method. Thus, the present LC-3ECD method for determining 11 compounds from SHL preparations has been validated by the standard substances.

Table 1-5 Comparison of LOD of 11 compounds studied by various analytical methods

Method	Elution	Detection limit (ng/ml, *S/N* = 3)										
		NCHA	CHA	CCHA	CFA	FTA	STL	ICHA B	ICHA C	BC	WGD	BCE
LC-DAD [53]	Gradient	-	6	-	9	30	6	-	-	40	-	3
LC-DAD [55]	Gradient	27	33	31	22	57	43	29	32	29	1	-
LC-MS [67]	Gradient	-	1.1	-	-	0.5	-	-	-	0.5	-	-
LC-MS [68]	Gradient	-	0.3	-	-	-	-	-	-	0.3	-	-
LC-MS [69]	Gradient	-	-	-	-	-	-	-	-	0.8	-	10.4
LC-DAD-ECD [58]	Gradient	-	1000 [a]	-	200 [a]	-	-	-	-	9000 [b]	-	-
LC-ECD [70]	Isocratic	-	-	-	-	-	-	-	-	1.78	-	1.08
LC-3ECD (Present method)	Isocratic	0.22	0.33	0.39	0.17	0.48	0.90	0.69	0.46	0.11	0.53	0.67

[a] The analytes were detected by ECD.
[b] The analyte was detected by DAD.

Chapter 2 System repeatability estimation in quantitative HPLC-UV and LC-3ECD for herbal medicines by ISO 11843-7

As many components act as active compounds and/or matrixes exist in herbal medicines, an HPLC method is attractive to perform a separated determination of various components for the quantitative evaluation of herbal medicines [71-75]. In order to verify the "System suitability" of the HPLC for the accurate determination of these compounds, the precision, expressed as SD and/or RSD of the peak area or peak height, must be estimated by six repetitive measurement of the test solution as criteria for "System repeatability", which has been recorded into the pharmacopoeia of each country, such as Japanese, United State, Europe, British, and Chinese [9, 62-65]. In a quantitative HPLC for herbal medicines, it usually takes a long analytical time to obtain a chromatogram, because the samples for analyses often contain various compounds of different properties including late eluting components [76]. An analyst or researcher would spend a lot of time, effort, and chemicals to estimate system repeatability by a statistical way of repetitive measurement.

A new methodology called FUMI theory, which has been published in ISO 11843-7 in 2012, has been developed to estimate the RSD for the system repeatability from stochastic properties of signal and noise in a chromatogram without repetitive measurement. Furthermore, the limit of detection and quantification can also be calculated directly from the measurement RSD by ISO 11843-7. In the earlier studies, some analysts tried to use this methodology to estimate the precision in HPLC-UV/DAD [77, 78], atomic absorption spectrometry (AAS) [79], atomic fluorescence spectrometry (AFS) [80], GC-MS [80], and CE [81]. The prominent advantage of this methodology is that with a chromatogram by a single injection rather than repetitive measurement, the measurement RSD could be estimated. It means the less consume of chemicals, energy, and experimental time. Although ISO 11843-7 is recently applied to examine detection limits and estimate the precision in quantitative HPLC for synthetic drugs [82], the other application of ISO 11843-7 has hardly been reported to estimate "System repeatability" in quantitative HPLC for herbal medicines.

In chapter 1, the novel LC-3ECD system has been developed by the new design of channel connections, in which, each detection channel kept the isocratic elution in the whole analytical process. However, the mobile phases flowed from the pre-column were alternately changed when the two SVs were rotated. The residual mobile phase in pre-column with different compositions which flowed into another detection channel would slightly affect the respective electrical double layer on the working electrode. The characteristic features of the chromatographic baseline noise would differ from that of the absolute isocratic LC-ECD, which only consists of one-way flow. Therefore, the system repeatability of the present LC-3ECD is especially important, which should be evaluated, comprehensively.

In this chapter, an HPLC-UV for determining baicalin from *Scutellaria Radix* was taken as an example to verify the applicability of ISO 11843-7 for the system repeatability of quantitative HPLC for herbal medicines, firstly. Then, ISO 11843-7 was examined whether it

can estimate the system repeatability of the present LC-3ECD for determining 7 caffeoylquinic acids (NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, and ICHA C) and 2 flavonoids (LTG and LT) from *Chrysanthemi Flos*.

2.1 Scope and principle of ISO11843-7 for the system repeatability estimation

ISO 11843-7, titled as "Methodology based on stochastic properties of instrumental noise" , is concerned with mathematical methodologies for estimating the SD and the minimum detectable value in case that the most predominant source of measurement uncertainty is background noise. The SD and minimum detectable value can directly and mathematically be derived from the stochastic characteristics of the background noise. It involves with basic methods to extract the stochastic properties of the background noise, use the stochastic properties to estimate the SD of the response variable, and calculate the minimum detectable value based on the SD obtained above. The methods described in ISO 11843-7 are useful for checking the detection of a certain substance by various types of measurement equipment such as HPLC-UV, AAS, AFS, and GC-MS, in which the background noise of the instrumental output predominates over the other sources of measurement uncertainty.

According to ISO 11843-7, the chromatographic background noise, often formulated as 1/*f* noise, was approximated by a mixed stochastic process comprised of white noise and Markov process. The white noise is a random process with a horizontal power spectrum, whereas the Markov process has a monotonously decreasing power spectrum. In the ISO 11843-7, the SD of white noise ($\tilde{w}$), and the SD of the Markov process ($\tilde{m}$), and the retention parameter (ρ) of the Markov process are utilized as noise parameters. $\tilde{m}$ means the SD of the white noise included in the Markov process, often called the driving force of the process. ρ denotes the extent to which the random path retains the last position of it. If $\rho = 0$, the Markov process is the same as the white noise. If $\rho = 1$, it is called the Brownian motion.

The noise parameters of $\tilde{w}$ $\tilde{m}$, and ρ were determined by least squared fitting of the model power spectrum of the white noise and Markov process to the real power spectrum of chromatographic baseline noise in HPLC, by the following equation (*Eq.* 1) [83]:

$$P(f) = \frac{\tilde{m}^2}{1-\rho^2} \times \frac{2\alpha}{\alpha^2 + 4\pi^2 f^2} + \tilde{w}^2 \qquad (1)$$

Where *f* is the frequency, $\alpha = (1 - \rho)/\Delta t$, and Δt is the sampling interval. Thus, the power spectrum derived from the baseline noise in frequency space is utilized for the parameterization of $\tilde{w}$, $\tilde{m}$, and ρ. A measurement RSD of chromatographic signal of analyte, the expression of precision/ system repeatability, which has a peak area (*A*) and width (*i.e.*, integration domain, k_f), is obtained based on the noise parameters of $\tilde{w}$, $\tilde{m}$, and ρ, respectively, by the following equation (*Eq.* 2) [83]:

$$\mathrm{RSD}^2 = \frac{k_f \tilde{w}^2}{A^2} + \frac{\tilde{m}^2}{(1-\rho)^2 A^2}\left(k_f - 2\rho\frac{1-\rho^{k_f}}{1-\rho} + \rho^2\frac{1-\rho^{2k_f}}{1-\rho^2}\right) + I^2 \qquad (2)$$

Where I is the RSD of the injection volume error of the sample injector. While using peak height (H) for determination, A was substituted by H.

2.2 System repeatability estimation of quantitative HPLC-UV by ISO 11843-7

2.2.1 HPLC-UV for determining baicalin from *Scutellaria Radix*

The instrument for HPLC-UV analysis was composed of a pump (L-2130, Hitachi, Tokyo), a UV detector (L-2400, Hitachi), an auto sample injector (L-2200, Hitachi), an ODS column (Mightysil RP-18GP, 150×4.6 mm, 5 μm, Kanto Chemical Co., Inc.), a column oven (CTO-10ASvp, Shimadzu, Kyoto, Japan), and a recorder (SS420X, Scientific Software, Inc., California, USA). The chromatographic conditions for determining baicalin from *Scutellaria Radix* were as according to the Japanese Pharmacopoeia 16th edition (JP 16). An MeCN-H_2O-H_3PO_4 (35:90:0.6, v/v/v) mixture was used as a mobile phase. The flow rate of the mobile phase, column temperature, and detection wavelength were set at 0.8 ml/min, 50 ℃, and 277 nm, respectively.

2.2.2 Precision profile of baicalin in quantitative HPLC-UV by ISO 11843-7

As shown in Fig. 2-1, a chromatogram of baicalin in standard solution (80 ng/ml) shows that the chromatographic peak of baicalin was obtained at 6.5 min. The peak and baseline noises on the chromatogram were analyzed to obtain measurement RSD of chromatographic peak area in HPLC-UV for determining baicalin by ISO 11843-7, which provides a measurement SD and RSD of chromatographic peak from stochastic aspects of the signal and noise in a chromatogram.

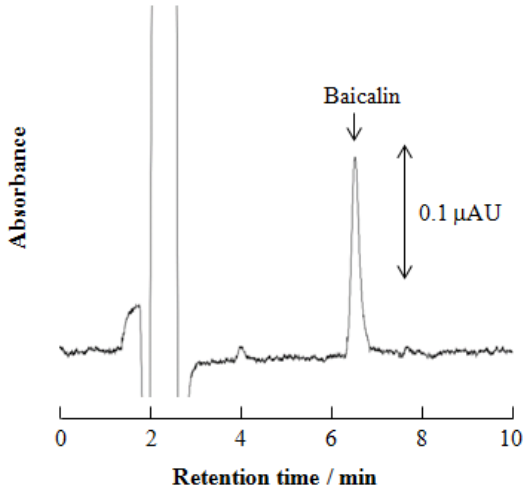


Fig. 2-1 Chromatogram of standard solution of 80 ng/ml baicalin by HPLC-UV.

A real power spectrum was obtained from consecutive 512 digital data points of the chromatographic baseline (Fig. 2-1) between 8.0 and 9.7 min (0.2 s/point, 512 point) by Fourier transform as shown in Fig. 2-2 (zigzag line). The noise parameters of $\tilde{w}$, $\tilde{m}$, and ρ in the chromatographic baseline noise were determined by the least squares fitting of *Eq.* 1 to real power spectrum, respectively. The fit of the least squares between the model power spectrum (Fig. 2-2, smooth line) and real power spectrum (Fig. 2-2, zigzag line) from the chromatographic baseline noise in HPLC-UV was satisfactory, indicating the prerequisite for a successful use of ISO 11843-7. By the least squared fitting, the noise parameters were determined as $\tilde{w}$ =0.837, $\tilde{m}$ =0.370, and ρ =0.935, respectively. By reference to the specification sheet of auto sample injector studied, 0.12% RSD at 10 μl in HPLC-UV for determining baicalin was substituted for *I*. Three noise parameters, *A*, k_f, and *I* were substituted for *Eq.* 2, and therefore a precision profile of baicalin over a wide concentration range was obtained as shown in Fig. 2-3 (solid line).

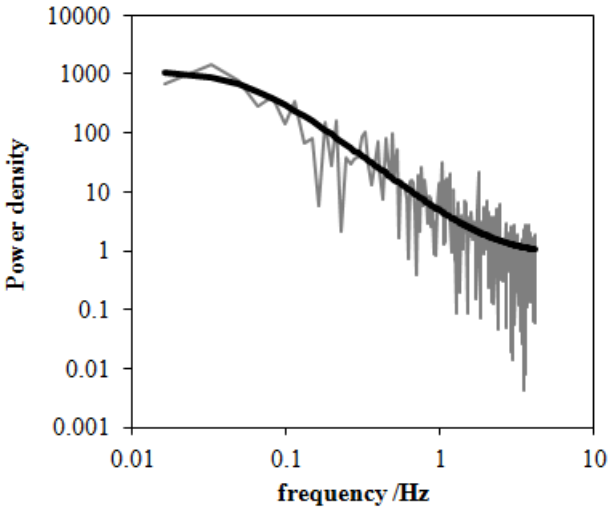


Fig. 2-2 Power spectrum converted from the baseline noise by Fourier transform in HPLC-UV. The smooth line means the best fit of the model power spectrum. The zigzag line means the real power spectrum.

In order to verify whether ISO 11843-7 can be applied to obtain measurement precision in the present HPLC-UV, the measurement RSDs by ISO 11843-7 were compared with the experimentally observed RSDs by repetitive measurement in the statistical ways. 95% confidence intervals of the statistically obtained SDs (n = 5, 6, and 7) are ±0.70%, ±0.60%, and ±0.55%, respectively, against population standard deviation. The repetition number (n) of chromatographic experiments was fixed here at n = 6 considering the analytical time and statistical reliability of SD estimates. The experimentally observed RSDs at 16, 40, 80, and 160 ng/ml baicalin were 8.4%, 5.9%, 2.0%, and 0.53% (each n=6), respectively. As shown in Fig. 2-3, the measurement RSD by ISO 11843-7 was within the 95% confidence interval of the statistically obtained RSD at 16, 40, 80, and 160 ng/ml baicalin (each n=6). Thus, it was

found that the measurement RSD by ISO 11843-7 is applicable to estimate the system repeatability in HPLC-UV for determining baicalin without repetitive measurement of real samples.

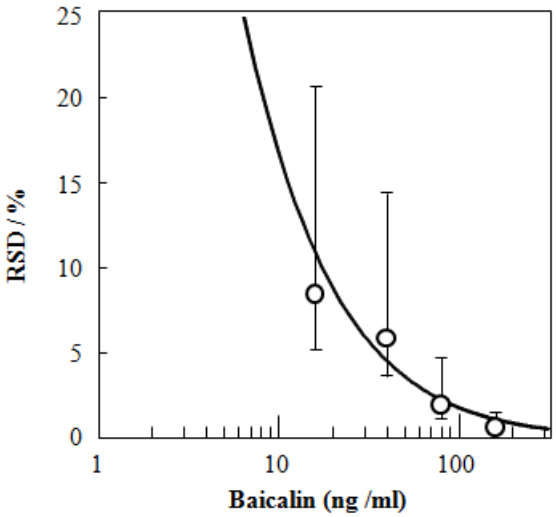


Fig. 2-3 Precision profile of baicalin using HPLC-UV. Open circles mean observed RSDs of chromatographic peak areas by repetitive measurement (n=6 each). The error bars show 95% of the confidence intervals for the observed RSDs. Solid line means measurement RSDs by ISO 11843-7 using one chromatogram of 80 ng/ml baicalin.

2.2.3 System repeatability test of HPLC-UV

ISO 11843-7 was also applied to examine an allowable limit of "System repeatability" in JP 16. In JP 16, "System repeatability" of system suitability testing for an HPLC-UV to determine baicalin in *Scutellariae Radix* was provided as follows [62]:

"System repeatability: When the test is repeated 6 times with 10 μl of the standard solution under the above operating conditions, the RSD of the peak area of baicalin is not more than 1.5%." where concentration of baicalin in the standard solution is 50 μg/ml, and the above operating condition is described in the section 2.2.1.

The experimentally observed RSD at 50 μg/ml baicalin by repetitive measurement was 0.15% (n=6). Meanwhile, the RSD by ISO 11843-7 was 0.12% (n=1). The present HPLC-UV for determining baicalin was compliant in JP 16. The RSD by ISO 11843-7 was within the 95% confidence interval of the statistically obtained RSD (n=6), ranging from 0.094% to 0.37%. Thus, it was found that ISO 11843-7 can be applied to examine "System repeatability" in HPLC-UV for determining baicalin in *Scutellariae Radix*. Although *Scutellariae Radix* is also listed in official monographs of ChP 2010 [9] and the quality is tested using an HPLC-UV, *Scutellariae Radix* is not listed in that of current United State Pharmacopoeia 37th edition (USP 37), Europe Pharmacopoeia 6th edition (EP 6), and British Pharmacopoeia 2014 edition (BP 2014). In these pharmacopoeias, however, many herbal medicines are listed in official monographs and the quality and purity are examined using HPLC [9, 62-65]. And the

precision of quantitative HPLC for herbal medicines was assessed based on “System suitability” in “Liquid chromatography” by repetitive measurement of the statistical ways. Thus, the present system repeatability estimation method by ISO 11843-7 would be applied to examine the allowable limit test of “System repeatability” in “Liquid chromatography” based on various countries’ pharmacopoeias.

2.2.4 Determination of baicalin in JP *Scutellaria Radix*

The HPLC-UV, compliance the JP 16, for determining baicalin in *Scutellaria Radix* was applied to the real sample analysis using JP *Scutellaria Radix*. The baicalin content in JP *Scutellaria Radix* studied was 102 mg/g, and this reproducibility was 1.1% RSD (n=6). In JP 16, the baicalin content in *Scutellaria Radix* is regulated to be not less than 100 mg/g, calculated on the basis of dried material. By the JP compliant HPLC-UV, the *Scutellaria Radix* studied is found to conform to JP 16.

2.3 System repeatability estimation of quantitative LC-3ECD by ISO 11843-7

As the system repeatability was one of the key factors to obtain reproducibly quantitative results in LC-3ECD, ISO 11843-7 was applied to estimate system repeatability of the present LC-3ECD for determining caffeoylquinic acids and flavonoids from *Flos Chrysanthemi*.

Chromatograms of standard mixture (30 ng/ml for CHA, CCHA, ICHA B, ICHA A, LTG, and LT, 25 ng/ml for BG (IS_3), 20 ng/ml for NCHA, 10 ng/ml for PAD (IS_1), EG (IS_2), CFA, and ICHA C) concerned with *Flos Chrysanthemi* were shown in Fig. 2-4. Chromatographic peaks of the analytes and the baseline noises in each detector were analyzed to obtain the measurement RSD by ISO 11843-7.

By Fourier transform, three real power spectra were obtained from consecutive 512 digital data points of chromatographic baseline in D_1, D_2, and D_3, respectively. The noise parameters of $\tilde{w}$, $\tilde{m}$, and ρ in each chromatographic baseline noise were determined by the least squared fitting of *Eq.* 1 to each real power spectrum, respectively. The fitting of the least squares between the model and real power spectrum from each chromatographic baseline noise in LC-3ECD was satisfactory. In chromatographic baseline noise from D_1, the noise parameters were determined as $\tilde{w}$ =0, $\tilde{m}$ =4.53×10^{-3}, and ρ =0.716, respectively. In chromatographic baseline noise from D_2, the noise parameters were determined as $\tilde{w}$=0, $\tilde{m}$ =2.85×10^{-3}, and ρ =0.581, respectively. In chromatographic baseline noise from D_3, the noise parameters were determined as $\tilde{w}$ =5.96×10^{-5}, $\tilde{m}$ =1.38×10^{-3}, and ρ =0.803, respectively.

Based on these three noise parameters and the chromatographic signal of the analytes, the measurement RSDs for system repeatability was obtained by the *Eq.* 2 mentioned above, in which, A was substituted by H, as the determination of these analytes by the present LC-3ECD were performed on the measurement of each chromatographic peak height. By reference to the specification sheet of the manual sample injector studied, 0.3% RSD at 5 μl in the present LC-3ECD was substituted for I.

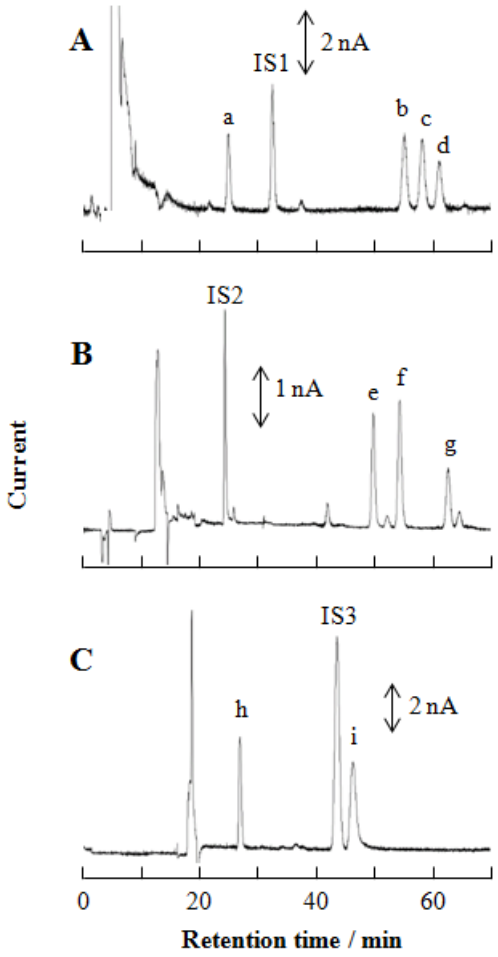


Fig. 2-4 Chromatograms of standard mixture concerned with *Flos Chrysanthemi* by LC-3ECD. Chromatograms were measured in D_1 (A), D_2 (B), and D_3 (C), respectively. Peaks: (A) a, 20 ng/ml NCHA; IS_1, 10 ng/ml PAD; b, 30 ng/ml CHA; c, 30 ng/ml CCHA; d, 10 ng/ml CFA; (B) IS_2, 10 ng/ml EG; e, 30 ng/ml LTG; f, 30 ng/ml ICHA B; g, 30 ng/ml ICHA A; (C) h, 10 ng/ml ICHA C; IS_3, 25 ng/ml BG; i, 30 ng/ml LT.

By ISO 11843-7, the measurement RSDs of chromatographic peak heights in LC-3ECD for determining 7 caffeoylquinic acids (NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, and ICHA C) and 2 flavonoids (LTG and LT) at each concentration were estimated, and they are listed in Table 2-1. The measurement RSDs by ISO 11843-7 were compared with the experimentally observed RSD by repetitive measurement in the statistical ways. As shown in Table 2-1, the measurement RSDs by ISO 11843-7 were within the 95% confidence intervals of the statistically obtained RSDs at each concentration of caffeoylquinic acids and flavonoids, respectively (n=6). Thus, it was found that the measurement RSD by ISO 11843-7 was applicable to estimate the precision of LC-3ECD for determining caffeoylquinic acids and flavonoids without repetitive measurement of the real samples.

In the case of the system repeatability estimation method by repetitive measurement (n=6), six runs of chromatographic experiments were required. But in an experiment to examine the

precision using ISO 11843-7, only one single run was performed. It shows that the experimental time to estimate the system repeatability was remarkably reduced by the application of ISO 11843-7. Since the confidence interval of the RSD by ISO 11843-7 was ±20%, which is equal to SD error from repetitive measurement (n=40) [66], a RSD by ISO 11843-7 is more reliable than that of repetitive measurement (n=6).

Table 2-1 Measurement RSD of chromatographic peak height by ISO 11843-7 and repetitive measurement with 95% confidence interval

Analyte			ISO11843-7	Repetitive measurement		
Peak	Name	Concentration (ng/ml)	RSD (%, n=1)	Lower limit of 95%CI *	RSD (%, n=6)	Upper limit of 95%CI *
a	NCHA	20	2.32	1.15	1.84	4.51
b	CHA	30	2.41	1.55	2.48	6.08
c	CCHA	30	2.54	1.44	2.30	5.64
d	CFA	10	3.57	2.78	4.45	10.9
e	LTG	30	0.80	0.59	0.95	2.33
f	ICHA B	30	0.73	0.62	0.99	2.43
g	ICHA A	30	1.45	0.97	1.55	3.80
h	ICHA C	10	1.60	0.63	1.01	2.48
i	LT	30	2.01	1.16	1.86	4.56
IS_1	PAD	10	1.47	0.71	1.13	2.77
IS_2	EG	10	0.50	0.24	0.39	0.96
IS_3	BG	25	0.86	0.52	0.84	2.06

*, 95% confidence interval for RSD of chromatographic peak height.

Chapter 3 Quantitative evaluation of *Flos Chrysanthemi* and SHL preparations by LC-3ECD

In chapter 1, a novel LC-3ECD system was established by the new design of channel connections and the technique of alternate rotation of the switching valves. By the present LC-3ECD method, the separation effect, precision, linearity, and sensitivity has been investigated by the standard substances concerned with *Flos Chrysanthemi* and SHL preparations. In chapter 2, the precision of the present LC-3ECD was reconfirmed by ISO 11843-7, deeply. The positive results have been obtained under these investigations. Thus, it is urgent to verify the applicability of the present LC-3ECD by the real samples of herbal medicines. In this chapter, the specificity, repeatability, and accuracy of the present LC-3ECD were investigated by the real samples. Then, the bioactive components in *Flos Chrysanthemi* and SHL preparations were simultaneously determined by the present LC-3ECD and the quality of these two kinds of herbal medicines was evaluated, comprehensively.

Principle component analysis (PCA) [84] is a statistical method for reducing the dimensions of multivariate problems without losing much information, which could be used to interpret complex data by classifying samples that show similar characteristics [18, 26, 85]. PCA can be applied to explore the natural interrelationship including classifying, clustering, and outliers among numerous complicated samples. Thus, PCA would be useful for quality analysis of plant materials by processing complex original data. In this chapter, PCA was carried out using the results of caffeoylquinic acids and flavonoids determination by the present LC-3ECD to investigate the classification of *Flos Chrysanthemi* and their sulfur-fumigated products.

3.1 Quantitative evaluation of *Flos Chrysanthemi* by LC-3ECD

3.1.1 Determination of caffeoylquinic acids and flavonoids in the real samples of *Flos Chrysanthemi*

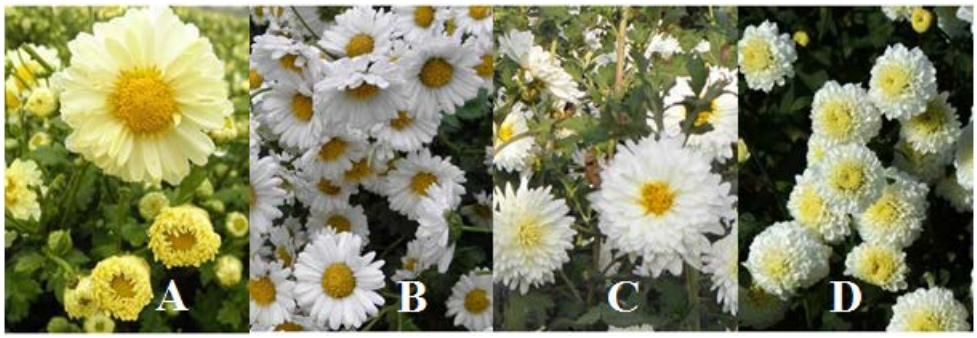


Fig. 3-1 Four standard cultivars of *Flos Chrysanthemi* in China. A: ‘HJ’; B: ‘BJ’; C: ‘CJ’; D: ‘GJ’.

There are many cultivars of *Flos Chrysanthemi* available, in China. In this study, four cultivars of *C. morifolium* cv. ‘Hangju’ (HJ), ‘Boju’ (BJ), ‘Chuju’ (CJ), and ‘Gongju’ (GJ) as shown Fig. 3-1 were studied, which have been recorded into ChP 2010 as standard varieties of

Flos Chrysanthemi. Seven caffeoylquinic acids (NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, and ICHA C) and 2 flavonoids (LTG and LT) in four cultivars of *Flos Chrysanthemi* would be determined by the present LC-3ECD, simultaneously. In chapter 1, the present LC-3ECD method has been validated by using the standard mixtures of these 9 compounds. However, for the determinations, some aspects of the present LC-3ECD should be investigated by the real samples.

In ChP 2010, the *Flos Chrysanthemi* sample solution was prepared by an ultrasonication extraction method to determine the contents of CHA, ICHA A, and LTG, respectively [9]. The same extraction method was used for the sample preparation. However, the extraction conditions including extraction solvent and extraction time were optimized in the present study. The extraction solvents including 50% MeOH, 70% MeOH, and MeOH and the extraction time including 10, 20, 30, 40, and 50 min were investigated, respectively. The results were shown in Fig. 3-2. The contents of caffeoylquinic acids and flavnoids were highest when using 70% MeOH as the extraction solvent. The contents of caffeoylquinic acids and flavnoids almost kept invariable when the extraction time was up to 20 min. Thus, 70% MeOH was selected as extraction solvent and 20 min was selected as extraction time.

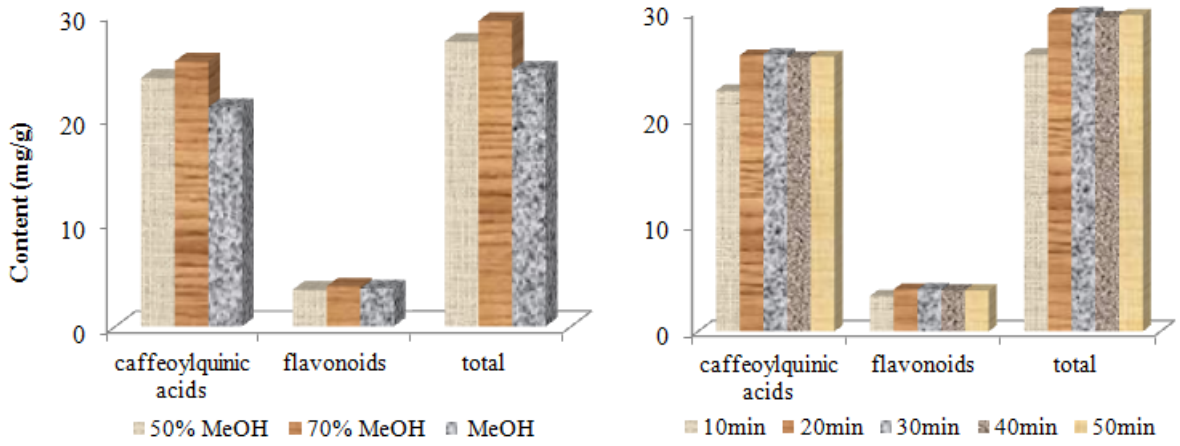


Fig. 3-2 Contents of caffeoylquinic acids, flavnoids and the total content of 9 analytes in *Flos Chrysanthemi* of 'HJ' sample (No. 111) under the different extraction conditions.

A test solution was prepared and stored at room temperature. Then it was analyzed at 0, 3, 9, 24, 36 and 48 h after sample preparation. The RSDs (n = 6) of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT in sample solution analyzed within 48 h were 2.0%, 0.88%, 1.7%, 1.7%, 0.76%, 3.3%, 2.6%, 1.4%, and 2.9%, respectively. It suggests that the sample solutions were stable within two days.

A mixture *Flos Chrysanthemi* sample ('HJ'-No.113**:**'S-BJ'-No.223 =1**:**1, *w*/*w*) was used to validate the repeatability and accuracy. The repeatability was assessed by analyzing six samples independently prepared by the sample preparation method. The accuracy (recovery experiment) was tested in sextuplicate by spiking the samples with nearly equal amounts of the standard substances. The chromatograms of the mixed standard solution and mixture *Flos*

Chrysanthemi sample were shown in Fig. 3-3. In the real sample, a high peak was observed after the peak of ICHA A (Fig. 3-3B, peak g) in CN_2. To identify the compound of this peak, some reference substances (*e.g.* apigenin-7-*O*-*β*-D-glucoside, diosmetin-7-*O*-*β*-D-rutinose, and diosmetin-7-*O*-*β*-D-glucoside) were injected, which might be eluted after ICHA A and contained in *Flos Chrysanthemi*. However, the chromatographic peaks of these reference substances were not overlapped on this peak, thus it was not identified.

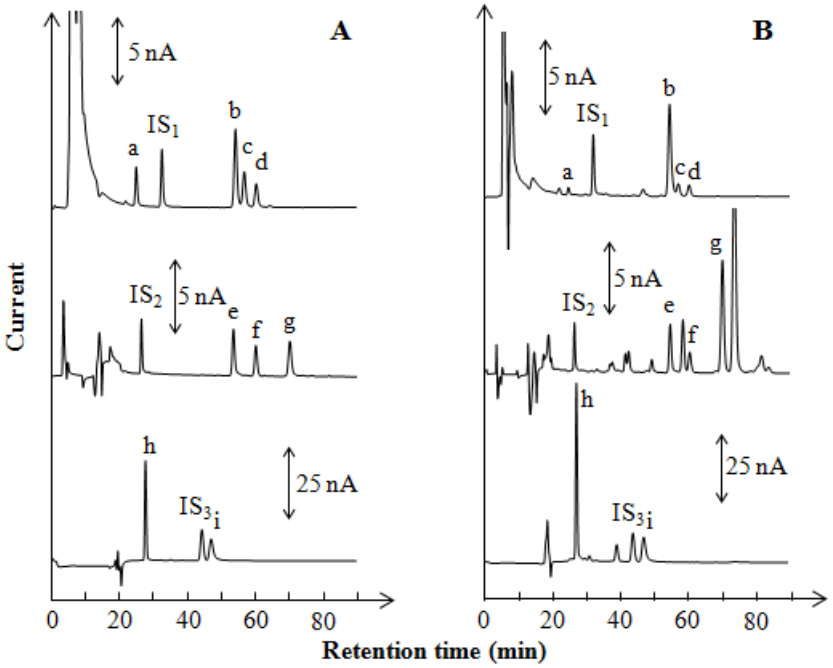


Fig. 3-3 Chromatograms of mixed standard solution (A) and mixture *Flos Chrysanthemi* sample (B). ['HJ' (No. 113):'S-BJ' (No. 223) =1:1, *w*/*w*]. Peaks: NCHA (a), CHA (b); CCHA (c), CFA (d); LTG (e), ICHA B (f), ICHA A (g), ICHA C (h), LT (i), PAD (IS_1), EG (IS_2), BG (IS_3).

Table 3-1 Repeatability and recovery of caffeoylquinic acids and flavonoids in *Flos Chrysanthemi*

	Repeatability (n = 6)		Recovery (n = 6)		
Analyte	Content (mg/g)	RSD (%)	Spiked (mg/g)	Recovery (%)	RSD (%)
NCHA	0.170	1.4	0.170	95.8	2.0
CHA	3.12	1.9	3.00	100.2	1.4
CCHA	0.474	2.1	0.500	99.1	2.5
CFA	0.204	1.7	0.200	97.7	1.9
ICHA A	13.2	3.1	13.0	101.0	1.9
ICHA B	0.665	2.3	0.700	102.4	3.7
ICHA C	5.93	1.6	6.20	104.9	2.2
LTG	1.95	0.21	1.80	96.4	3.0
LT	2.64	0.39	2.60	96.9	3.3

The data obtained in the repeatability and accuracy studies were shown in Table 3-1. The RSD (n = 6) of each analyte for the repeatability were below 3.1%. The recovery for the spiked sample solutions was in the range of 95.8% to 104.9% with the RSD (n = 6) of each analyte less than 3.7%. These results demonstrate that the present LC-3ECD provide accurate determination of caffeoylquinic acids and flavonoids in *Flos Chrysanthemi*.

3.1.2 Quantitative comparison of caffeoylquinic acids and flavonoids in *Flos Chrysanthemi*

After the present LC-3ECD was validated, the contents of 7 caffeoylquinic acids and 2 flavonoids in *Flos Chrysanthemi* and their sulfur-fumigated products were determined, simultaneously. Typical chromatograms of four varieties of *Flos Chrysanthemi* are shown in Fig. 3-4. The contents of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT in 6 lots of four varieties of *Flos Chrysanthemi* were shown in Table 3-2 and summarized in Table 3-3. For the ‘sameness’ analyses of caffeoylquinic acids, the compounds of CHA, ICHA A, and ICHA C were the main forms of caffeoylquinic acids in four varieties of *Flos Chrysanthemi* analyzed. On the other hand, the content of CFA was so low that it was hard to be determined in ‘HJ’, ‘CJ’, and ‘GJ’. The compound of NCHA was not detected in the ‘BJ’ samples analyzed. The average contents of CCHA and ICHA B were less than 0.85 and 1.25 mg/g, respectively. For the ‘sameness’ analyses of flavonoids, LTG was quantified in four varieties of *Flos Chrysanthemi*. Nevertheless, there was no detection of LT in some of the *Flos Chrysanthemi* samples.

Concerning the quantitative profiles of caffeoylquinic acids and flavonoids, the ‘differences’ were observed obviously among the four varieties of *Flos Chrysanthemi*. As the main form of caffeoylquinic acids in these four varieties, the average content of CHA was in the range of 3.89 to 5.65 mg/g. The content of ICHA A existing in the ‘BJ’ samples was more than twice as much as that of other varieties, while the highest content of ICHA C was found in ‘HJ’ samples. The average content of the total caffeoylquinic acids were in the order of ‘BJ’ > ‘HJ’ > ‘CJ’ > ‘GJ’. Regarding the flavonoids analyzed, the content of LTG was obviously higher than that of LT in ‘HJ’, ‘CJ’, and ‘GJ’. In spite of this, the samples of ‘BJ’ exhibited a significant variation, in which the content of LT [2.23±0.22 mg/g (mean ± SD)] was remarkably higher than that of LTG [0.82±0.07 mg/g (mean ± SD)]. The average contents of the sum of the flavonoids (LTG and LT) analyzed were in the order of ‘GJ’ > ‘HJ’ > ‘BJ’ > ‘CJ’. These results demonstrated that there were significant differences in the quantities of caffeoylquinic acids and flavonoids among the present four varieties of *Flos Chrysanthemi*.

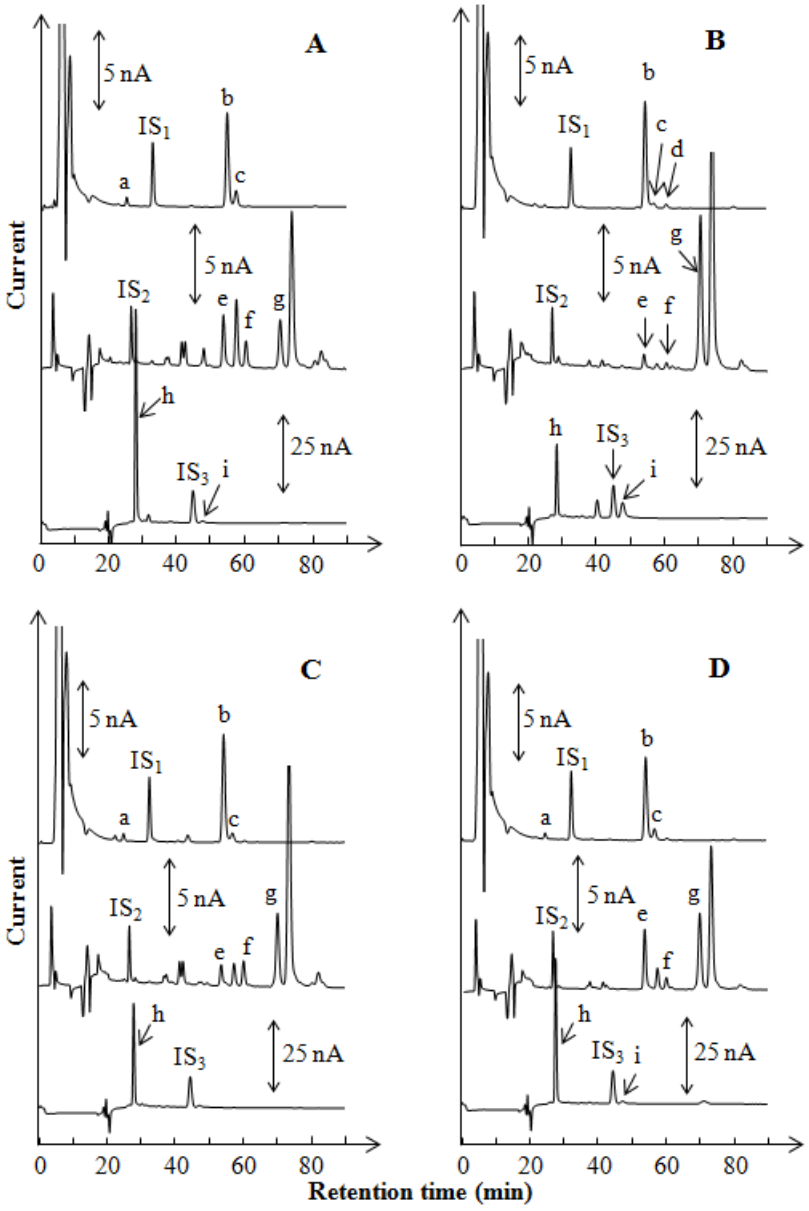


Fig. 3-4 Typical chromatograms obtained from *Flos Chrysanthemi*. A: 'HJ'; B: 'BJ'; C: 'CJ'; D: 'GJ'. Peaks: NCHA (a), CHA (b); CCHA (c), CFA (d); LTG (e), ICHA B (f), ICHA A (g), ICHA C (h), LT (i), PAD (IS_1), EG (IS_2), BG (IS_3).

Table 3-2 Content of caffeoylquinic acids and flavonoids in *Flos Chrysanthemi* and their sulfur-fumigated products

Sample	No.	Content (mg/g)								
		Caffeoylquinic acid							Flavonoid	
		NCHA	CHA	CCHA	CFA	ICHA A	ICHA B	ICHA C	LTG	LT
HJ	111	0.23	4.59	0.81	ND	10.1	1.18	9.06	3.28	0.31
	112	0.27	4.76	0.88	ND	9.31	1.27	9.18	3.06	0.30
	113	0.32	4.48	0.92	ND	10.2	1.16	9.95	3.45	0.29
	114	0.26	4.44	0.86	ND	6.69	1.03	8.53	3.14	ND
	115	0.24	4.36	0.74	ND	7.87	1.00	8.39	2.93	ND
	116	0.34	4.87	0.87	ND	7.47	1.57	9.45	3.12	ND
BJ	121	ND	6.08	0.31	0.13	24.6	0.29	3.23	0.82	2.58
	122	ND	5.28	0.27	0.11	18.1	0.95	3.55	0.87	2.15
	123	ND	5.45	0.27	0.13	20.4	1.02	3.31	0.77	2.24
	124	ND	5.68	0.28	0.11	20.5	1.03	3.47	0.90	2.28
	125	ND	5.55	0.29	0.11	20.9	0.51	3.10	0.70	1.89
	126	ND	5.85	0.30	0.12	21.8	0.56	3.25	0.85	2.24
CJ	131	0.24	4.36	0.38	ND	9.07	0.93	3.49	0.76	ND
	132	0.27	5.66	0.53	ND	10.8	1.16	5.09	1.28	ND
	133	0.29	5.22	0.47	ND	10.7	1.32	4.86	1.04	ND
	134	0.28	5.25	0.49	ND	9.73	1.34	3.88	0.82	ND
	135	0.28	5.96	0.57	ND	10.5	1.62	4.93	0.87	ND
	136	0.27	5.42	0.45	ND	10.4	1.15	4.51	1.03	ND
GJ	141	0.20	4.08	0.61	ND	9.61	0.57	6.28	3.67	0.38
	142	0.17	3.60	0.56	ND	9.31	0.52	5.89	3.41	ND
	143	0.22	4.21	0.66	ND	9.43	0.61	7.23	3.87	ND
	144	0.18	3.71	0.58	ND	9.30	0.51	6.13	3.36	0.16
	145	0.18	3.93	0.63	ND	9.47	0.52	6.50	3.54	ND
	146	0.20	3.82	0.60	ND	9.28	0.64	6.23	3.38	0.19
S-HJ	211	ND	1.27	0.24	0.10	4.10	0.18	3.35	1.35	6.02
	212	ND	2.55	0.42	0.17	6.99	0.20	4.37	1.24	6.50
	213	ND	1.32	0.19	0.14	5.86	ND	3.23	1.33	5.76
	214	ND	1.14	0.24	0.10	3.45	0.15	3.53	1.56	5.82
	215	ND	1.43	0.20	0.12	6.10	ND	3.25	1.26	5.75
	216	ND	2.19	0.44	0.20	6.33	0.22	4.42	0.80	7.87
S-BJ	221	ND	1.66	ND	0.32	12.6	0.15	1.41	0.40	3.87
	222	ND	1.85	ND	0.46	15.9	0.19	1.74	0.41	4.45
	223	ND	1.76	ND	0.40	16.0	0.18	1.88	0.44	4.96
	224	ND	1.32	ND	0.48	12.6	0.16	1.33	0.34	5.17
	225	ND	1.01	ND	0.33	10.7	ND	1.10	0.37	4.57
	226	ND	1.05	ND	0.34	10.2	ND	1.23	ND	4.98

ND: not detected.

Table 3-3 Summarized results of the caffeoylquinic acid and flavonoid content in *Flos Chrysanthemi* and their sulfur-fumigated products

Sample	Content [mean (mg/g) ± SD (n = 6)]											
	Caffeoylquinic acid								Flavonoid			
	NCHA	CHA	CCHA	CFA	ICHA A	ICHA B	ICHA C	Subtotal	LTG	LT	Subtotal	Total
HJ	0.28±0.04	4.58±0.20	0.85±0.06	ND	8.61±1.47	1.20±0.21	9.09±0.58	24.6±2.04	3.16±0.18	0.15±0.16	3.31±0.31	27.9±2.31
BJ	ND	5.65±0.26	0.29±0.02	0.12±0.01	21.1±2.13	0.73±0.31	3.32±0.17	31.2±2.09	0.82±0.07	2.23±0.22	3.05±0.27	34.2±2.26
CJ	0.27±0.02	5.31±0.54	0.48±0.07	ND	10.2±0.67	1.25±0.23	4.46±0.64	22.0±2.00	0.97±0.19	ND	0.97±0.19	22.9±2.13
GJ	0.19±0.02	3.89±0.21	0.61±0.03	ND	9.40±0.12	0.56±0.05	6.34±0.42	21.0±0.74	3.54±0.18	0.12±0.14	3.66±0.22	24.7±0.92
S-HJ	ND	1.65±0.58	0.29±0.11	0.14±0.04	5.47±1.38	0.13±0.10	3.69±0.56	11.4±2.43	1.26±0.25	6.29±0.83	7.54±0.61	18.9±2.86
S-BJ	ND	1.44±0.37	ND	0.39±0.07	13.0±2.48	0.11±0.09	1.45±0.30	16.4±3.24	0.33±0.16	4.67±0.48	4.99±0.44	21.4±3.34

ND: not detected.

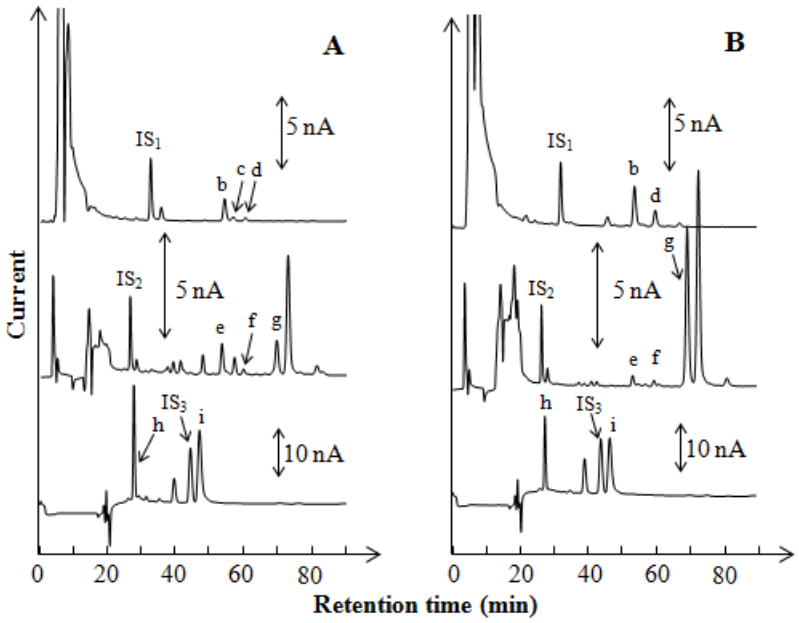


Fig. 3-5 Typical chromatograms obtained from sulfur-fumigated *Flos Chrysanthemi* of 'S-HJ' (A), and 'S-BJ' (B). Peaks: CHA (b), CCHA (c), CFA (d), LTG (e), ICHA B (f), ICHA A (g), ICHA C (h), LT (i), PAD (IS_1), EG (IS_2), BG (IS_3).

Significant differences were also found between the *Flos Chrysanthemi* and their sulfur-fumigated products. Typical chromatograms of the sulfur-fumigated *Flos Chrysanthemi* of 'S-HJ' and 'S-BJ' are shown in Fig. 3-5. The quantitative results are also shown in Table 3-2 and summarized in Table 3-3. Compared with the non-fumigated samples of 'HJ', the average contents of NCHA, CHA, CCHA, ICHA A, ICHA B, and ICHA C were reduced from 0.28 mg/g to ND, 4.58 to 1.65 mg/g, 0.85 to 0.29 mg/g, 8.61 to 5.47 mg/g, 1.20 to 0.13 mg/g, and 9.09 to 3.69 mg/g, respectively, in the sulfur-fumigated samples of 'HJ', whereas the average content of CFA was increased from ND to 0.14 mg/g. For the contents of the flavonoids, a lower amount of LTG (reduced from 3.16 to 1.26 mg/g) and a much higher amount of LT (increased from 0.15 to 6.29 mg/g) were observed in the sulfur-fumigated 'HJ' samples. The similar chemical variability existed between the non-fumigated and sulfur-fumigated 'BJ' samples. Compared with the non-fumigated 'BJ' samples, the average contents of CHA, CCHA, ICHA A, ICHA B, ICHA C, and LTG in the sulfur-fumigated 'BJ' samples were reduced from 5.65 to 1.44 mg/g, 0.29 mg/g to ND, 21.1 to 13.0 mg/g, 0.73 to 0.11 mg/g, 3.32 to 1.45 mg/g, and 0.82 to 0.33 mg/g, respectively. However, the average content of CFA and LT was increased from 0.12 to 0.39 mg/g and 2.23 to 4.67 mg/g, respectively, in the sulfur-fumigated 'BJ' samples. It was found that nearly 60% of LTG and more than 47% of caffeoylquinic acids were lost due to sulfur fumigation. Therefore, sulfur fumigation showed a destructive effect on *Flos Chrysanthemi*. These results may provide some evidence for the advantages and disadvantages of sulfur fumigation on plant materials.

For the chemical changes that occurred in *Flos Chrysanthemi* during the sulfur-fumigation

processing, it could be deduced that the caffeoylquinic acids were partly destroyed by oxidization or/and hydrolyzation and the flavonoid glycosides were partly converted to flavonoid aglycones. From the quantitative results, the higher content of CFA and the lower content of other caffeoylquinic acids were observed in the sulfur-fumigated samples compared with non-fumigated samples. As the CFA was the hydrolysate of other caffeoylquinic acids, it revealed the presence of the hydrolysis of caffeoylquinic acids during the sulfur-fumigation processing. Literature showed that there were several kinds of flavonoid glycosides related to LT in *Flos Chrysanthemi*, such as Luteolin-7-*O*-rutinoside, Luteolin-7-*O*-glucuronide, Luteolin-7-*O*-pentosylhexoside, Luteolin-7-*O*-6'-malonylglucoside, *etc*. [23, 25] These compounds might also be hydrolyzed to LT during the sulfur-fumigation processing. It partly explains why the content of LT in the sulfur-fumigated *Flos Chrysanthemi* samples was much more than that of the non-fumigated samples.

3.1.3 PCA for the classification of *Flos Chrysanthemi*

After the content determination of caffeoylquinic acids and flavonoids in *Flos Chrysanthemi* and their sulfur-fumigated samples, the content data were further analyzed by PCA to classify the samples. The content results of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT in 'HJ', 'BJ', 'CJ', and 'GJ' samples were set as a data set and imported into the software of SIMCA-P +12.0. NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT were set as 9 original variables, and the present *Flos Chrysanthemi* samples were set as observations. The summary of the fit of the model was displayed with eigenvalues, R2X (fraction of the variation of the data set explained by each component), and cumulative R2X (cum) for each component. Hereon, the components are new variables computed as linear combinations of all the original variables for a good summary of original information. Nine components could be obtained from 9 original variables. The eigenvalue shows the weight/contribution of the respective component for the summary of the original information. Generally, when the eigenvalues of the components are more than 1.0, the combination of these components could explain the most variance of the observations. Concerning the present data set of the 'HJ', 'BJ', 'CJ', and 'GJ' samples, the eigenvalues of the first and second components were greater than 1.0 as shown in Table 3-4. These two components were considered as the first and second principle component (PC_1 and PC_2), respectively. PC_1 contained a 68.8% variance and PC_2 contained a 19.1% variance. The two PCs explained 87.9% of the total variation. Therefore, PC_1 and PC_2 were selected for identifying the inhomogeneity of the present *Flos Chrysanthemi* samples in the scores plot. As shown in Fig. 3-6A, based on PC_1 and PC_2, it showed four groups of samples, the first group with 'HJ' samples, the second with 'CJ' samples, the third with 'BJ' samples, and the fourth with 'GJ' samples. Thus, the classification of these *Flos Chrysanthemi* samples was obviously exhibited in the PCA scores plot. In addition, the loadings are the weights with which the original variables are combined to form the PCA scores. The loadings plot shows which original variables describe the similarity and inhomogeneity among the observations. As

shown in Fig. 3-6B, the locked positions of the original variables of ICHA A, CFA, and LT were close to the respective positions of the observations of ‘BJ’ samples in Fig. 3-6A. It means ICHA A, CFA, and LT were the main variables for distinguishing the ‘BJ’ samples from ‘HJ’, ‘CJ’, and ‘GJ’ samples. The locked positions of NCHA, CCHA, and ICHA C were close to the respective positions of ‘HJ’ samples. It means NCHA, CCHA, and ICHA C were the main variables for distinguishing the ‘HJ’ samples from ‘BJ’, ‘CJ’, and ‘GJ’ samples. The locked position of CHA was located at the middle position between ‘CJ’ and ‘BJ ’samples. It means the similar CHA contents were present in ‘CJ’ and ‘BJ ’samples. By the same way, the correlation between the original variables of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, LT and the observations of the ‘HJ’, ‘BJ’, ‘CJ’, and ‘GJ’ samples can be obtained from the Fig 3-6A and Fig 3-6B.

Table 3-4 Eigenvalue, variance, and cumulative variance from each data set

Content data set	Component	Eigenvalue	R2X (% of variance)	R2X(cum) (% of cumulative variance)
‘HJ’, ‘BJ’, ‘CJ’,‘GJ’	**1**	**6.19**	**68.8**	**68.8**
	2	**1.72**	**19.1**	**87.9**
	3	0.824	9.15	97.0
	4	0.176	1.95	99.0
	5	0.0397	0.44	99.4
	6	0.0242	0.27	99.7
	7	0.0108	0.12	99.8
	8	0.00921	0.10	99.9
	9	0.00661	0.07	100.0
‘HJ’, ‘BJ’, ‘S-HJ’,‘S-BJ’	**1**	**6.29**	**69.9**	**69.9**
	2	**1.93**	**21.4**	**91.3**
	3	0.48	5.33	96.6
	4	0.135	1.50	98.1
	5	0.116	1.29	99.4
	6	0.0286	0.32	99.7
	7	0.0142	0.16	99.9
	8	0.00404	0.04	100.0
	9	0.00181	0.02	100.0

Moreover, the content results of NCHA, CHA, CCHA, CFA, LTG, ICHA B, ICHA A, ICHA C, and LT in the ‘HJ’, ‘BJ’, ‘S-HJ’, and ‘S-BJ’ samples were also imported into the software of SIMCA-P +12.0 and analyzed by PCA. As shown in Table 3-4, the eigenvalues of PC_1 and PC_2 were also greater than 1.0 concerning the content data set of the ‘HJ’, ‘BJ’, ‘S-HJ’, and ‘S-BJ’ samples, so these two components were selected for identifying the inhomogeneity of the present samples. PC_1 contained a 69.9% variance and PC_2 contained a

21.4% variance. The two PCs explained 91.3% of the total variation. On the PCA scores plot shown in Fig. 3-7A, the ‘HJ’, ‘BJ’, ‘S-HJ’, and ‘S-BJ’ samples were also clearly classified into four groups. On the loadings plot shown in Fig. 3-7B, NCHA, CCHA, ICHA C and LTG were the main variables for distinguishing the ‘HJ’ samples from ‘BJ’, ‘S-BJ’, and ‘S-HJ’ samples. CFA and LT were the main variables for distinguishing the ‘S-BJ’ and ‘S-HJ’ samples from ‘BJ’ and ‘HJ’ samples.

The above results obtained by PCA demonstrated that there were significant differences of the caffeoylquinic acids and flavonoids among the four cultivars of *Flos Chrysanthemi* and their sulfur-fumigated products. Alternatively, the present LC-3ECD was an applicable method to determine the caffeoylquinic acids and flavonoids content in *Flos Chrysanthemi* which can be dealt with by PCA.

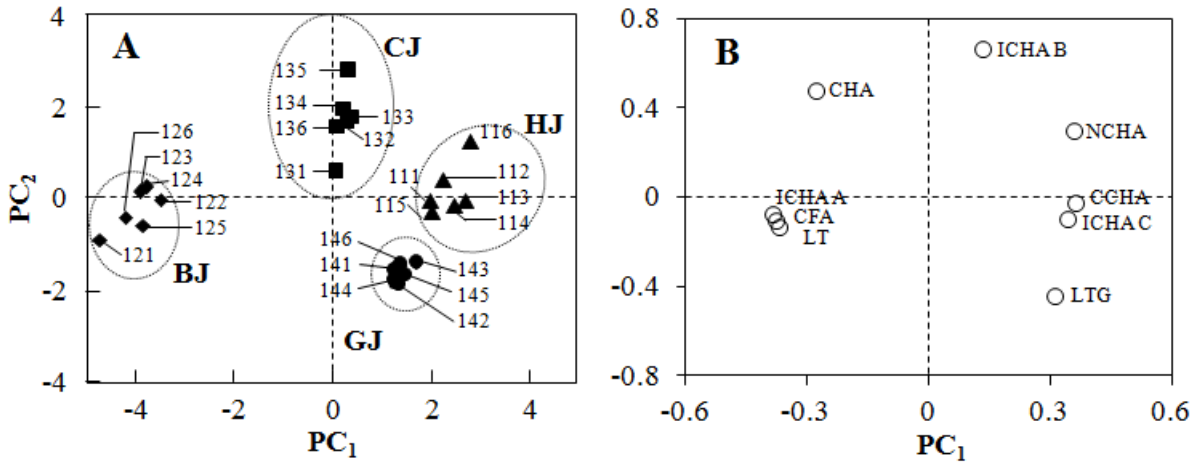


Fig. 3-6 Analysis results obtained from the content data set of ‘HJ’, ‘BJ’, ‘CJ’ and ‘GJ’ samples by PCA. (A) the scores plot; (B) the loadings plot.

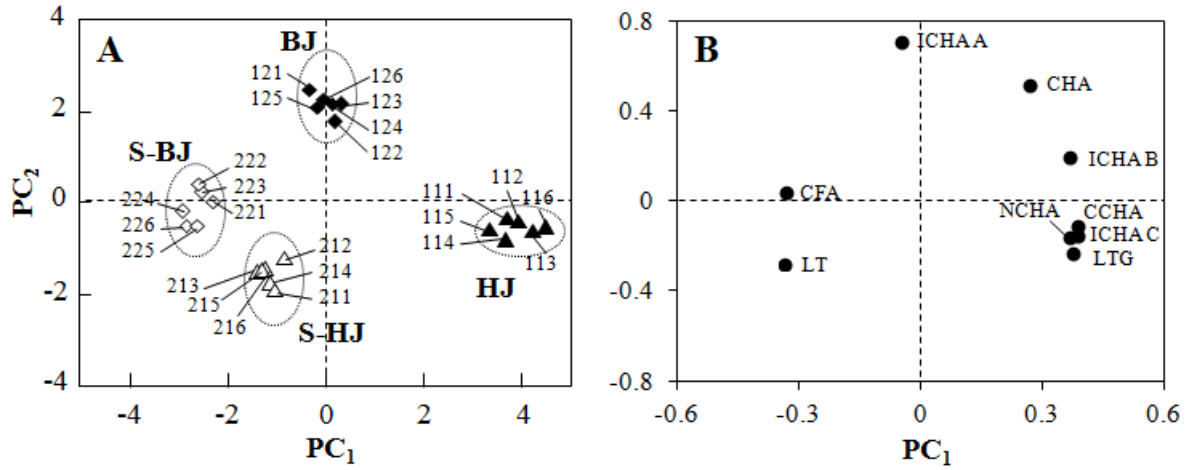


Fig. 3-7 Analysis results obtained from the content data set of ‘HJ’, ‘BJ’, ‘S-HJ’ and ‘S-BJ’ samples by PCA. (A) the scores plot; (B) the loadings plot.

3.2 Quantitative evaluation of SHL preparations by LC-3ECD

SHL is a typical composite formula of TCM comprised of three herbs: *Flos Lonicerae*, *Radix Scutellariae*, and *Fructus Forsythiae*. There are many kinds of SHL preparations, such as oral liquid, lyophilized powder for injection, capsule, granule, tablet, and suppository. In this study, the preparations of SHL oral liquid and SHL lyophilized powder for injection were studied for the quantification of various bioactive components. Eleven compounds including 6 caffeoylquinic acids (NCHA, CHA, CCHA, CFA, ICHA B, and ICHA C), 4 flavonoids (STL, BC, WGD, and BCE) and 1 phenylethanoid glycoside (FTA) would be simultaneously determined by the present LC-3ECD in 14 batches of SHL oral liquid and 12 batches of SHL lyophilized powder for injection produced by different manufacturers in China. In chapter 1, the present LC-3ECD method has been validated by using the standard mixtures of these 11 compounds. However, the specificity, repeatability, and accuracy of the real samples and the stabilities of sample solution haven't been investigated yet.

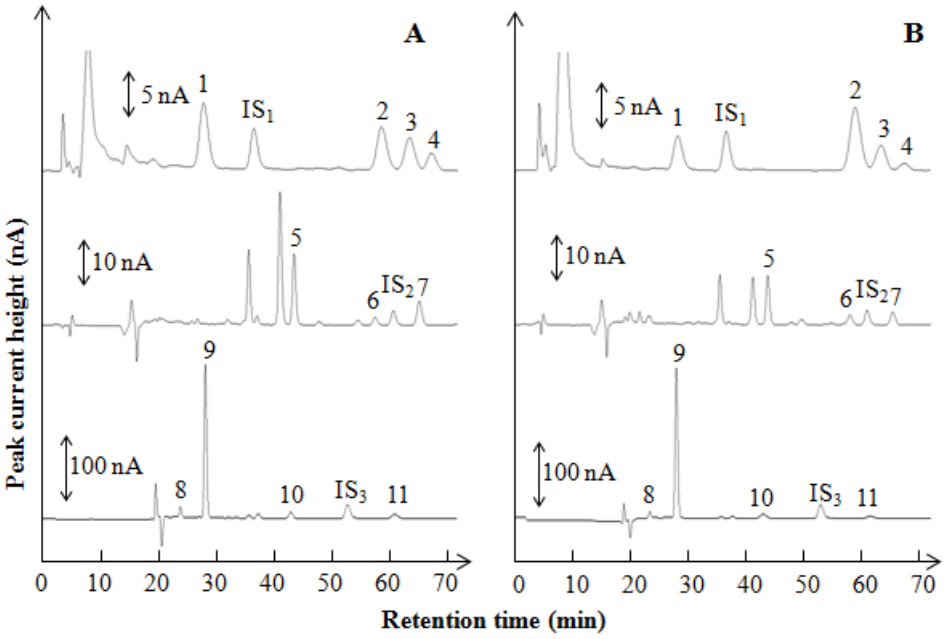


Fig. 3-8 Typical chromatograms of SHL oral liquid (A) and SHL lyophilized powder for injection (B). Peaks: 1, NCHA; 2, CHA; 3, CCHA; 4, CFA; 5, FTA; 6, STL; 7, ICHA B; 8, ICHA C; 9, BC; 10, WGD; 11, BCE; IS_1, PAD; IS_2, PG; IS_3, KF.

The chromatograms of the real samples were obtained by the present LC-3ECD. Fig. 3-8 shows the typical chromatograms of SHL oral liquid and SHL lyophilized powder for injection. The chromatographic peaks of NCHA, PAD (IS_1), CHA, CCHA and CFA appeared at 28.0, 36.4, 57.9, 62.5, and 66.6 min in CN_1, respectively. The chromatographic peaks of FTA, STL, PG (IS_2), and ICHA B appeared at 44.2, 58.1, 61.2, and 65.8 min in CN_2, respectively. The chromatographic peaks of ICHA C, BC, WGD, KF (IS_3), and BCE appeared at 24.0, 28.3, 43.0, 52.8, and 61.0 min in CN_3, respectively. The R_s between CHA and CCHA

was greater than 1.70. The R_s between CCHA and CFA was greater than1.80. The tailing factors of all analytes were in the range of 0.95 to 1.05, so the peak height quantification made sense. Each compound displayed good separation under the present system conditions by LC-3ECD.

The specificity of the present LC-3ECD method was demonstrated by the completed separation of each analyte and IS in the real samples of SHL preparations as shown in Fig. 3-8. Besides the check of peaks in real SHL samples, the negative samples of "LF" (*Flos Lonicerae* plus *Fructus Forsythiae*), "LS" (*Flos Lonicerae* plus *Radix Scutellariae*), and "FS" (*Fructus Forsythiae* plus *Radix Scutellariae*) were also analyzed to check the peak interference or overlapping of each analyte and IS as the further study of specificity/ selectivity.

One component of FTA, six components of NCHA, CHA, CCHA, CFA, ICHA B, and ICHA C, and four components of STL, BC, WGD, and BCE in SHL preparations are originated from crude herbs of *Fructus Forsythiae*, *Flos Lonicerae*, and *Radix Scutellariae*, respectively [46, 86-88]. That is to say, in negative samples of "LF" (without *Radix Scutellariae*), there would be no peaks in the retention time of STL, BC, WGD, and BCE. In negative samples of "LS" (without *Fructus Forsythiae*), there would be no peak in the retention time of FTA. In negative samples of "FS" (without *Flos Lonicerae*), there would be no peaks in the retention time of NCHA, CHA, CCHA, CFA, ICHA B, and ICHA C. Otherwise, the peak interferences exist to the corresponding analyte. The chromatograms of the negative samples of "LF", "LS", and "FS" were shown in Fig. 3-9. Compared the chromatograms of the negative samples with that of the real samples (Fig. 3-8), the peaks in "LF" (Fig. 3-9A) had no interference or overlapping to the peaks of STL, BC, WGD, and BCE in the SHL samples. The same results existed in the "FS" (Fig. 3-9B) and "LS" (Fig. 3-9C) compared with the SHL samples. The peaks in "FS" had no interference or overlapping to the peaks of NCHA, CHA, CCHA, CFA, ICHA B, and ICHA C in the SHL samples. The peaks in "LS" also had no influence to the peak of FTA. It indicated that the present LC-3ECD method has good specificity and/or selectivity.

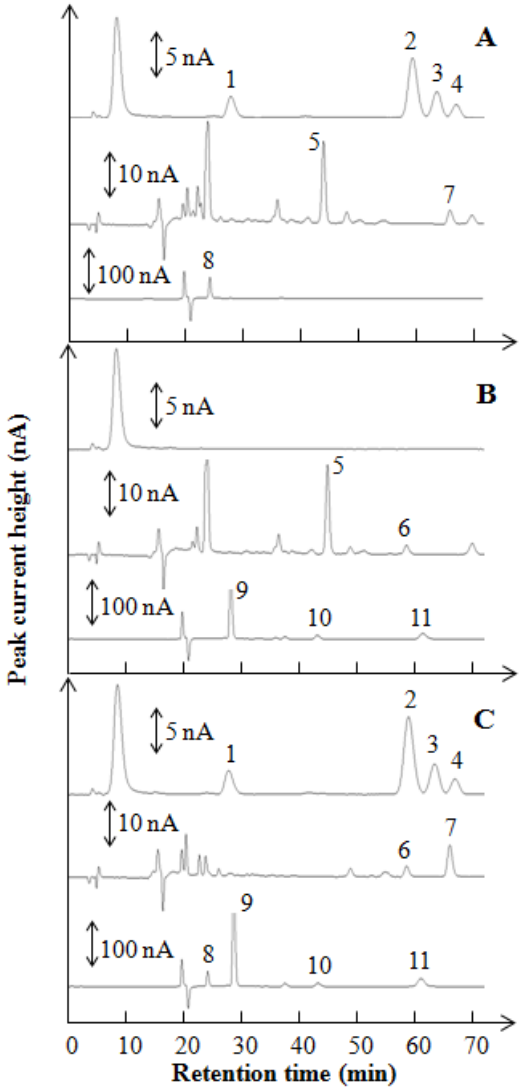


Fig. 3-9 Chromatograms of negative samples of "LF"(A), "FS"(B), and "LS"(C). Peaks: 1, NCHA; 2, CHA; 3, CCHA; 4, CFA; 5, FTA; 6, STL; 7, ICHA B; 8, ICHA C; 9, BC; 10, WGD; 11, BCE.

For the stability test, the test solution was prepared according to sample preparation method and stored at room temperature. Then it was analyzed at 0, 4, 10, 24, 36 and 48 h by the present LC-3ECD. The stabilities of each analyte in the SHL sample solution within 48 h were ranging from 1.1% to 2.8% RSD (Table 3-5). It suggested that it was feasible to analyze samples within two days.

The repeatability of the present LC-3ECD was obtained by analyzing six samples of the same batch of SHL preparation using the same preparation procedure. The accuracy of the present LC-3ECD method was evaluated using recovery tests. Each standard compound of the analytes was directly spiked into the SHL samples, and then pretreated according to the sample preparation method to prepare the test solutions in sextuplicate. The recovery of the analyte was 100% when the increased amount of each analyte was equal to the calculated amount which was equivalent to the amount of the spiked standard compound. The results

were shown in Table 3-6.

Table 3-5 The RSD of the peak height ratios of each analyte within two days (*n* = 6)

Analyte	$H_{standard}/H_{IS}$						RSD/%
	0 h	4 h	10 h	24 h	36 h	48 h	
NCHA	2.020	2.013	2.036	1.981	1.975	1.955	**1.6**
CHA	1.186	1.192	1.174	1.182	1.160	1.136	**1.8**
CCHA	1.028	1.013	1.066	1.031	1.007	1.000	**2.3**
CFA	0.331	0.329	0.343	0.333	0.327	0.316	**2.7**
FTA	0.831	0.841	0.811	0.805	0.802	0.796	**2.2**
STL	0.149	0.156	0.151	0.146	0.147	0.150	**2.4**
ICHA B	0.631	0.621	0.629	0.626	0.615	0.616	**1.1**
ICHA C	0.706	0.693	0.675	0.665	0.665	0.658	**2.8**
BC	11.00	10.76	10.34	10.56	10.34	10.74	**2.5**
WGD	0.435	0.428	0.427	0.412	0.415	0.412	**2.3**
BCE	0.444	0.438	0.426	0.434	0.420	0.419	**2.4**

Table 3-6 Repeatability and recovery of each analyte in SHL preparations studied by LC-3ECD

Analyte	Oral liquid (Lot No. 130204)					Lyophilized powder for injection (Lot No. 1206222)				
	Repeatability (*n* = 6)		Recovery (*n* = 6)			Repeatability (*n* = 6)		Recovery (*n* = 6)		
	Con. (mg/ml)	RSD (%)	Spiked (mg/ml)	Rec. (%)	RSD (%)	Content (mg/g)	RSD (%)	Spiked (mg/g)	Rec. (%)	RSD (%)
NCHA	1.70	1.8	1.70	100.8	2.4	9.16	0.38	9.23	98.2	0.96
CHA	1.13	1.9	1.15	97.3	1.4	18.6	0.87	18.7	99.7	0.88
CCHA	1.07	1.8	1.05	98.2	1.8	2.81	2.0	2.86	97.8	1.4
CFA	0.16	2.8	0.15	99.4	2.6	0.51	1.4	0.51	96.5	1.5
FTA	0.31	0.83	0.31	103.6	3.1	16.9	1.4	16.9	100.6	1.1
STL	0.088	1.6	0.086	96.7	1.4	3.36	2.7	3.30	101.6	2.1
ICHA B	0.30	1.7	0.30	103.0	1.3	2.86	1.9	2.86	99.3	1.8
ICHA C	0.42	3.2	0.42	97.4	2.7	5.21	1.6	5.27	95.6	2.2
BC	11.7	3.2	11.5	95.6	2.9	270	1.2	273	102.1	0.47
WGD	0.81	2.0	0.81	100.6	1.7	2.13	2.5	2.15	99.8	2.8
BCE	0.41	2.5	0.42	102.0	2.4	4.22	2.0	4.18	98.3	1.5

Concentration (Con.); Recovery (Rec.)

For the repeatability test, the RSDs of the contents (*n* = 6) of each analyte were ranging from 0.83% to 3.2% and ranging from 0.38% to 2.7% in SHL oral liquid and SHL lyophilized powder for injection, respectively. The recoveries of the analytes for the spiked sample

solutions were between 95.6% and 103.6% and the RSD (n = 6) were less than 3.1% in SHL oral liquid. The recoveries of the analytes in SHL lyophilized powder for injection were between 95.6% and 102.1% with the RSD (n = 6) less than 2.8%. These results demonstrate that the present LC-3ECD method is characterized by high repeatability and accurate measurement of these 11 redox compounds.

Since the present LC-3ECD was validated by the real samples, these 11 bioactive redox components in 14 batches of SHL oral liquid and 12 batches of SHL lyophilized powder for injection produced by different manufacturers were simultaneously determined by the present LC-3ECD. The contents of NCHA, CHA, CCHA, CFA, FTA, STL, ICHA B, ICHA C, BC, WGD, and BCE in each batch of SHL oral liquid and SHL lyophilized powder for injection were listed in Table 3-7 and Table 3-8, respectively.

The contents of these bioactive components varied greatly among the different samples. For example, the content of FTA was 0.29 mg/ml in SHL oral liquid of Lot no. 12112717 while 1.20 mg/ml in that of Lot no.112111828 produced by another manufacturer, a four-fold variation. The variation might be the consequence of many aspects, such as plant resource, climate, cultivation regions, harvesting time of crude herbs and preparation procedure of products. The multiple components determination by the present LC-3ECD could reflect the quality of herbal medicines, comprehensively.

In ChP 2010, the contents of CHA and BC in SHL oral liquid are regulated to be not less than 0.6 mg/ml and 10.0 mg/ml, respectively. The contents of CHA and BC in SHL lyophilized powder for injection are regulated to be not less than 14.2 mg/g and 213 mg/g, respectively. As shown in Table 3-7 and Table 3-8, each batch of SHL oral liquid and SHL lyophilized powder for injection studied was found to comply with the limits regulated by ChP 2010.

Table 3-7 Content of 11 compounds in SHL oral liquid determined by the present LC-3ECD

Sample	Lot No.	Concentration (mg/ml) [a]										
		NCHA	CHA	CCHA	CFA	FTA	STL	ICHA B	ICHA C	BC	WGD	BCE
SHL1	112101127	1.77	0.96	0.86	0.29	1.31	0.11	0.57	0.75	13.1	0.65	0.41
	112111828	1.83	1.03	0.93	0.31	1.20	0.11	0.63	0.84	13.5	0.55	0.34
	112110426 [b]	1.72	1.16	0.97	0.23	1.68	0.25	0.72	0.83	24.4	0.82	0.61
	112103022	1.78	1.18	1.05	0.39	1.38	0.099	0.70	0.79	12.6	0.41	0.37
	112112326	1.59	1.04	0.92	0.28	1.29	0.13	0.64	0.75	13.3	0.48	0.36
SHL2	12090517	1.09	0.80	0.68	0.067	1.11	0.074	0.28	0.40	12.8	0.29	0.41
	12102632	1.24	1.13	0.91	0.075	0.25	ND	0.30	0.46	13.2	0.30	0.31
	12102812	1.31	1.20	0.93	0.083	0.38	ND	0.30	0.42	13.6	0.31	0.41
	12112717	1.36	1.07	0.91	0.10	0.29	0.082	0.27	0.41	12.2	0.25	0.45
	12111317	1.29	1.05	0.86	ND	0.29	0.070	0.26	0.38	12.2	0.20	0.35
SHL3	20121037	1.31	1.07	0.80	0.20	0.68	0.078	0.48	0.61	12.3	0.38	0.42
	20121039	1.25	0.85	0.64	0.22	0.72	0.074	0.37	0.51	11.9	0.34	0.31
SHL4	130204	1.70	1.13	1.07	0.16	0.31	0.088	0.30	0.42	11.7	0.81	0.41
	130105	1.87	1.43	1.23	0.19	0.35	0.082	0.32	0.45	14.2	0.99	0.40

[a] The average of triplicate determinations from three independent experiments.

[b] A concentrated SHL oral liquid products.

ND, not detected.

Table 3-8 Content of 11 compounds in SHL lyophilized powder for injection determined by the present LC-3ECD

Sample	Lot No.	Content (mg/g) [a]										
		NCHA	CHA	CCHA	CFA	FTA	STL	ICHA B	ICHA C	BC	WGD	BCE
SHL5	1212016	8.45	16.4	4.47	0.34	10.3	2.16	3.59	4.68	237	2.87	2.43
	1211101	7.93	16.4	3.67	0.37	12.7	2.89	2.80	4.35	257	2.29	1.85
	1203105	7.09	17.1	3.05	0.53	16.3	3.10	2.37	4.81	253	2.11	2.03
	1209112	7.65	17.4	3.32	0.27	11.2	3.24	2.48	4.33	267	2.19	1.57
	1209105	8.40	19.1	4.22	0.41	11.8	3.14	3.13	5.13	260	2.20	1.58
	1209106	9.20	19.0	4.64	0.37	11.5	2.83	3.54	5.79	280	2.44	2.46
	1211106	7.36	18.0	4.05	0.39	11.5	3.36	3.11	5.02	254	2.77	2.06
	1211112	8.80	18.1	4.04	0.38	10.7	2.55	3.02	4.84	268	2.43	2.15
	1206104	9.08	17.9	4.59	0.39	13.3	3.43	3.81	5.53	277	ND	1.14
	1206222	9.16	18.6	2.81	0.51	16.9	3.36	2.86	5.21	270	2.13	4.22
	1206223	12.0	18.9	2.89	0.65	21.4	3.65	2.48	4.80	274	2.13	4.73
SHL6	20111001	10.4	22.1	7.80	1.17	20.4	2.30	5.56	7.42	266	ND	1.59

ND, not detected.

[a] The average of triplicate determinations from three independent experiments

GENERAL DISCUSSION AND CONCLUSIONS

An LC-ECD is a useful analytical method for redox compounds owing to its high sensitivity and selectivity. In order to satisfy the needs of quantitative analyses of various bioactive components in herbal medicines, a novel LC-3ECD system has been developed by the new design of channel connections and the technique of alternately rotating the switching valves in the present study. There were several advantages for the present LC-3ECD method. Firstly, compared with other analytical methods such as LC-UV/DAD, LC-ELSD, and LC-MS/MS, the present LC-3ECD method has high selectivity for redox compounds such as caffeoylquinic acids and flavonoids, which are the main bioactive components in many kinds of herbal medicines. Secondly, by the original system design, three kinds of mobile phases could alternately flow through the same column (Pre-C) to elute the different polarity compounds into the different detection channels for separation. The analytical time was remarkably reduced compared with an isocratic LC-ECD. Thirdly, due to the stable electrochemical double layer on the working electrode surface maintained by an isocratic elution, the sensitivity of the present LC-3ECD was remarkably increased compared with a gradient LC-ECD. The LOD of the present LC-3ECD was up to pg or ng/ml level. Thus, it was highly sensitive. Therefore, the present LC-3ECD successfully provided a valuable analytical method for the simultaneous determination of various redox compounds that have remarkably different hydrophobic properties with high sensitivity and efficiency. It is reliable, applicable and superior for the quantitative evaluation of herbal medicines.

In this study, ISO 11843-7, which provides detection limits based on stochastic properties of instrumental noise, was applied to estimate the system repeatability of quantitative HPLC-UV for *Scutellaria Radix* and LC-3ECD for *Chrysanthemi Flos*, successfully. With the application of ISO 11843-7, the system repeatability estimation of quantitative HPLC-UV and LC-3ECD was performed using only single chromatographic measurement. The experimental time was remarkably reduced compared with the experimental repeatability estimation by repetitive measurement in statistical ways.

In the general information of JP 16, when a long time is required for one analysis, such as an analysis using a gradient method, or an analysis containing late eluting components, it may be acceptable to decrease the number of replication injections by adopting a new allowable limit of “System repeatability” equivalent to that at six replicate injections. It should be kept in mind that since a decreasing number of replicate injections results in an increase of the weight of each injection, it becomes more vital that the test is performed by an experienced operator, and that the equipment used is maintained in a suitable state. Similar rules are also described in USP 37, EP 6, and BP 2014. So the allowable limit of “System repeatability” becomes reduced, and the reliability of RSD in term of statistics becomes poor with a decreasing number of replicate injections. However, the reliability of measurement RSD (n=1) by ISO 11843-7 is statistically equal to that of measurement RSD from repetitive measurement (n=40) [66]. Therefore, the system repeatability estimation in quantitative

HPLC of herbal medicines by ISO 11843-7 has some advantages compared with that by repetitive measurement, providing a reliable method validation. Furthermore, ISO 11843-7 is also useful to examine the allowable limit test of “System repeatability” in “Liquid chromatography” by saving not only considerable chemical amounts but also experimental time.

The present LC-3ECD was applied to quantitatively analyze caffeoylquinic acids and flavonoids in *Flos Chrysanthemi* and their sulfur-fumigated products. The present LC-3ECD method provided high sensitivity and accurate measurement of 9 analytes (NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, ICHA C, LTG, and LT) for *Flos Chrysanthemi*. Significant chemical differences were found among four cultivars of *Flos Chrysanthemi* and their sulfur-fumigated ones by the content determination. Compared with the non-fumigated samples, nearly 60% of LTG and more than 47% of caffeoylquinic acids were lost in the sulfur-fumigated samples. Sulfur fumigation showed a destructive effect on *Flos Chrysanthemi*. It would be helpful for the standardization and quality control of *Flos Chrysanthemi*. A PCA was carried out using the content results of caffeoylquinic acids and flavonoids determined by the present LC-3ECD. The classification of *Flos Chrysanthemi* samples was revealed by PCA. On the PCA scores plots, the groups of ‘HJ’, ‘BJ’, ‘CJ’, ‘GJ’, ‘S-HJ’ and ‘S-BJ’ samples were clearly classified, where each group of samples were clustered together. Furthermore, on the PCA loadings plots, the similarity and inhomogeneity of 9 analytes in four cultivated varieties of *Flos Chrysanthemi* and their sulfur-fumigated varieties were clearly exhibited. It indicated that significant differences were also found among four cultivars of *Flos Chrysanthemi* and their sulfur-fumigated ones by PCA. Therefore, the present LC-3ECD coupled with PCA study was clearly applicable to elucidate the significant chemical differences for herbal medicines.

By the present LC-3ECD, 6 caffeoylquinic acids of NCHA, CHA, CCHA, CFA, ICHA B, and ICHA C, 4 flavonoids of STL, BC, WGD, and BCE, and 1 phenylethanoid glycoside of FTA were simultaneously determined in SHL preparations. The results demonstrated that the present LC-3ECD has achieved desired linearity ($r > 0.999$), precision (RSD <2.5%), accuracy (recovery, 95.6-103.6%), and high sensitivity (limit of detection, 0.11-0.90 ng/ml). The quality of SHL oral liquid and lyophilized powder for injection was evaluated by simultaneously determining these 11 bioactive components. In ChP 2010, the CHA and BC content in SHL oral liquid is regulated to be not less than 0.6 mg/ml and 10.0 mg/ml, respectively. The CHA and BC content in SHL lyophilized powder for injection is regulated to be not less than 14.2 mg/g and 213 mg/g, respectively. All batches of SHL samples analyzed were found to comply with these limits. However, the contents of these 11 components differed greatly among the different batches of SHL samples. Therefore, the multiple components determination by the present LC-3ECD could reflect the quality of herbal medicines, comprehensively.

EXPERIMENTAL SECTION

Chemicals and materials

Standard compounds

Neochlorogenic acid (NCHA, 5-*O*-caffeoylquinic acid, >98%) and cryptochlorogenic acid (CCHA, 4-*O*-caffeoylquinic acid, >98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Isochlorogenic acid A (ICHA A, 3, 5-dicaffeoylquinic acid, >98%), isochlorogenic acid B (ICHA B, 3, 4-dicaffeoylquinic acid, >98%), isochlorogenic acid C (ICHA C, 4, 5-dicaffeoylquinic acid, >98%), scutellarin (STL, >98), and wogonoside (WGD, >98%) were purchased from Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China). Forsythoside A (FTA, >98%) and protocatechuic aldehyde (PAD, >98%) were purchased from Shanghai Winherb Medical Science Co., Ltd. (Shanghai, China). Chlorogenic acid (CHA, 3-*O*-caffeoylquinic acid, >98%), caffeic acid (CFA, >98%), luteolin (LT, >98%), ethyl gallate (EG, >98%) and propyl gallate (PG, >98%) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Baicalin (BC, >95%), baicalein (BCE, >98%), kaempferol (KF, >95%), and butyl gallate (BG, >98%) were purchased from Wako Pure Chemical Industries, Co. Ltd. (Osaka, Japan). Luteolin 7-*O*-glucoside (LTG, >96%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPP, Beijing, China).

BC (Japanese Pharmacopoeia (JP) reference standard, >99%) used in the HPLC-UV system was obtained from the Pharmaceutical and Medical Device Regulatory Science Society of Japan (PMRJ).

Herbal medicines

The sample of *Scutellaria Radix*, the dried root of *Scutellaria baicalensis* Georgi (Fam. Labiatae), was purchased from Uchida Wakanyaku Ltd. (Tokyo, Japan).

Flos Chrysanthemi of *C. morifolium* cv. ‘Hangju’ (HJ) (No. 111, 112, 113, 114, 115, 116), ‘Boju’ (BJ) (No. 121, 122, 123, 124, 125, 126), ‘Chuju’ (CJ) (No. 131, 132, 133, 134, 135, 136), and ‘Gongju’ (GJ) (No. 141, 142, 143. 144, 145, 146) were directly collected from the cultivation farms in Tongxiang county (Zhejiang province, China), Bozhou city (Anhui province, China), Chuzhou city (Anhui province, China), and Huangshan city (Anhui province, China), respectively, which were harvested in bloom between October and November, 2012. Samples of the sulfur-fumigated ‘HJ’ (‘S-HJ’, No. 211, 212, 213, 214, 215, 216) and the sulfur-fumigated ‘BJ’ (‘S-BJ’, No. 221, 222, 223, 224, 225, 226) were the sulfur-fumigated products of HJ and BJ mentioned above. The sulfur fumigation treatments were operated by the local experienced herbal farmers following the following procedures. Fresh flowers were placed in the upper levels of a closed chamber and sulfur powder was burned at the bottom of the chamber overnight. Sulfur dioxide was released into the chamber during this process and penetrated into the flowers. After fumigation, the samples were dried

in the sun. All samples for the current study were authenticated by Prof. Zhimin Wang (China Academy of Chinese Medical Sciences). The voucher specimens were deposited in the Herbarium of the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, PR China.

Commercial Shuang-Huang-Lian (SHL) oral liquid products of SHL1 [Lot no. 112110426 (concentrated), 112111828, 112112326, 112103022, 112101127], SHL2 (lot no. 12090517, 12102632, 12102812, 12111317, 12112717), SHL3 (lot no. 20121037, 20121039), and SHL4 (lot no. 130204, 130105) were purchased from Tailong Pharmaceutical Co., Ltd. (Zhengzhou, China), Sanjing Pharmaceutical Co., Ltd. (Harbin, China), Dongguan Asia Co., Ltd. (Dongguan, China), and Fusen Pharmaceutical Co., Ltd. (Nanyang, China), respectively. Commercial SHL lyophilized powder for injection products of SHL5 (lot no. 1206222, 1206223, 1212016, 1206104, 1209105, 1211112, 1211101, 1203105, 1209112, 1211106, 1209106) and SHL6 (lot no. 20111001) were purchased from Harbin Pharmaceutical Co., Ltd. second Chinese medicine factory (Harbin, China) and Songhua River Pharmaceutical Co., Ltd. (Harbin, China), respectively. *Flos Lonicerae*, *Radix Scutellariae*, and *Fructus Forsythiae* raw herbs were collected from Shandong, Heilongjiang, and Hunan provinces (China), respectively.

Others

The water used was distilled and purified with a NANO Pure II filtering system (Barnstead Co., Ltd., Boston, MA, USA). Other chemical reagents were of analytical or HPLC grade.

Chapter 1

Preparation of mixed standard solutions for *Flos Chrysanthemi*

Primary stock solutions of NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, ICHA C, LTG and LT were separately prepared by dissolving the accurately weighed standard compounds in MeOH. A series of mixed standard solutions were prepared by diluted each stock solution to the desired concentration by an MeCN-H_2O-H_3PO_4 mixture (8.4:91.6:0.5, v/v/v). Each mixed standard solution contains constant concentrations of three ISs (10, 10, and 25 ng/ml for PAD, EG, and BG, respectively) by adding the appropriate amount of 3 ISs before diluting to volume.

Preparation of mixed standard solutions for SHL preparations

Primary stock solutions of NCHA, CHA, CCHA, CFA, ICHA B, ICHA C, STL, BC, WGD, BCE and FTA were separately prepared by dissolving the accurately weighed standard compounds in MeOH. A series of mixed standard solutions were prepared by diluted each stock solution to the desired concentration by an MeCN-H_2O-H_3PO_4 mixture (8.6:91.4:0.5, v/v/v). Each mixed standard solution contains constant concentrations of three ISs (0.05, 0.02, and 0.08 μg/ml for PAD, PG, and KF, respectively) by adding the appropriate amount of 3 ISs

before diluting to volume.

Chapter 2

Preparation of standard solution of baicalin for HPLC-UV

A stock solution of BC was prepared at a concentration of 0.5 mg/ml in MeOH. The stock solution was diluted by 10 fold with 70% MeOH as the standard solution of BC. For the precision plots, the standard solution of BC was diluted to 16, 40, 80, and 160 ng/ml by 70% MeOH.

Preparation of test solutions of *Scutellariae Radix* for HPLC-UV

A test solution of *Scutellariae Radix* was prepared according to the JP 16. A pulverized *Scutellariae Radix* (0.5 g) was accurately weighed and extracted by reflux in a water bath with 30 ml of 70% MeOH for 30 min. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant was collected. Then the residue was washed twice by 30 ml of 70% MeOH with a 5 min shake and centrifuged at 3000 rpm for 5 min. All of the supernatants were combined together and diluted to 100 ml by 70% MeOH. Then the extract solution was diluted by 10 fold with 70% MeOH. The diluted solution was filtered through a 0.45 μm membrane, and this filtrate was used as a test solution.

Preparation of mixed standard solution for LC-3ECD

Primary stock solutions of NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, ICHA C, LTG, LT, PAD (IS_1), EG (IS_2), and BG (IS_3) were separately prepared by dissolving the accurately weighed standard compounds in MeOH. The mixed standard solution containing 30 ng/ml of CHA, CCHA, ICHA B, ICHA A, LTG, and LT, 25 ng/ml of BG, 20 ng/ml of NCHA, and 10 ng/ml of PAD, EG, CFA, and ICHA C were prepared by diluted each stock solution for several fold by an MeCN-H_2O-H_3PO_4 mixture (8.4:91.6:0.5, v/v/v).

Software to obtain measurement RSD based on ISO 11843-7

The digital data of chromatogram was required to obtain measurement RSD of chromatographic peak area and/or height in HPLC for determining the analytes by ISO 11843-7. The digital data of a chromatogram were recorded by a personal computer at sampling intervals of 0.2 s/point. The calculation of measurement RSD based on the ISO 11843-7 was performed with the software (TOCO, Institute of FUMI Theory, Chiba, Japan).

Chapter 3

Preparation of test solutions of *Flos Chrysanthemi*

The dried *Flos Chrysanthemi* was milled and sieved by using an 80 mesh sieve. An accurately weighted amount of the *Flos Chrysanthemi* powder (0.10 g) was diluted by 70% MeOH to 40 ml with the addition of 3 ISs (80 μg of PAD, 80 μg of EG, and 200 μg of BG). The mixture was extracted by ultrasonication (180 W, 47 kHz) at room temperature for 20

min. The extracted solution was prepared by the method of weight relief, by which the weight lost in the extraction procedure was compensated. Then after filtering the mixture, the supernatant was diluted by 200 fold with an MeCN-H_2O-H_3PO_4 mixture (8.4:91.6:0.5, v/v/v). The diluted solution was filtered through a 0.22 μm membrane, and 5 μl was injected.

Preparation of test solutions of SHL preparations

A 0.1 ml amount of SHL oral liquid or approximate 50 mg of SHL lyophilized powder for injection was dissolved with 50% MeOH in a 10 ml volumetric flask. 100 μg of PAD, 40 μg of PG, and 160 μg of KF were added into it as ISs. The mixture was pretreated in an ultrasonic water bath for 20 min. After ultrasonication, the mixture was placed to room temperature and filled to the volume of 10 ml. Then the mixture solution was diluted by 200 fold with an MeCN-H_2O-H_3PO_4 mixture (8.6:91.4:0.5, v/v/v). The diluted solution was filtered through a 0.22 μm membrane as sample solution, and 5 μl was applied to the LC-3ECD system. The negative samples of “LF”, “LS”, and “FS” for LC-3ECD analyses were prepared with the same procedure above with no addition of 3 ISs.

Preparation of negative SHL samples

The preparation of SHL oral liquid was recorded in ChP 2010 [9] as follows: 375 g of *Radix Scutellariae*, 375 g of *Flos Lonicerae*, and 750 g of *Fructus Forsythiae* were decocted, concentrated, extracted with ethanol, adjusted pH to 7 with HCl and NaOH, distilled to eliminate solvent and the residues were dissolved and diluted with water to 1000 ml. The negative SHL liquid extract samples of “LF” (*Flos Lonicerae* plus *Fructus Forsythiae*), “LS” (*Flos Lonicerae* plus *Radix Scutellariae*), and “FS” (*Fructus Forsythiae* plus *Radix Scutellariae*) were prepared in laboratory according to the above method with no addition of *Radix Scutellariae*, *Fructus Forsythiae*, or *Flos Lonicerae*, respectively.

Procedure of principle component analysis

The software of SIMCA-P +12.0 was applied for PCA in the present study. The primary data were prepared in an Excel file containing the contents of the 9 analytes (NCHA, CHA, CCHA, CFA, ICHA A, ICHA B, ICHA C, LTG, and LT) in each sample analyzed. Then the data set (primary data mentioned above) was imported to the software in a new project, in which 9 analytes were set as variables and the samples was set as observations. Various components were obtained when ‘Autofit’, ‘Next Component’, or ‘First Two Components’ under the menu bar of ‘Analysis’ was performed. According to the parameter values, such as ‘R2X’, ‘R2X (cum)’, and ‘Eigenvalues’, several components were selected as a fitting PC model. Then, the scores plot and the loadings plot could be obtained from the PC model results.

ACKNOWLEDGEMENTS

The studied described herein were carried out in the Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science. I would like to express my gratitude to the international exchange program between the China Academy of Chinese Medical Science and the School of Pharmacy, Tokyo University of Pharmacy and Life Sciences. I sincerely thank Tokyo University of Pharmacy and Life Sciences for accepting me and research support.

I sincerely thank my supervisor, Professor Hideki Hakamata for his unwavering support, patient guidance, and warm encouragement throughout my graduate studies and the preparation of the dissertation. I also sincerely thank Emeritus Professor Fumiyo Kusu for her accepting me to the Department of Analytical Chemistry, kind guidance, and unreserved support.

I gratefully acknowledge Ph.D. Akira Kotani for his kind guidance, advice, and warm assistance. I would like to acknowledge Ph.D. Kouji Takahashi and teacher Kazuhiro Yamamoto for their help during my period in Japan. Many thanks go to Risa Tsutsumi and other students in the Department of Analytical Chemistry, for their generous help.

I sincerely thank Professor Akira Dobashi, Professor Yoshihiro Mimaki, and Professor Kinzo Watanabe for their expert advice and guidance in the process of preparing my dissertation.

Thanks also go to the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences and Professor Zhimin Wang for supporting my continuing studies.

Finally, I would like to thank my family members for their tremendous support.

REFERENCES

[1] Jiang W.Y., *Trends Pharmacol. Sci.*, **26,** 558–563 (2005).

[2] Normile D., *Science,* **299,** 188–190 (2003).

[3] Di X., Chan K. K. C., Leung H.W., Huie C.W., *J. Chromatogr. A,* **1018,** 85–95 (2003).

[4] Janeiro P., Brett A., *Anal. Chim. Acta,* **518,** 109–115 (2004).

[5] Hotta H., Sakamoto H., Nagano S., Osakai T., Tsujino Y., *Biochim. Biophys. Acta*, **1526,** 159-167 (2001).

[6] Sun Z., Ma Z., Zhang W., Wang X., Fan C., Li G., *Anal. Bioanal. Chem.*, **379,** 283-286 (2004).

[7] Chen X. C., Kotania A., Hakamata H., Wang J., Du S. Y., Kusu F., *J. Chromatogr. A,* **1256,** 105–113 (2012).

[8] Lai J. P., Lim Y.H., Su, J., Shen H. M., Choon N. O., *J. Chramatogr. B,* **848,** 215–225 (2007).

[9] Chinese Pharmacopoeia Commission, Pharmacopoeia of the People's Republic of China 2010 Vol. 1, 2010.

[10] Kim H. J., Lee Y. S., *Planta Med.*, **71,** 871–876 (2005).

[11] He D. X., Ru X. C., Wen L., Wen Y. C., Jiang H. D., Bruce I. C., Jin J., Ma X., Xia Q., *J. Ethnopharmacol.*, **139,** 68–73 (2012).

[12] Miyazawa M., Hisama M., *Biosci., Biotechnol., Biochem.*, **67,** 2091–2099 (2003).

[13] Peng Y. R., Shi L., Luo Y. H., Ding Y. F., *Lishizhen Med. Mater. Med. Res.*, **17,** 1131–1132 (2006).

[14] Hua B., Lv G. Y., *Qingdao Med. J.*, **38,** 282–283 (2006).

[15] Xie Y. Y., Yuan D., Yang J. Y., Wang L. H., Wu C. F., *J. Asian Nat. Prod. Res.*, **11,** 771–778 (2009).

[16] Lee J. S., Kim H. J., Lee Y. S., *Planta Med.*, **69,** 859–861 (2003).

[17] Chu Q., Fu L., Guan Y., Ye J., *J. Agric. Food Chem.*, **52,** 7828–7833 (2004).

[18] Liang X., Wu H., Su W., *Food Anal. Methods,* **7,** 197–204 (2014).

[19] Wang T., Zhu Z., Guo Q., Mao P., *Biochem. Syst. Ecol.*, **47,** 74–79 (2013).

[20] Wang Y. -J., Yang X. -W., Guo Q. -S., Liu H.-T., *Chromatographia,* **70,** 109-116 (2009).

[21] Wang S., Hao L.J., Zhu J.J., Zhang Q.W., Wang Z. M., Zhang X., Song X. M., *Phytomedicine,* **21,** 773–779 (2014).

[22] Clifford M. N., Wu W., Kirkpatrick J., Kuhnert N., *J. Agric. Food. Chem.*, **55,** 929–936 (2007).

[23] Cao G., Cai H., Cong X., Liu X., Ma X., Lou Y., Qin K.M., Cai B., *Analyst,* **137,** 3828–3838 (2012).

[24] Sun Q. -L., Hua S., Ye J. -H., Zheng X. -Q., Liang Y.- R., *Afr. J. Biotechnol.*, **9,** 3817–3821 (2010).

[25] Lin L. -Z., Harnly J. M., *Food Chem.*, **120,** 319–326 (2010).

[26] Avula B., Wang Y. H., Wang M., Avonto C., Zhao J., Smillie T. J., Rua D., Khan I. A., *J.*

Pharm. Biomed. Anal., **88,** 278–288 (2014).
[27] Kan W. L., Ma B., Lin G., *Front. Pharmacol.*, **2,** 1–7 (2011).
[28] Jiang X., Huang L. F., Zheng S. H., Chen S. L., *Phytomedicine*, **20,** 97–105 (2013).
[29] Yang M., Sun J. H., Lu Z. Q., Chen G. T., Guan S. H., Liu X., Jiang B. H., Ye M., Guo D. A., *J. Chromatogr. A*, **1216**, 2045–2062 (2009).
[30] Kim J. Y., Cho J. Y., Ma Y. K., Park K. Y., Lee S. H., Ham K. S., Lee H. J., Park K. H., Moon J. H., *Food Chem.*, **125,** 55–62 (2009).
[31] Lou Z. X., Wang H. X., Zhu S., Ma C. Y., Wang Z. P., *J. Food Sci.*, **76,** 398–403 (2011).
[32] Li Y. L., But P. P., Ooi V. E., *Antivir. Res.*, **68**, 1–9 (2005).
[33] Iwai K., Kishimoto N., Kakino Y., Mochida K., Fujita T., *J. Agric. Food Chem.*, **52,** 4893–4898 (2004).
[34] Gao Z. H., Huang K. X., Yang X. L., Xu H. B., *Biochim. Biophys. Acta*, **1472,** 643–650 (1999).
[35] Hong H., Liu G. Q., *J. Asian Nat. Prod. Res.*, **9,** 135–143 (2007).
[36] Woo K. J., Lim J. H., Suh S., Kwon Y. K., Shin S. W., Kim S. C., Choi Y. H., Park J. W., Kwon T. K., *Immunobiology*, **211,** 359–368 (2006).
[37] Luo P., Tan Z. H., Zhang Z. F., Zhang H., Liu X. F., Mo Z. J., *Yakugaku Zasshi*, **128,** 1293–1299 (2008).
[38] Ikemoto S., Sugimura K., Yoshida N., Yasumoto R., Wada S., Yamamoto K., Kishimoto T., *Urology*, **55,** 951–955 (2000).
[39] Li C. R., Lin G., Zuo Z., *Biopharm. Drug Dispos.*, **32,** 427–445 (2011).
[40] Pan Z. W., Feng T. M., Shan L. C., Cai B. Z., Chu W. F., Niu H. L., Lu Y. J., Yang B. F., *Phytother. Res.*, **22,** 1428–1433 (2008).
[41] Liao J. F., Hung W. Y., Chen C. F., *Eur. J. Pharmacol.*, **464,** 141–146 (2003).
[42] Qu H. H., Zhang Y. M., Wang Y., Li B. X., Sun W. J., *J. Pharm. Pharmacol.*, **60,** 261–266 (2008).
[43] Qu H, H., Zhang Y. M., Chai X. Y., Sun W. J., *Bioorg. Chem.*, **40,** 87–91 (2012).
[44] Nishibe S., Okabe K., Tsukamoto H., Sakushima A., Hisada S., Baba H., Akisada T., *Chem. Pharm. Bull.*, **30,** 4548–4553 (1982).
[45] Kimura Y., Okuda H., Nishibe S., Arichi S., *Planta Med.*, **53,** 148–153 (1987).
[46] Nishibe S., Okabe K., Tsukamoto H., Sakushima A., Hisada S., *Chem. Pharm. Bull.*, **30,** 1048–1050 (1982).
[47] Kim J. M., Kim S., Kim D. H., Lee C. H., Park S. J., Jung J. W., Ko K. H., Cheong J. H., Lee S. H., Ryu J. H., *Eur. J. Pharmacol.*, **660,** 326–333 (2011).
[48] Chen G., Zhang H. W., Ye J. N., *Talanta*, **53,** 471–479 (2000).
[49] Li X. J., Zhang Y. P., Yuan Z. B., *Chromatographia*, **55,** 453–456 (2002).
[50] Ma Y. C., Wang X. Q., Hou F. F., Ma J., Luo M., Chen A., Jin P., Lu S., Xu I., *J. Pharm. Biomed. Anal.*, **54,** 265–272 (2011).
[51] Su Q., Liu Q. F., Wang Y., Bi K. S., Luo G. A., *Chem. Res. Chinese U.*, **25,** 122–124 (2009).

[52] Zhang Y. M., Yan D., Zhang P., Ren Y. S., Zhang S. F., Feng X., Xiao X. H., *Acta Pharm. Sinica*, **45,** 93–97 (2010).
[53] Yang D. Z., An Y. Q., Jiang X. L., Tang D. Q., Gao Y. Y., Zhao H. T., Wu X. W., *Talanta*, **85,** 885–890 (2011).
[54] Wu J., Chen X., An Y. Q., Tang D. Q., Yin X. X., Jiang X. L., Zhao Z. M., *Lat. Am. J. Pharm.*, **30,** 2048-2056 (2011).
[55] He B., Yang S. Y., Zhang Y., *Acta Pharm. Sinica*, **47,** 1653–1659 (2012).
[56] Han J., Ye M., Guo H., Yang M., Wang B. R., Guo D. A., *J. Pharm. Biomed. Anal.*, **44,** 430–438 (2007).
[57] He X., Shi C. W., Feng Y., *China J. Chin. Mater. Med.*, **22,** 604–605 (1997).
[58] Liu L., Suo Z. R., Zheng J. B., *Chinese J. chromatogr.*, **24,** 247–250 (2006).
[59] Matsumoto D., Kotani A., Hakamata H., Takahashi K., Kusu F., *J. Chromatogr. A*, **1217,** 2986–2989 (2010).
[60] Day R. A., Underwood A. L., "Quantitative analysis", Prentice Hall, Englewood Cliffs, NJ, 1991.
[61] Miller J.C., Miller J.N., *Analyst*, **113,** 1351 (1988).
[62] The Ministry of Health, Labour and Welfare, The Japanese Pharmacopoeia 16th ed., 2011.
[63] The United States Pharmacopeial Convention, The United States Pharmacopeia 37th ed., 2014.
[64] The Convention on the Elaboration of a European Pharmacopoeia, European Pharmacopoeia 6th ed., 2007.
[65] The British Pharmacopoeia Commission Secretariat of the Medicines and Healthcare Products Regulatory Agency, British Pharmacopoeia 2014, 2014.
[66] Hayashi Y., Matsuda R., Poe P. B., *Analyst*, **121,** 591 (1996).
[67] Ye J. X., Wei W., Quan L. H., Liu C. Y., Chang Q., Liao Y. H., *J. Pharm. Biomed. Anal.*, **52,** 625–630 (2010).
[68] Li Z. X., Qiu F., Yin X. B., Zou H. Y., Gong M. X., Zhai Y. S, Ni J., *Anal. Methods*, **5,** 2748–2792 (2013).
[69] Boyle S. P., Doolan P. J., Andrews C. E., Reid R. G., *J. Pharm. Biomed. Anal.*, **54,** 951–957 (2011).
[70] Kotani A., Kojima S., Hakamata H., Kusu F., *Anal. Biochem.*, **350,** 99–104 (2006).
[71] Xie Y., Qu J., Wang Q., Wang Y., Yoshikawa M., Yuan D., *J. Agric. Food Chem.*, **60,** 12574 (2012).
[72] Yin L., Lu B., Qi Y., Xu L., Han X., Xu Y., Peng J., Sun C., *J. Pharm. Biomed. Anal.*, **49,** 1101 (2009).
[73] Okamura N., Miki H., Harada T., Yamashita S., Masaoka Y., Nakamoto Y., Tsuguma M., Yoshitomi H., Yagi A., *J. Pharm. Biomed. Anal.*, **20,** 363 (1999).
[74] Wua X., Zhou Y., Yin F., Mao C., Li L., Cai B., Lu T., *Phytomedicine*, **21,** 551 (2014).

[75] Song G., Zhou L., Sheng N., Zhang X., Xu Y., Zhang L., Li X., *J. Pharm. Biomed. Anal.*, **107,** 304 (2015).
[76] Chen H., Chen X., Han Q., Wu J., Tang D., Du Q., Yin X., and Yang D., *Anal. Bioanal. Chem.*, **404,** 1851 (2012).
[77] Hayashi Y., Matsuda R., *Anal. Sci,*. **10,** 725–730 (1994).
[78] Poe R. B., Hayashi Y., Matsuda R., *Anal. Sci.*, **13,** 951–962 (1997).
[79] Matsuda R., Hayashi Y., Sasaki K., Saito Y., Iwaki K., Harakawa H., Satoh M., Ishizuki Y., Kato T., *Anal. Chem.*, **70,** 319–327 (1998).
[80] Hayashi Y., Matsuda R., Haishima Y., Yagami T., Nakamura A., *J. Pharm. Biomed. Anal.*, **28,** 421–429 (2002).
[81] Yomota C., Matsumoto Y., Okada S., Hayashi Y., Matsuda R., *J. Chromatogr. B*, **703,** 139 (1997).
[82] Kotani A., Hayashi Y., Kusu F., *Chromatography*, **33,** 145 (2012).
[83] The International Organization for Standardization, ISO 11843 Part 7 Methodology based on stochastic properties of instrumental noise, 2012.
[84] Jolliffe, I.T., “Principal Component Analysis”, Springer, Berlin, 1986.
[85] Sun J., Chen P., *J. Pharm. Biomed. Anal.*, **61,** 142–149 (2012).
[86] Zhao G. L., Liu J. J., Lin D., Zhang X. H., Wang H., *Chinese J. Chin. Trad. Pat. Med.*, **24,** 973–976 (2002).
[87] Li C. R., Zhou L. M., Lin G., Zuo Z., *J. Pharm. Biomed. Anal.*, **50,** 298–306 (2009).
[88] Horvath C. R., Martos P. A., Saxena P. K., *J. Chromatogr. A*, **1062,** 199–207 (2005).