Systematic, computer-assisted development of high performance liquid chromatography for multi-component analysis

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Abstract: The aim of this study was to develop a multi-component determination analytical method. The analytical Quality by Design (QbD) concept was used in the beginning of high performance liquid chromatography (HPLC) method establishment for compound traditional Chinese medicine (TCM) preparation using Diode-array detector (DAD) and Evaporative Light Scattering Detector (ELSD) in series. The QbD workflow was discussed and demonstrated with systematic HPLC method development, including the risk assessment, the design of experiments (DOEs), and assessment of the data to provide a method operable design space (DS). Modeling software Drylab was employed to set up experiments for development of a simple and robust separation method and visually to achieve required criteria as an initial DS of the analytical method based on simulation. To improve the method development and optimization step, the statistical software JMP@ (SAS Institute) was applied to simultaneously optimize the chromatographic conditions such as the gradient time, the concentration of aqueous phase, the column temperature, the flow rate and the ELSD parameters. Finally, a successful HPLC method was developed and validated to verify the robustness of QbD system. The use of QbD workflows streamlines the development of methods as compared to traditional approaches. With the addition of systematic DOEs, the optimization resulted in critical resolution Rs,crit ≧1.5 for all the six compounds researched . As a result, robust and reliable method operable design region was established. The method had fewer issues and failures throughout the lifecycle due to the knowledge gained via the QbD process.

Keywords: Quality by Design; Drylab; JMP; HPLC-DAD/ELSD; multi-component assay; Longjiatongluo capsule

1. Introduction

High performance liquid chromatography (HPLC) is the most commonly used analytical method in quality assessment of compound Traditional Chinese Medicine (TCM) preparation. It has been employed to analyze various substances by combining with different detectors. However, for the lack of systematization and normativity in both theoretical and operational views, its repeatability and robustness are not very well. On one hand, small changes in experimental conditions often result in confused peak movements.[1] On the other hand, the trial and error approach that examines the resolution of peaks until the best method often results in a non-robust performance when transferred into another lab because interactions between factors are not considered.[2] Therefore, if this method is simply applied to the quality control of high-risk drugs, it probably causes errors or faults to some extent. What`s worse, it may lead to adverse drug events. On the other hand, the International Conference on Harmonization (ICH) Q2 lists the characteristics for method validation, such as linearity, precision, accuracy, repeatability and so on.[3] But these robustness tests are typically carried out during the final stages of a method development process in the validation stage, which often leads to undesired results found later on and the developed method has to be redeveloped and revalidated. To avoid these costly repetitions, there is an increasing tendency to include thorough, multifactorial robustness evaluations at an early stage of development, that is to say, to build in quality from the outset.[4]

Quality by design (QbD) is a key principle that has gained much discussion since its initiation as part of the U.S. Food and Drug Administration's (FDA) vision for the 21st century Current Good Manufacturing Practices (cGMPs) guidance on pharmaceutical development. QbD is a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management.[2] Regulatory authorities (FDA, ICH, etc.)

nowadays are promoting and requesting the application of QbD principles to ease the exchange of complex information to support better method control.[5] Although its initiative is primarily intended for pharmaceutical product development, its use in the development of an integrated control strategy that involves analytical technology and methods should not be underestimated. In fact, many of the terms used in the QbD initiative are very familiar to analytical chemists when put into the context of method development activities for new pharmaceutical ingredients.[6] Furthermore, the ICH Q8 made a clear illustration that the QbD concept can be employed to the development, validation and assessment of analytical methods. The appearance of terms such as QbD and Design Space (DS) are an indication of this growing trend which requires a high level of understanding of the basic roles of HPLC.

In QbD concept, wider operating ranges may be possible which can provide greater production or method flexibility, but this often requires more up front effort than a traditional approach. Changes within these ranges and limits do not require prior approval. The application of QbD principles to analytical method development is focused on the concept of building quality into methods during development, instead of testing methods for quality after development. The QbD concept may reduce the effort needed to gain desired information. Compared to traditional one-factor-at-a-time (OFAT) experimentation, the QbD approach leads to a better understanding of the factor influencing chromatographic separation and hence has the potential for development of analytical method. A very useful component of QbD is the understanding of factors and their interaction effects by a desired set of experiments.[2]

Five key steps of QbD, are commonly described:[7] first, objectives of the analytical target method performance criteria are clearly defined (method intent), especially the critical quality attributes (CQAs); second, the critical process parameters are identified by quality risk assessment, which can safely eliminate a large number of parameters by using Ishikawa diagrams that segregate risks into different categories on the basis of prior knowledge and initial experimentation; third, knowledge space is systematically investigated by design of experiments (DoEs), which provides an effective way to simultaneously evaluate the effects of factors and their interactions. With modeling software or statistical software, the relationship between the critical parameters and the CQAs was simulated and predicted. The modeling and simulation Drylab software is demonstrated to be a useful tool for optimizing the separation of model drug candidates. The Drylab method development wizard (MDW) can assist new chromatographers in setting up experiments for development of a simple and robust separation method. Drylab software comes equipped with a number of sample data files that allow new users to familiarize themselves with the variables affecting a separation. With a minimum number of experimental runs, accurate predictions for a broad range of separation conditions can be achieved. This leads to substantial time-saving and more effective use of staff and resources.[8] Furthermore, the statistically software is used to identify the optimal operating conditions as well as evaluate the range of several important method parameters.[6] Data were analyzed in JMP using analysis of variance method (ANOVA) by least-square fit which realized statistical evaluation of data obtained from chromatographic analyses of multiple compounds in TCM. Fourth, the DS for analytical methods is defined as a multidimensional space which includes every combination of the variables that have been demonstrated to provide assurance of quality of the data produced by the method and finally the selected method is thoroughly assessed (method evaluation) and a control strategy is implemented (method control) in order to guarantee method robustness.

The Longjiatongluo capsule (LJ) is compound TCM preparation made from *Acanthopanax senticosus* (Rupr. Et Maxim.) Harms and *Dioscorea nipponica* Makino. This medicine is used for curing Apoplexy (mild-to-moderate Brain infarction) by means of promoting blood circulation, removing blood congestion and freeing the network vessels in the theory of Traditional Chinese Medicine.[9] Its active ingredients include Dioscin, Pseudoprotodioscin, Trillin, Protodioscin, Chlorogenic acid, Syringin, Eleutheroside E, Rutin, Isofraxidin, etc.[10] Until now, the content of saponin hydrolyzate Diosgenin is the only index of quantitative assessment for the production process of LJ preparation. However, the single component control method is incapable of monitoring the content changes of other active ingredients in TCM. On the other hand, multi-component assay has been widely researched and accepted in recent years, which improves the quality control standard of TCM. However, few of literature on quantitative assay of these two natural products has been reported. In this paper, according to the QbD concept, we developed the HPLC method of six main active compounds in LJ, the Steroidal saponins compounds Dioscin, Pseudoprotodioscin and Protodioscin from *Dioscorea nipponica* Makino and Chlorogenic acid, Syringin, Isofraxidin from *Acanthopanax senticosus* (Rupr. Et Maxim.) Harms. Since the Steroidal saponins compounds have terminal or even no ultraviolet absorption, [11] we used Evaporative Light Scattering Detector (ELSD) cooperated in series with Diode-array detector (DAD) to simultaneously determine the six analytes.

Recently, a number of articles have been published describing general strategies for the application of QbD principles to analytical measurements[12, 13] and to the development of HPLC methods.[14, 15] Nevertheless, these papers utilized either the computer simulation program or the statistical software to design experiments, while in this article, we combined these two mathematical tools to develop the ultimate method operable control space. We also attempted to explore the combination of two HPLC detectors in use of QbD concept, which provides guidance for method development of multi-dimensional analytical equipments.

2. Experimental

2.1 Chemicals & Eluents

Methanol (HPLC gradient grade) and acetonitrile (HPLC gradient grade) were purchased from Concord Technology, formic acid (HPLC gradient grade) was purchased from Tianjin Kermel, Ultrapure water was obtained from a Milli-Q Plus 185 water purification system from Millipore (Billerica, MA, USA).

Dioscin (100.0%, Batch No.11707), Pseudoprotodioscin (94.8%, Batch No.111855), Protodioscin (94.9%, Batch No.111937), Chlorogenic acid (96.6%, Batch No.110753), Syringin (100.0%, Batch

No.111574) and Isofraxidin (99.5%, Batch No.110837) were purchased from National Institutes for Food and Drug Control (Beijing, China). The Longjiatongluo capule was provided by Shijitianlong Pharmacy (Tianjin, China).

2.2 Equipment & Column

Chromatographic experiments were performed on an Agilent 1260 separation module coupled to an Agilent Guat Pump 1260, an Agilent Diode-array detector (DAD) 1260 and an Agilent Evaporative Light Scattering Detector (ELSD)380 all controlled by ChemStation software (Agilent, USA). PH measurements were performed with a SevenEasy S20 pH meter (Mettler Toledo, Columbus, OH, USA).

The choice of the chromatographic column was based on two main reasons. First, according to the Chinese Pharmacopoeia (2010, Part I), the content assay of both *Acanthopanax senticosus* (Rupr. Et Maxim.) Harms and *Dioscorea nipponica* Makino requires the C_{18} column. The chemistry of the C_{18} column allows a relatively good retention for most of the organic compounds. The most common size C_{18} (250×4.6mm, 5μm) was chosen in our research. Second, Kromasil C_{18} column is covered by unique silicon hydroxyl group, which offers high coverage of Silane. This makes the column available to tolerate a wide range of pH (1.5-9.5). What`s more, by means of screening experiments, the Kromasil C_{18} (250×4.6mm, 5μm) column shows the properties of higher column efficiency, better peak symmetry and higher separative capacity than the other C_{18} columns selected. Therefore, this column was selected to separate the multi-compounds in TCM preparations.

2.3 Software / Data Treatment

DryLab 2000 plus (Rheodyne LLC, CA, USA) software was used for simulating and optimizing the chromatography conditions. Further optimizing and robustness of the method was evaluated with statistical design created by using JMP@ (SAS Institute) software.

2.4 Procedure

All chromatographic experiments were performed in the linear gradient mode, which is the easiest choice and the most commonly used in analytical laboratories.[16] According to several pre-experiments, Acetonitrile (Phase A) and formic acid solution(Phase B) were chosen as the mobile phase. The formic acid was determined as the aqueous component which was applicable to the ELSD detector and optimized the performance of chromatographic peaks. The lowest proportion of eluent A was 5% and the highest was 100%. The DAD detection was carried out at 254 nm (Syringin) and 350nm (Chlorogenic acid, Isofraxidin) simultaneously.

2.5 Standard & Sample preparation

A mixed standard solution containing Dioscin, Pseudoprotodioscin, Protodioscin, Chlorogenic acid, Syringin and Isofraxidin were prepared with methanol as solvent and protected from light by use of amber volumetric flask. Final concentration corresponded to 145, 231, 245, 81, 57 and 95μg/mL, respectively.

A sample solution of LJ was prepared in 85% methanol solution with ultrasonic dissolving for 50 minutes. The sample solution was filtered through a 0.22μm filter membrane and the resulting clear solution was used for the HPLC determination.

3. Results and Discussion

3.1 Definition of analytical target profiles (ATP)

The quality of a chromatographic method is generally defined by some CQAs. These CQAs should be representative of the separation quality between peaks (e.g. difference between retention times, selectivity and resolution) or consider various chromatographic parameters (e.g. asymmetry, efficiency and peak height). These CQAs (i.e. measuring the performance of a chromatographic method) are generally investigated during the method development phase. In addition, some quantitative parameters (e.g. repeatability, trueness, precision and accuracy) are representative of the ability of a method to accurately estimate the compounds concentration in a given sample and are evaluated during the method validation.[17] The primary goal of developing a HPLC multi-compound content method is generally to separate the different components (resolution Rs≧1.5). Here, the critical resolution[18] (Rs,crit≧1.5) - resolution between the least well separated peak pair –was cited.

3.2 Quality risk assessment

In an early risk assessment the critical parameters should be identified. That could be method factors which may affect extraction of the compounds of interest in sample preparation as well as settings in the instrumental analysis. From all the influencing factors, the critical parameters in the overwhelming majority of HPLC separations were the gradient time (t_G/min), the column temperature (Tc/□), the pH of the aqueous phase (eluent B), and the flow rate(V_{liquid}/ mL·min^{-1}). Specially, in this research, we used ELSD connected in series with DAD, so the influencing factors also included the evaporation temperature (T_{Eva}/□), the gas flow (v_{gas} /L·min) and the nebulization temperature (T_{Neb}/□). We suggest a generic Ishikawa diagram (in Fig .1) for the HPLC-DAD/ELSD method.

3.3 Method development strategy using Drylab

3.3.1 Mode 1: LC-RP Gradient / Temperature (4-Run)

The present most successful strategy by starting method development is to study on the influence of the column temperature (Tc) and the elution force of organic eluents (t_G) in a simultaneous mode.[19] We started with four gradient runs at two different temperatures both 10℃ lower than the temperatures suggested by the Drylab MDW, which considering the tolerance of the chromatographic column. Four experiments were carried out at 25℃ and 40℃ with linear gradients and with gradient times of 18 and 53 minutes as the Drylab MDW suggested. The same amount of standard solution (20μL) was injected to keep peak areas constant. All experimental data, such as retention time and peak area of components, dwell volume, column information and gradient conditions were input into Drylab (see Table 1).

The table was inserted into the input data table for Drylab. A 3-dimensional dynamic resolution map of gradient time t_G against column temperature Tc was generated (see Fig. 2). The color code in the resolution map represents the value of the critical resolution (Rs,crit), with warm "orange" colors showing large resolution values (Rs,crit > 1.90) and cold "blue" colors showing low resolution values (Rs,crit < 0.54). The 3-D isoheight solution map shows a robust region that gradient time ranges from 20.0min to 135.0min and the column temperature ranges from 25.0℃to 35.0℃.

The 3-D resolution map shows simulative resolution maps under different combinations of t_G and Tc. To verify the accuracy of this model, two working points (t_G = 30.0min, Tc =25.1℃; t_G = 70.0min, Tc =24.9℃) were chosen in the map. The predicted and experimental chromatograms are shown in Fig.3. Good agreement was found between experimentally obtained and computer-predicted retention times, which indicates that the predicted results could be confirmed experimentally and the accuracy is excellent with a relative error.

3.3.2 Mode 2: LC-RP Gradient / pH (6-Run)

In an additional set of experiments the influence of pH was studied. It is critical to select a pH in which the method is robust, to ensure that minor changes in the aqueous phase will not have a negative influence on the resolution of peaks. Since the wide range of pH, it is difficult to define a region for pH. In this study, we adopted the Drylab software to estimate the influences between gradient time and pH of aqueous phase. Based on prior experiments, we knew that with a formic acid solution (0.1%) at pH of 3.0 the method was robust. So we investigated the variation of pH of the aqueous eluent between pH 2.0 and pH 7.0 and created the 3-D resolution map of Rs,crit vs. pH and gradient time. The result shows that the method is robust in the pH range between 1.9 and 3.0 and a gradient time between 18.0 and 110.0 min.

In conclusion, we can achieve an initial and partial design space (DS) with only eight runs in total. The modeling software Drylab leads to substantial time-saving and more effective use of staff and resources. In addition, the software is an excellent instructional tool for users who are new to chromatography.[10]

3.4 Further optimization strategy using JMP

The Drylab software offers the influences of given factors such as column temperature (Tc), gradient time (t_G) and pH in gradient elution programme, while other parameters that may also affect the quality attributes are limited to be investigated. For example, the ELSD detecting conditions may also have impacts on the chromatogram. Therefore, further optimization was implemented with statistical design by using JMP software. The design was completed with the fractional factorial design to distinguish the significant factors, and then the operable design region was built with the aid of model fitting.

The strategies employ the following workflow:[13]

(1).Factor and response selection: Select the process parameters (factors) identified from the risk assessment and set low and high factor limits for experimentation. According to the initial design space and the prior knowledge about the instrument and the column, factors are presented in Table 2 along with the experimental ranges investigated. For the convenience of control, we took the factor of formic acid concentration (c_{FA}/%) in aqueous phase instead of pH.

(2). DOE Design Selection and Design Layout: Select and generate a statistical design for the factors that allows the determination of important main effects and two-factor interactions using an appropriate statistical software package (e.g. JMP)

(3).DOE HPLC analysis: Translate the design points into a sample set for HPLC analysis. Instrument methods were generated in ChemStation to support factor variations for each of the design points. Samples were evaluated under each design point. Response results were gathered and summarized for statistical analysis.

(4). DOE Statistical Response Analysis: Statistical analysis software was used to evaluate the data from the DOE.

Based on the number of factors, the Plackett-Burman screening design was selected and run codes were generated (see Table 2). HPLC experiments were set according to the code. The critical resolutions were gathered in Table 2. Standard least-squares method was employed to fit the model and analyzed the response.

Table 2 shows that the critical resolutions of runs 3, 6, 9, 10, 11are less than 1.5, which means all those experiments failed. Therefore, we should find out the significant characters that led to the failure, and relocate the factors to meet the Chinese Pharmacopeia (CP) requirement. The statistical results has shown that in the fitting model the p value (Prob>F) is less than 0.05, which means there must be at least one significant factor in the model. By means of model fitting, factors that influence the response (Rs,crit) are figured out in order of the significance. The significant factors are gradient time, column temperature and flow rate, successively. Furthermore, the R square of fitting model is 0.96, which means 96% variation of response can be explained by the model, and the mean line intersects the 95% confidence interval which indicates that the model is significant. In consequence, the fitting model has been verified to be reliable and accurate.

Fig. 4 shows the results of the designed experiment as prediction traces from a least squares fit of the data. The dynamic profiling figure shows the prediction of how the response for critical resolution (y-axis) changes as the input parameters (x-axis) varies. In viewing Fig. 4, horizontal responses indicate that the output is relatively unaffected by changes to the input and can be interpreted as being robust over the range studied. Sloping lines indicate an impact of the x-variable on the response as the parameter varies. Specifically, the data indicates that changes to the formic acid concentration and the ELSD conditions have minor impact relative to the gradient time, column temperature and the flow rate. The use of the predicted responses from the design experiment enables the method developer to optimize the conditions quite readily and observe predicted impact of the changes being made. The three significant factors were reset. Similar to the steps mentioned, we gained a more rigorous range to fit for the HPLC method. Thus, a more robust and reliable design space was achieved (see Table 3).

In viewing Table 3, data in the column of “Factor design” were the initially designed value. It is obviously that the design ranges were wider than the regular control space except for the factors of the formic acid concentration and the ELSD conditions. The adjusted space was verified to be a robust and reliable operating space, which means that variations happened in the operating space can totally be neglected. Note that the regular control spaces of the column temperature (Tc) and the flow rate (v_{liquid}) are +/-5℃ and +/-20% respectively, where failure results happened. In other words, the operating spaces of Tc and v_{liquid} were smaller than the regular ones, which indicates that the regular control space can`t satisfy the robustness requirement.

3.5 Method determination and validation

Based on the operating space built with the PB design, the HPLC-DAD/ELSD method of TCM preparations was developed. Particularly, the gradient elution programme was adjusted to maximize the baseline separation and minimize the elution time in consideration of the various and analogous impurities in the sample solution. Finally, a multi-component determination method was established by using acetonitrile-water (with 0.1% formic acid) as the mobile phase for gradient elution (0-10min, 12%-15%A; 10-20min, 15%-25%A; 20-30min, 25%-30%A; 30-50min, 30%-40%A; 50-55min, 40%-55%A; 55-60min, 55%A; 60-80min, 55%-80%A). The flow rate was 1.0mL/min and the column temperature was 26℃. Dual wavelengths (Syringin, 254nm; Chlorogenic acid, Isofraxidin, 350nm) were selected to get maximal specific detection with a diode-array detector. The other three Steroidal saponins were detected with evaporative light scattering detector at evaporation temperature of 100℃, gas flow of 1.8L/min and nebulization temperature of 40℃. The final method conditions were assessed against the 2010 CP validation characteristics, specifically examining linearity, precision, accuracy (recovery), repeatability and stability in 24 hours. Good linearities of six analytes were obtained with the correlation coefficients ranging from 0.9997 to 0.9999, and the average recoveries (n=9) were 99.7-100.6% with RSD from 0.94% to 1.35%. What`s more, the RSDs of precision, repeatability and stability of the sample were less than 2%. In conclusion, this method is accurate, reliable and repeatable.

The established method was applied to the determination of the six compounds in three batches of LJ, which guaranteed the consistency for different batches.

4. Conclusion

This study has developed a novel HPLC method for multi-compound in Traditional Chinese Medicine preparations made from *Acanthopanax senticosus* (Rupr. Et Maxim.) Harms and *Dioscorea nipponica* Makino. The development has been described in terms of several key concepts of the quality by design paradigm. The QbD workflow starts with understanding the method needs (that is, ATP), identifying risk assessments and implementing DOEs to alleviate experimental risk factors. The approach leverages structural knowledge of the samples, method development wizard, chemometric data reduction, and software-based decision support. The end result is a robust chromatographic method with a well-understood method operable design region. The use of statistical tools to design robustness experiments and optimize method parameters has led to a sensitive yet well-controlled, validated analytical method for multi-component content analysis. The cooperation of the modeling software Drylab and the statistical software JMP has been perfectly and firstly introduced into the analytical method development via QbD concept. Furthermore, this paper is also a first attempt to explore the combination of two HPLC detectors in use of QbD system, which can provide some guidance for analytical method development that use more comprehensive and progressive equipments.

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References

1 L. Didaoui, U. Lewin-Kretzschmar, A. Touabet and W. Engewald, *Chromatographia Supplement*, 2003, 57, 129-135.

2 A. Ayre, P. Mane, K. Ghude, M. Nemade and P. Gide, *Int. J. of .Adv. in. Pharmac. Ana*, 2014, 4, 1-6.

3 ICH Q8 (R2)-Guidance for Industry, Pharmaceutical Development, 2009.

4 K. Monksa, I. Molnára, H. -J. Riegera, B. Bogátib and E. Szabób, *J. Chromatogr. A*, 2012, 1232, 218-230.

5 I. Molnár, H.-J. Rieger and K. E. Monks, *J. Chromatogr. A*, 2010, 1217, 3193–3200.

6 P. F. Gavin and B. A. Olsen, *J. Pharm. Biomed. Anal*, 2008, 46, 431–441.

7 S. Orlandini, S. Pinzauti and S. Furlanetto, *Anal. Bioanal. Chem*, 2013, 405, 443–450.

8 T. H. Hoang, D. Cuerrier, S. McClintock and M. Di Maso, *J. Chromatogr. A*, 2003, 991, 281–287.

9 L. Z. Huang, H. F. Zhao, B. K. Huang, C. J. Zheng, W. Peng and L. P. Qin, *Pharmazie*, 2011, 66, 83-97.

10 S. H. Lin, D. M. Wang, D. P. Yang, J. H. Yao, Y. Tong and J. P. Chen, *Anal. Chim. Acta*, 2007, 599, 98-106.

11 C. Z. Liu, H. Y. Zhou and Q. Yan, *Anal. Chim. Acta*, 2007, 582, 61-68.

12 T. Kawabea, T. Tomitsukab, T. Kajirob, N. Kishib and T. Toyo'okaa, *J. Chromatogr. A*, 2013, 1273, 95-104.

13 F. G. Vogt and A. S. Kord, *J. PHARM. SCI-US*, 2011, 100, 797.

14 G. L. Reid, G. L. Cheng, D. T. Fortin, J. W. Harwood, J. E. Morgado, J. Wang and G. Xue, *J. LIQ. CHROMATOGR. R. T*, 2012, 36, 2612-2638.

15 M. L. Jadhav and S. R, *Chromatography Research International*, 2013, 1-9.

16 J. García-Lavandeira, J. A. Martínez-Pontevedra, M. Lores and R. Cela, *J. Chromatogr. A*, 2006, 1128, 17–26.

17 B. Debrus, D. Guillarme and S. Rudaz, *J. Pharm. Biomed. Anal*, 2013, 84, 215-223.

18 K. Monksa, I. Molnára, H.-J. Riegera, B. Bogátib and E. Szabób, *J. Chromatogr. A*, 2012, 1232, 218-230.

19 A. H. Schmidta and I. Molnarb, *J. Chromatogr. A*, 2002, 948, 51–63.

Figures & Tables

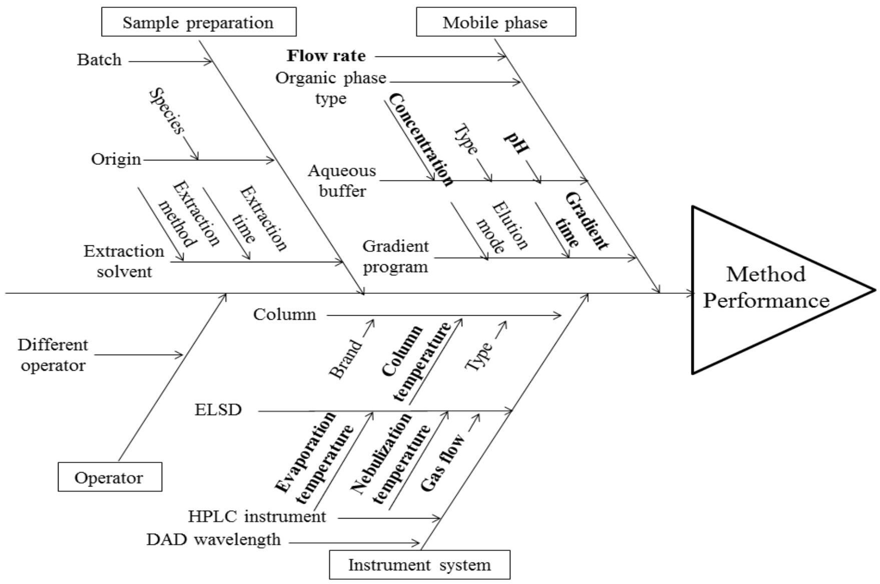

Fig.1 Proposed Ishikawa diagram for a generic HPLC-DAD/ELSD method.

The CPPs which are typically evaluated by a DoE during method development are indicated in bold. The other conditions are usually fixed by preliminary experiments and/or prior knowledge

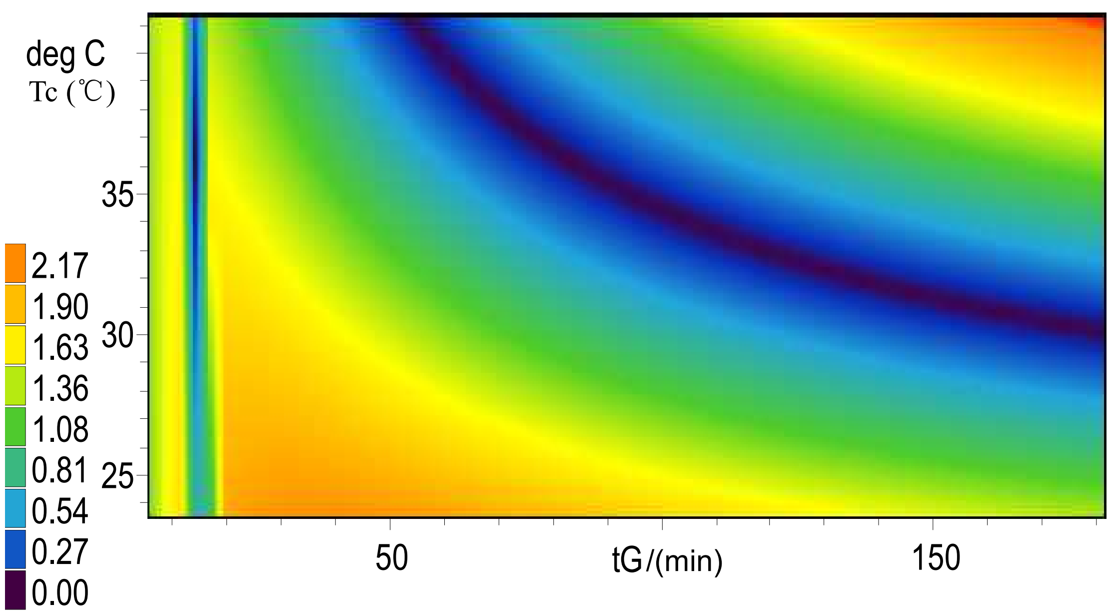

Fig. 2 The chromatic isoheight Solution Map

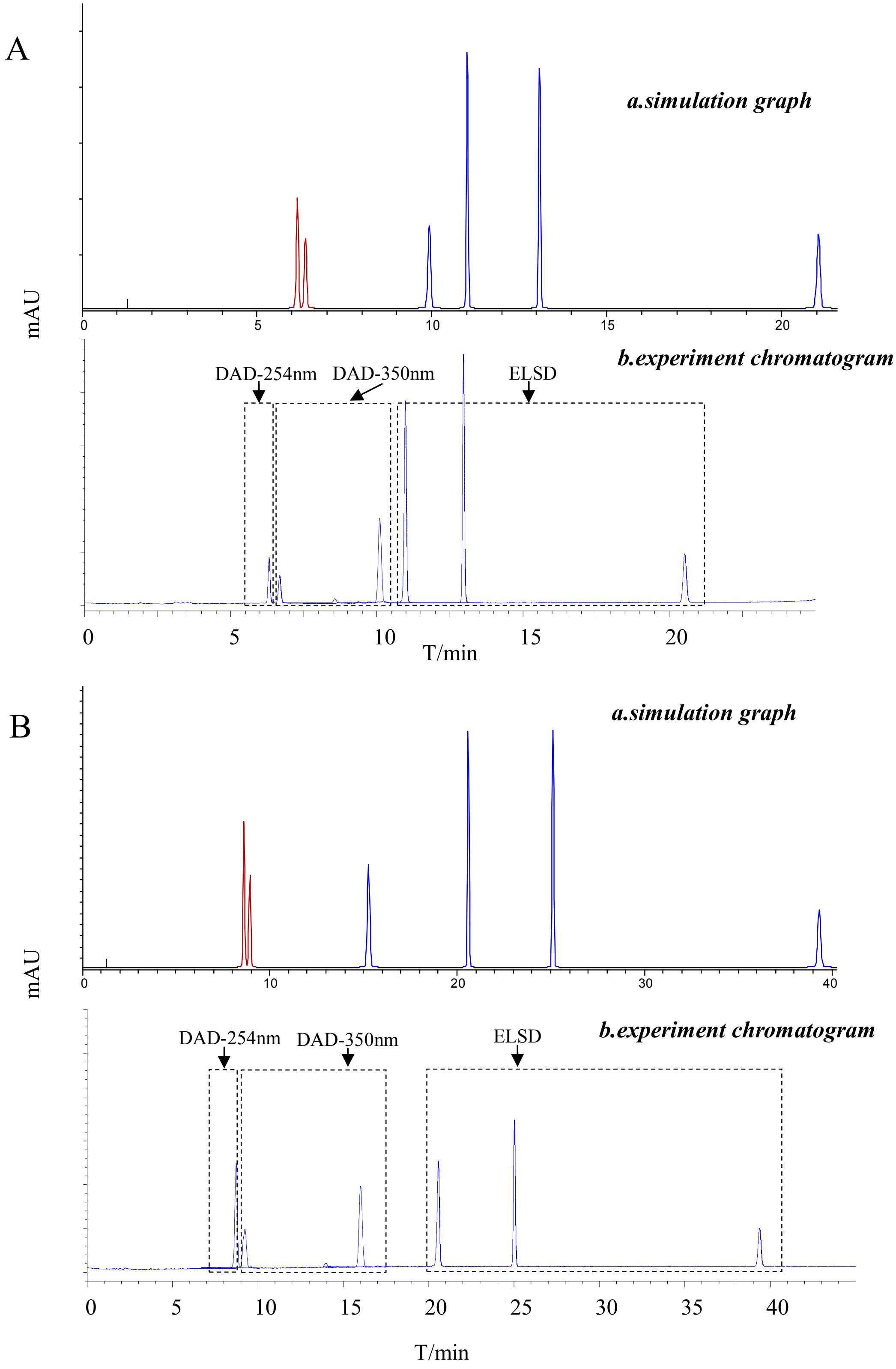

Fig. 3 Chromatograms of Validation

(A. t_G = 30.0min, Tc =25.1℃； B. t_G = 70.0min, Tc =24.9℃)

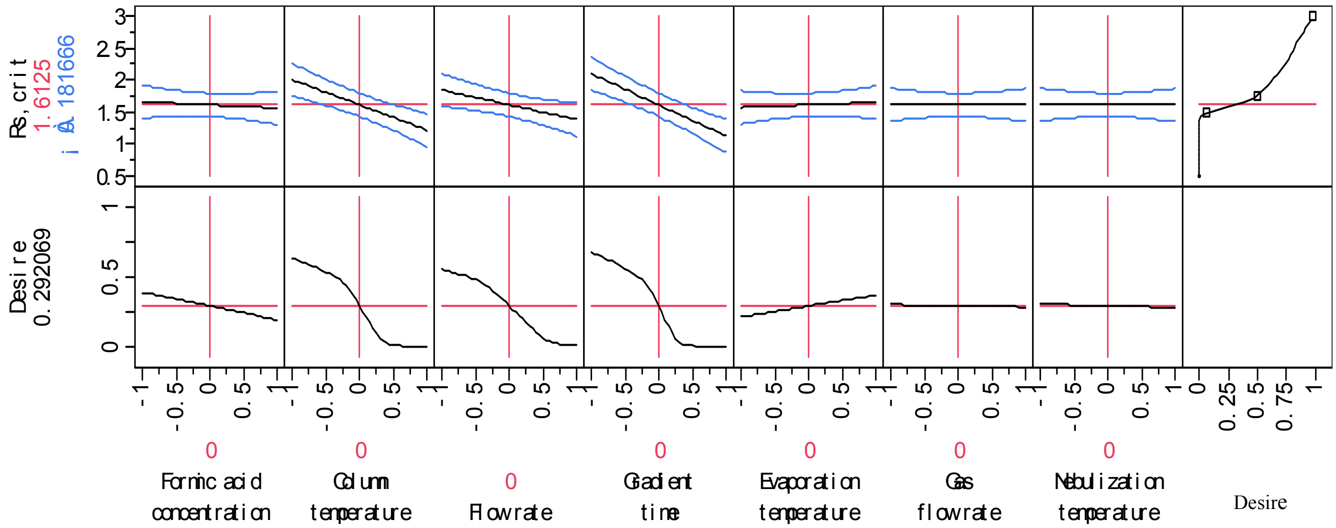

Fig. 4 The factor profiling figure

Table1. Experimental data collected with four gradient runs

No.	Compound name	Tc=25℃				Tc=40℃			
		t_G=18min		t_G=53min		t_G=18min		t_G=53min	
		t_R	Area	t_R	Area	t_R	Area	t_R	Area
1	Syringin	5.065	1200.6	7.698	1178.8	5.027	1202.5	7.479	1786.2
2	Chlorogenic acid	5.248	790.5	7.981	774.7	5.162	787.1	7.509	772.6
3	Isofraxidin	7.674	1184.7	13.256	1186.3	7.578	1181.3	12.727	1184.4
4	Protodioscin	7.860	2782.2	16.631	1596.3	7.892	2705.2	16.782	1510.8
5	Pseudoprotodioscin	9.147	2675.4	20.127	1732	9.213	2642.9	20.406	1621.6
6	Dioscin	14.616	1454.1	31.985	853.6	14.729	1310.4	32.655	763.4

Gradient: 5%-100%A; aqueous phase (phase B): 0.1% formic acid solution; flow rate: 1.5mL/min; evaporation temperature: 100℃; gas flow: 1.8L/min; nebulization temperature: 30℃.

Table2. Plackett-Burman design table

Items \ Factors		c_{FA}(%)	Tc(℃)	v_{liquid}($mL \cdot min^{-1}$)	t_G(min)	T_{Eva}(℃)	v_{gas}($L \cdot min^{-1}$)	T_{Neu}(℃)	
Level	-1	0.30	25.0	0.8	40.0	60.0	1.2	25.0	
	0	0.20	30.0	1.0	80.0	80.0	1.8	35.0	
	1	0.10	35.0	1.2	120.0	100.0	2.4	45.0	
									Rs,crit
Mode	++———+—	1	1	-1	-1	-1	1	-1	1.89
	——+—+++	-1	-1	1	-1	1	1	1	2.30
	—+—+++—	-1	1	-1	1	1	1	-1	0.82
	——+——+—	-1	-1	1	-1	-1	1	-1	2.31
	+———+——	1	-1	-1	-1	1	-1	-1	2.83
	—+++———	-1	1	1	1	-1	-1	-1	0.68
	+——+—++	1	-1	-1	1	-1	1	1	1.66
	———+——+	-1	-1	-1	1	-1	-1	1	1.75
	+++———+	1	1	1	-1	-1	-1	1	1.15
	+—+++——	1	-1	1	1	1	-1	-1	1.18
	+++++++	1	1	1	1	1	1	1	0.67
	—+——+—+	-1	1	-1	-1	1	-1	1	2.11

Table3. Control space table

Factors	Factor design	Operating space	Control space
Fomic acid concentration(c_{FA})/%	0.10-0.30	0.10-0.30	+/-10%
Column temperature (Tc)/℃	25.0-35.0	25.0-30.0	+/-5℃
Flow rate(v_{liquid})/$mL\cdot min^{-1}$	0.8-1.2	0.8-1.0	+/-20%
Gradient time(t_G)/min	40.0-110.0	40.0-90.0	+/-5%
Evaporation temperature(T_{Eva})/℃	60.0-100.0	60.0-100.0	+/-5℃
Gas flow(v_{gas}) /L·min	1.2-2.4	1.2-2.4	+/-20%
Nebulization temperature(T_{Neb})/℃	25.0-45.0	25.0-45.0	+/-5℃