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Rapid optimization of dual-mode gradient high performance liquid chromatographic separation of Radix et Rhizoma Salviae Miltiorrhizae by response surface methodology

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ABSTRACT

An approach for rapid optimization of dual-mode gradient high performance liquid chromatography (HPLC) by response surface methodology (RSM) was developed for fast simultaneous separation of hydrophilic and hydrophobic components in Radix et Rhizoma Salviae Miltiorrhizae (Danshen) and its preparations. The aim of this study was to achieve a high throughput RSM optimization using a short ultra-high performance liquid chromatographic (UHPLC) column to simultaneously optimize flow rate and solvent gradient, and then transfer the optimized method to conventional HPLC for routine analytical purposes. The optimization was designed with Box Behnken design (BBD) and the global Derringer's desirability was used for describing the multicriteria response variables. Sixty-two designed experiments were performed by UHPLC with a short sub-2 μm column (2.1 mm × 50 mm, 1.7 μm) and a total running time of only 5 h. The predicted gradient profile was further transferred to a long UHPLC column (2.1 mm × 100 mm, 1.7 μm) and a conventional HPLC columns (2.1 mm × 100 mm, 3.5 μm and 4 mm × 100 mm, 5 μm, respectively). Compared to the published methods, the newly developed dual-mode gradient is faster and more efficient at simultaneously separating hydrophilic and hydrophobic components in Danshen and its preparations.

1. Introduction

Gradient elution in liquid chromatography, which is based on programmed separation, is a powerful technique to separate samples that contain complex components such as natural products. The main purpose of gradient elution is to accelerate the elution when the compounds possess a broad polarity range. The advantages of gradient elution are that it can improve sensitivity, enhance peak capacity and shorten running time [1]. Its drawbacks are mainly related to the particular requirements on the instrumentation and the laborious method development, even though optimization software can tackle this difficulty [2,3]. Currently, there are four gradient elution modes available: mobile phase composition, stationary phase (couple-column operation), flow rate and column temperature programming [4]. In each mode, only one of the above parameters is varied with time. Although solvent programming is the best and the most common operation of gradient elution since it is easier to control, it can suffer from poor front-end resolution [1,5]. Therefore, the coupling of solvent and flow or temperature programming which is called "dual-mode gradient" can become an alternative tool in many situations to improve resolution and shorten the analysis time. However, due to the difficulty in the parameter optimization of dual-mode gradient, only few papers have been reported on it [6–11]. Recently, studies on establishing appropriate theoretical expressions for the chromatographic behavior of the solutes inside the column under dual-mode elution have been reported [4,5,12–14]. Although these fundamental equations are useful for predicting the retention factors of known analytes, since the prediction depends on the system geometry (gradient time, flow rate and column dimensions, etc.), the estimation is more complex, and is not reliable for the optimization of samples with complex matrices such as natural products, which contain a large amount of unknown components. Alternatively, response surface methodology (RSM) combined with multicriteria decision making (MCDM) such as Derringer's desirability function can be used for this type of optimization.

RSM is a collection of statistical techniques which explores relationships between a number of explanatory factors and one or more response variables to obtain an optimal response by using a set of designed experiments. The second-order model of RSM designs such as 3-level factorial design, central composite design (CCD), Box Behnken design (BBD) and D-optimal designs is widely

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used since they can approximate the true response surface, estimate the parameters and work well in solving real response surface problems [15–18]. However, these designs are inflexible for large designed experimental sizes. For example, a 3-factor experimental design, BBD only requires 15 runs; while a 7-factor design, BBD requires 62 runs (contained 6 central runs) and an orthogonal central composite design requires 177 runs. Even for 2-level designs, a 7-factor full factorial experimental design requires 128 runs. Therefore, it is tedious, costly and time-consuming for conventional high performance liquid chromatography (HPLC) optimization on a column with 3–5-μm particle size, since a single run takes more than an hour. In recent years, a high throughput chromatographic analysis usually refers to ultra-high performance liquid chromatography (UHPLC) with a sub-2 μm column. Compared to conventional HPLC, UHPLC has shorter running times, higher efficiency and lower solvent consumption. This high throughput technique makes it easier to perform RSM and allows more factors to be explored.

Radix et Rhizoma Salviae Miltiorrhizae (Danshen in Chinese), the dried root of *Salviae Miltiorrhizae* Bunge, is frequently used in China and Southeast Asia for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhosis, chronic renal failure, dysmenorrheal and neurosasthenic insomnia [19–27]. Danshen is also widely used as a dietary supplement due to its high content of polyphenolic compounds with high antioxidant activity [28]. Many herbal medicine preparations that contain Danshen, such as the extract granule of Danshen, Danshen injection and Compound Danshen Tablets, are available in the market with variable quality. Previously reported analytical methods for the quality control of Danshen products include HPLC and capillary electrophoresis; however their running times are too long [29–34]. An UHPLC method was reported [35], but the running time was longer than 18 min. Therefore, a fast analytical method for high throughput sample analysis is necessary for the quality control of materials, in-process inspections, and final products release testing as well as shelf life evaluation. Our lab has previously developed an improved UHPLC method for the quality assessment of the extract granule of Danshen, but the separation efficiency was not satisfactory. In this study, an approach was developed for rapid optimization of dual-mode gradient elution for the fast determination of hydrophilic and hydrophobic components in Danshen and its products by combining the high optimization efficiency of RSM with the high throughput sample analysis of UHPLC. Furthermore, since most laboratories are not usually equipped with the UHPLC instrument, the UHPLC scale used in the newly developed method was converted to the conventional HPLC scale [2,3] to accommodate routine quality control practice.

2. Experimental

2.1. Chemicals and materials

Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were from Tedia (Tedia Company, Fairfleld, USA). 1,4-Dioxane of HPLC grade was purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, USA). Formic acid of MS grade was from Fluka (Steinheim, Germany). Ultra-pure water was produced by Milli-Q system (Millipore, Bedford, USA) and other chemicals were analytical grade.

Reference standards of danshensu (**1**), protocatechuic aldehyde (**2**), rosmarinic acid (**3**), lithospermic acid (**4**), salvianolic acid B (**5**), salvianolic acid A (**6**), dihydrotanshinone I (**7**), cryptotanshinone (**8**), tanshinone I (**9**) and tanshinone IIA (**10**) were provided by Hong Kong Jockey Club Institute of Chinese Medicine (Shatin, Hong Kong, China). The purity of all these reference standards was ≥98.0% (HPLC). Samples of Danshen, extract granule of Danshen, Compound Danshen Tablets (each tablet contained 0.45 g of Danshen, 0.14 g of Radix et Rhizoma Notoginseng and 0.08 g of Borneolum Syntheticum) were purchased in local drug stores of Hong Kong and Shenzhen, China.

2.2. Chromatographic instrumentations and conditions

All data for RSM study were acquired from Waters Acquity UPLC system which consisted of a binary solvent manager, sample manager, photodiode array detector (Waters Co., Milford, USA). The designed experiments were performed on a Waters Acquity UPLC BEH C18 (2.1 mm × 50 mm, 1.7 μm, Ireland) column at 35 °C. The column used for transferred dual-gradient profile was a Waters Acquity UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm, Ireland). The column temperature was set at 50 °C. The temperature of autosampler was maintained at 10 °C and the injection volume was 2.0 μl. The chromatogram was recorded at 280 nm. The initial mobile phase was A: 5% 1,4-dioxane/water (containing 0.2% formic acid), and B: 10% 1,4-dioxane/ACN (containing 0.2% formic acid).

The conventional HPLC analysis was performed on a Waters 2695 Separation Module (Waters, Milford, USA), which comprised a quaternary solvent delivery system with online degasser, autosampler, column compartment and Waters 2996 photodiode array detector (PDA). A Waters SunFire C18 (2.1 mm × 100 mm, 3.5 μm, Ireland) and a Zorbax SB-C18 StableBond Analytical (4.6 mm × 250 mm, 5 μm) were used and the column temperature was set at 50 °C. Detailed gradient profiles were listed in Table 1. The detection wavelength was 280 nm.

2.3. Solutions preparation

2.3.1. Sample solutions

A 0.2 g of powered sample was accurately weighed and ultrasonicated with 10 ml of methanol for 20 min. After being cooled down to room temperature, the supernatant was collected and fil-

Table 1
Predicted and transferred method and parameters of four columns. Mobile phase A and B were the same as in text.

Column and parameters		Gradient profile			
		Time (min)	Flow rate (ml/min)	A%	B%
1. Acquity UPLC BEH C18					
Diameter	2.1 mm	0.0	0.50	95.0	5.0
Length	50 mm	1.0	0.67	87.4	12.6
Particle size	1.7 μm	2.5	0.92	76.0	24.0
		3.0	1.00	54.3	45.7
		3.7	1.00	24	76
		4.0	1.00	0.0	100
2. Acquity UPLC BEH C18					
Diameter	2.1 mm	0.0	0.50	95.0	5.0
Length	100 mm	2.0	0.67	87.4	12.6
Particle size	1.7 μm	5.0	0.92	76.0	24.0
		6.0	1.00	54.3	45.7
		7.4	1.00	24.0	76.0
		8.0	1.00	0.00	100
3. SunFire C18					
Diameter	2.1 mm	0.0	0.24	95.0	5.0
Length	100 mm	4.1	0.32	87.4	12.6
Particle size	3.5 μm	10.3	0.44	76.0	24.0
		12.4	0.48	54.3	45.7
		15.2	0.48	24.0	76.0
		16.5	0.48	0.0	100
4. Zorbax SB-C18					
Diameter	4.6 mm	0.0	0.82	95.0	5.0
Length	250 mm	14.7	1.09	87.4	12.6
Particle size	5 μm	36.8	1.50	76.0	24.0
		44.1	1.63	54.3	45.7
		54.4	1.63	24.0	76.0
		58.8	1.63	0.0	100

tered through a membrane filter (0.2 μm) for further analysis. All the solutions were stored at 0–4 °C refrigerator prior to analysis.

2.3.2. Testing solution

The testing solution for designed experimental optimization was prepared by mixing 1 ml of a sample solution of Danshen (20 mg/ml) and 1 ml of a sample solution of the extract granule of Danshen (20 mg/ml), which contain major components of the herb and its preparation.

2.4. Experimental designs and data treatment

Data analysis and desirability function calculations were performed on Microsoft Excel 2002 (Microsoft Corporation). Experimental design and response surface optimization were performed on SAS® 9.0 (SAS Institute Inc., Cary, USA). Seven factors, namely, the initial composition of mobile phase (x_1, B%); trigger time of flow rate gradient (x_2, min); end time of flow rate gradient (x_3, min); gradient time (x_4, min) and the mobile phase composition (x_5, B%) for the end-point of first step linear solvent gradient; gradient time (x_6, min) and mobile phase composition (x_7, B%) for the end-point of second step linear solvent gradient. Detailed specification of variables and assignment of factors can be found in the Supplementary materials.

3. Results and discussion

3.1. Optimization

3.1.1. Factors and response criteria selection

A multilinear gradient profile was designed based on chromatographic theory knowledge and preliminary experimental results. With a multilinear gradient, separation is mainly affected by the slope and time of each step in which the composition of the mobile phase varies. Therefore optimization of the experimental conditions is a complicated process as many factors can affect retention behavior. The selected factors for optimization are listed in Table 2. The initial composition of mobile phase and the gradient profile was designed based on a previous study. For flow rate gradient optimization, the gradient of flow rate was varied from 0.5 to 1 ml/min according to the preliminary experimental results and system pressure limitation of the instrument. The trigger time and the end time of flow rate gradient were selected as variables for optimization.

Chromatographic optimization requires selecting suitable criteria for the evaluation of the resultant chromatograms in order to choose the optimum conditions. Furthermore, it is usually necessary to examine the very different qualitative aspects of a chromatogram and find a compromise between conflicting goals such as maximizing the separation while minimizing the analysis time. Therefore, the optimum condition defined according to only one response can be misleading. Multicriteria decision making (MCDM) is a useful approach and can be applied when more than one criterion needs to be taken into account. Derringer's desirability functions have been used in many fields as an approach of MCDM for simultaneous optimization of several goals [36]. In the Derringer's desirability function, each experimentally obtained response was scaled between 0 (unacceptable) and 1 (optimal). Since the measured response was transferred to a dimensionless desirability scale for each criterion, the values of several responses obtained from different scales of measurement may be combined. The major advantage of Derringer's desirability is that if one of the criteria was not fulfilled to an unacceptable value, then the overall product will also be unacceptable. In addition, this method offers the user flexibility in the definition of desirability functions. In this study, the chosen criteria include the resolution between the 10 selected target compounds (**1**–**10**, major components in Danshen) and their adjacent peaks, the running time and detected peak numbers. Peaks were identified by compared their retention times to those of each individual chemical marker. The individual criterion was transformed into desirability values according to the equations summarized in Table 2.

3.1.2. Model calculation, profile prediction and optimization research

Box Behnken design (BBD) is an independent, rotatable or nearly rotatable second-order design based on three-level incomplete factorial designs. BBD is more efficient compared to other response surface designs, such as central composite designs. In addition, BBD can provide sufficient information for testing the lack of fit, and therefore is one of the best quadratic models for RSM and has been widely used in analytical fields [16,17,37–39].

Because of the non-linearity of the model, a polynomial function to contain second-order model was postulated to describe the evolution phenomenon:

$$y_i = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{1 \le i \le j}^{n} b_{ij} x_i x_j + \varepsilon_i \quad (1)$$

where n is the numbers of variables, b_0 is the constant term, b_i, b_{ii} and b_{ij} represents the coefficient of the first order terms, quadratic terms and interaction terms, respectively, and ε_i is a term that represents other sources of variability not accounted for in the estimation, such as background noise, etc.

In this study, coefficients of the model were calculated using software SAS and the predicted model was

$$\begin{aligned}\hat{y} = {} & -2.09 + 9.75 \times 10^{-3} x_1 - 6.86 \times 10^{-1} x_2 + 5.04 \times 10^{-1} x_3 \\ & + 1.90 x_4 + 1.61 \times 10^{-2} x_5 + 1.28 \times 10^{-1} x_6 - 8.21 \times 10^{-3} x_7 \\ & - 5.44 \times 10^{-2} x_1 x_4 + 2.73 \times 10^{-3} x_1 x_5 + 2.18 \times 10^{-2} x_2 x_5 \\ & - 7.82 \times 10^{-2} x_3^2 - 3.91 \times 10^{-1} x_4^2 - 1.17 \times 10^{-3} x_5^2 \\ & + 2.28 \times 10^{-2} x_5 x_7 \end{aligned} \quad (2)$$

Table 2
Multicriteria decision and desirability limits for the simultaneous optimization of interacting variables through overall desirability response.

Variables optimization and Derringer desirability function	Individual response	Optimization criteria and desirability limits
$\hat{y} = (d_1 \times d_2 \times d_3 \times d_4 \times d_5 \times d_6 \times d_7 \times d_8 \times d_9 \times d_{10} \times d_{11} \times d_{12})^{1/12}$	d_{1-10} = resolution between the specified peak and its adjacent peaks ($RA_{i,i}$ = 1–10)	$d_i = \begin{cases} (RA_i - 0.5 \times 1.5)/1.5 \\ 0 \text{ if } RA_i < 0.5 \times 1.5 \\ 1 \text{ if } RA_i > 1.5 \end{cases}$
	d_{11} = retention time (R_t) of the last peak	$d_{10} = \begin{cases} Rt/4\,(Rt = 4) \text{ or } 4/Rt\,(Rt > 4) \\ 0 \text{ if } Rt < 2.5 \text{ or } Rt > 5 \\ 1 \text{ if } Rt = 4 \end{cases}$
	d_{12} = number of total determined peaks (Tps)	$d_{11} = \begin{cases} (Tps - 0.5 Tps_{(max)})/Tps(max) \\ 0 \text{ if } Tps < 0.5\, Tps_{(max)} \\ 1 \text{ if } Tps = Tps_{(max)} \end{cases}$

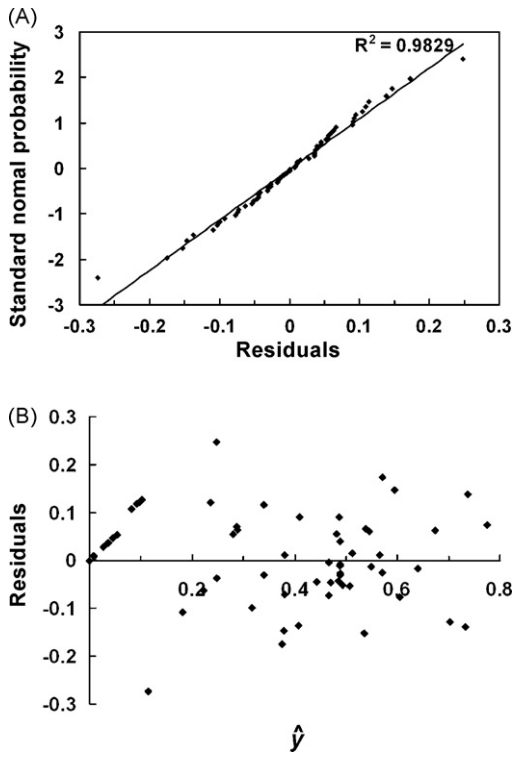

Fig. 1. Overview of residuals from the least squares fit: plots of residual versus normal probability (A) and residuals versus predicted for the global desirability (B).

Table 3
ANOVA of the model.

Source of variation	Degree of freedom	Sum of squares	Mean square	*F*	*p*-value
Model	35	2.668	0.0762	4.044	0.0001
Linear	7	1.488	0.2126	11.270	0.0001
Quadratic	7	0.381	0.0545	2.889	0.0226
Cross-product	21	0.799	0.0380	2.018	0.0452
Error	26	0.490	0.0189		
Lack of fit	21	0.344	0.0164	0.671	0.6498
Pure Error	5	0.146	0.0293		
Total	61	3.158			

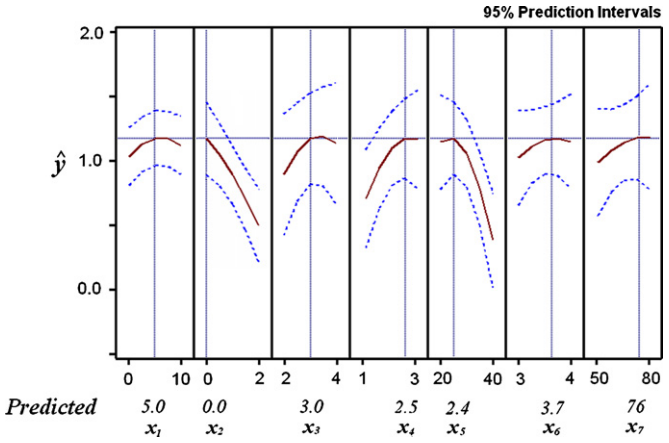

Fig. 2. The main effect plots of independent factors on global multicriteria desirability ($\hat{y}$) and the predicted gradient profile. The symbols of x_1–x_7 were the same as in the text.

$\hat{y}$ is the global Derringer desirability, which is defined in Table 2, and x_1–x_7 are variables described above. The R^2 and adjusted R^2 for the predictive model was 0.8937 and 0.8293, respectively, which indicated that the experimental data well fitted the second-order polynomial equation (within acceptable limits of $R^2 \geq 0.8$ [40]). To estimate the quality of the model and validate it, analysis of the variance and the values of residuals from the least squares fit were examined. The normality assumption was tested by constructing a normal probability plot of the residuals (Fig. 1-A) and then checked by a Kolmogorov–Smirnov test. It was shown that the data points have not deviated much from the fitted line, and the

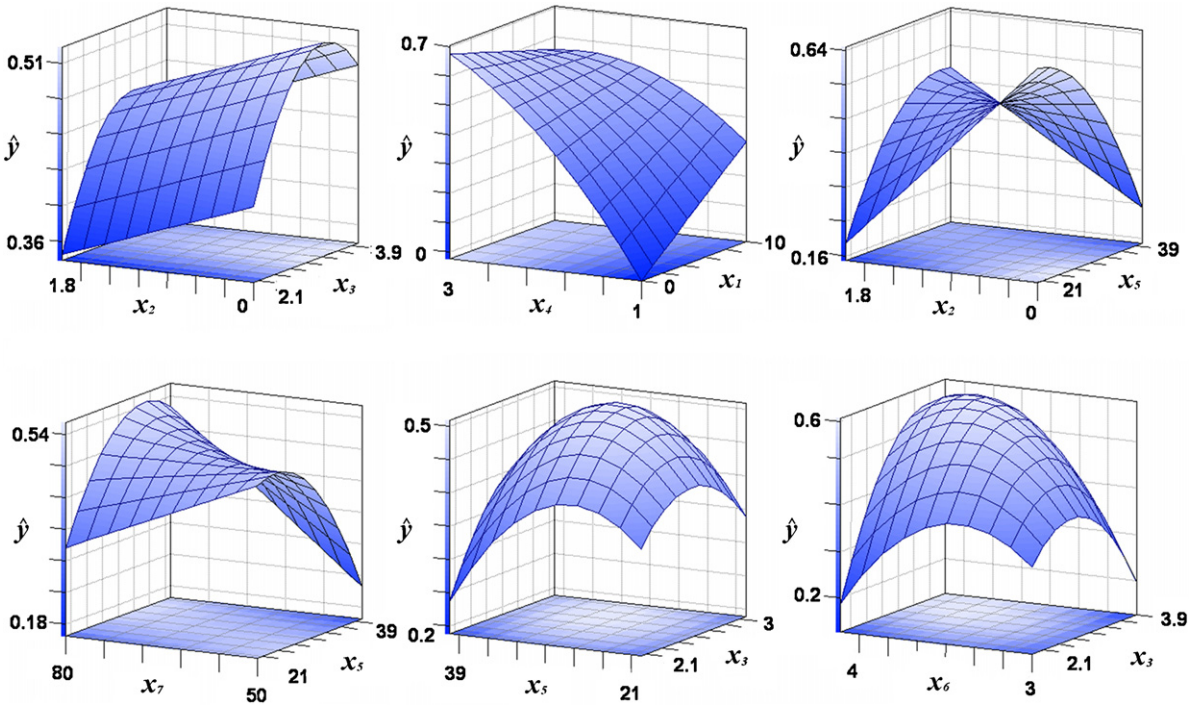

Fig. 3. Response surface plots representing the quadratic effects of interactions between factors on global multicriteria desirability ($\hat{y}$). The symbols of x_1–x_7 were the same as in the text.

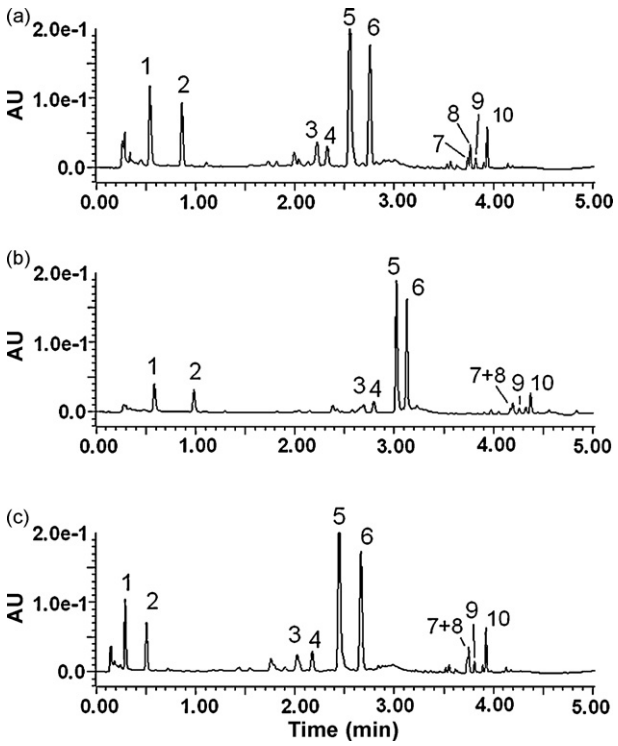

Fig. 4. Comparison of dual-mode gradient elution (a) and single solvent gradient elution at flow rate of 0.5 ml/min (b) and 1 ml/min (c). Chromatographic conditions were in Table 1 and peaks **1**–**10** were the same as in text.

Kolmogorov–Smirnov test value was 0.0557 < p-value (p = 0.150), indicating that the residuals were normally distributed. As shown in Fig. 1-B, which is a plot of residuals versus predicted response, the residuals scatter randomly, suggesting that the variance of the original observations is constant for all value of responses. The results indicated that the choice of model was appropriate.

Table 3 shows the analysis of variance for this model. The F-test value for the significance of the model is 4.044. Because the p-value is very small (0.0001), the hypothesis H_0: $b_1 = b_2 = \cdots \cdots = b_i = 0$ could be rejected, suggesting that some of these parameters were nonzero. Furthermore, the p-value for the linear terms and the quadratic terms is 0.0001 and 0.0226, respectively, indicating that both linear and quadratic terms contributed significantly to the model. The p-value for the lack of fit test is large (p = 0.6498) and so the hypothesis that the tentative model adequately described the data could not be rejected, implying that the quadratic model is adequate.

According to the predicted model, the optimized dual-gradient profile was estimated through prediction profiler by adjusting each parameter to get the highest desirability (Fig. 2). Fig. 3 shows the quadratic effects of interactions between factors on the global Derringer's desirability. The predicted dual-mode gradient profile was listed in Table 1. Fig. 4a is a typical chromatogram obtained with the optimized condition. Moreover, the effect of dual-mode gradient was compared to that of optimized solvent gradient elution at constant flow rate. As shown in Fig. 4, at a constant flow rate of 0.5 ml/min (Fig. 4b), the running time was long, resolution between peaks **3**/**4**, **5**/**6** and **7**/**8** decreased; at a constant flow rate of 1.0 ml/min (Fig. 4c), the running time was somewhat shorter, retention time of peak **1** was almost as short as the system peak, and peaks **7**/**8** were overlapped, indicating that dual-mode gradient is better than single solvent gradient elution.

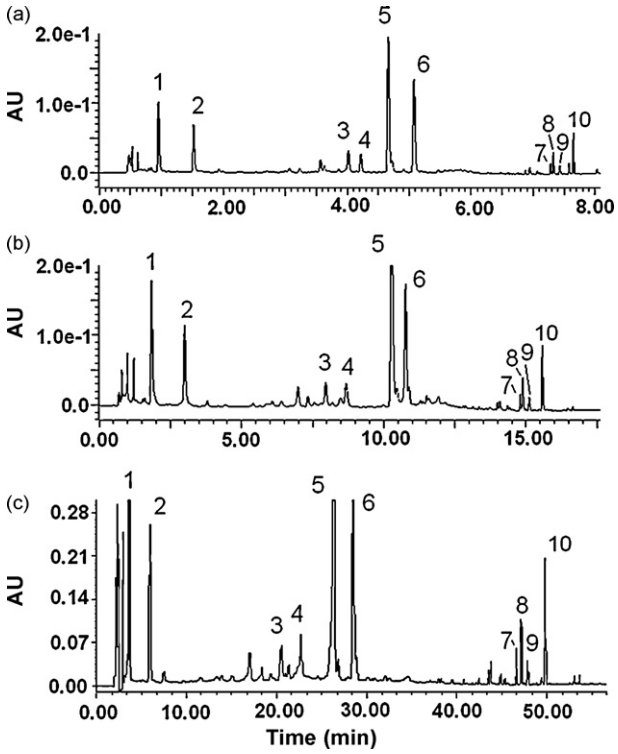

Fig. 5. Transferred dual-mode gradient elution on 100 mm UHPLC column (a), conventional small bore HPLC column (b) and conventional HPLC column (c). Chromatographic conditions were in Table 1 and peaks **1**–**10** were the same as in text.

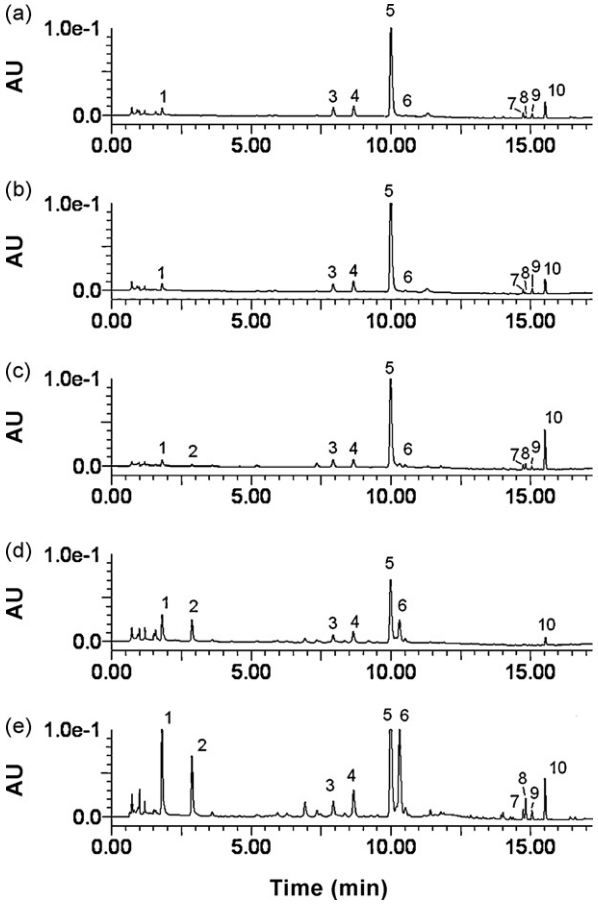

Fig. 6. Separation of sample of (a) Radix et Rhizoma Salviae Miltiorrhizae, (b–d) extract granule of Radix et Rhizoma Salviae Miltiorrhizae and (e) Compound Danshen Tablet by conventional small bore HPLC with the transferred method (**3**) in Table 1.

3.2. Method transferring and applications

The optimized gradient profile was transferred to a 100 mm UHPLC column (100 mm × 2.1 mm ID, 1.7 μm) and conventional HPLC columns (100 mm × 2.1 mm ID, 3.5 μm, and 250 mm × 4 mm ID, 5 μm, respectively) according to the published method [2,3]. The optimized and transferred conditions for the dual-mode gradient were listed in Table 1. When evaluating the equivalency of chromatographic behavior between optimized and transferred gradient profiles, the running time was within 8 min on the 100 mm UHPLC column (Fig. 5a), 16 min on the conventional small bore HPLC column (Fig. 5b), which is only 1/2 to 1/3 of the running time reported in published methods [29–32]. Even on a conventional 250 mm HPLC column, the analysis time was 50 min (Fig. 5c), which is much less than that reported in publications [31,34].

The transferred method was used for the routine quality control of Danshen and its preparations. Fig. 6 shows the chromatographic fingerprints of Danshen (a), extract granule of Danshen (b–d) and Compound Danshen Tablets (e) on the conventional small bore HPLC column. The major hydrophilic and hydrophobic components in both Danshen materials and its preparations could be eluted with baseline separation within 16 min, indicating that the newly developed method is a rapid and efficient approach for the routine quality control of Danshen and its preparations.

4. Conclusion

This paper described an approach for rapid optimization of dual-mode gradient chromatographic conditions for the separation of major hydrophilic and hydrophobic components in Danshen by response surface methodology. By using a high throughput analysis based on a short UHPLC column, large amounts of designed experiments were run with a short time. For example, it took approximately 5 h to run 62 designed experiments. The optimized method was transferred to conventional HPLC columns for routine fingerprinting analysis of Danshen and its medicinal preparations. It was proven that this approach is a fast, highly efficient and solvent conserving way to perform RSM optimization on a natural product with complex components with unknown matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.08.059.

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