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Article

Separation and Determination of Honokiol and Magnolol in Chinese Traditional Medicines by Capillary Electrophoresis with the Application of Response Surface Methodology and Radial Basis Function Neural Network

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A method for the separation and determination of honokiol and magnolol in *Magnolia officinalis* and its medicinal preparation is developed by capillary zone electrophoresis and response surface methodology. The concentration of borate, content of organic modifier, and applied voltage are selected as variables. The optimized conditions (i.e., 16 mmol/L sodium tetraborate at pH 10.0, 11% methanol, applied voltage of 25 kV and UV detection at 210 nm) are obtained and successfully applied to the analysis of honokiol and magnolol in *Magnolia officinalis* and Huoxiang Zhengqi Liquid. Good separation is achieved within 6 min. The limits of detection are 1.67 μg/mL for honokiol and 0.83 μg/mL for magnolol, respectively. In addition, an artificial neural network with "3-7-1" structure based on the ratio of peak resolution to the migration time of the later component (R_s/t) given by Box-Behnken design is also reported, and the predicted results are in good agreement with the values given by the mathematic software and the experimental results.

Introduction

The stem bark of *Magnolia officinalis* is a kind of traditional Chinese medicine. It has been widely used to treat thrombotic stroke, typhoid fever, and headache (1). Magnolol and honokiol, which are geometric isomers, are the major two pharmacologically active components in *Magnolia officinalis*. Their structures are shown in Figure 1. They possess activities against platelet aggregation, oxidation, anxiety, depression, and cancer (2). Recent studies demonstrated that the two compounds might be potential therapeutic agents for neurodegenerative diseases (3), acute promyelocytic leukemia (4) as well as atherosclerosis (5). The two active components are also included in some combination drugs. Therefore, efficient and sensitive analytical methods are required to determine their contents. There have been some reports about the analysis of magnolol and honokiol in different samples. Magnolol and honokiol in *Magnolia officinalis* as the raw herb and dried aqueous extract, as well as in rat plasma, have been determined by HPLC methods (6–9).

Capillary electrophoresis (CE), a separation technique with high efficiency, has rapidly developed since 1981 (10). It has been applied to the separation and determination of a variety of samples because of its minimal sample volume requirements, short analysis time, and high separation efficiency. Moreover, CE has been applied to the analysis of magnolol and honokiol in different samples. Magnolol and honokiol in *Magnolia officinalis* were separated and determined by capillary zone electrophoresis (CZE) with UV detection (11), nonaqueous capillary electrophoresis-UV (12), flow injection–CZE–UV (13), and CZE with electrochemical detection (14, 15). In addition, magnolol and honokiol in biological fluids were also analyzed using a CZE separation system coupled with a laser-induced fluorescence (LIF) detector (2).

In many cases, it is difficult to find suitable experimental conditions quickly for a given separation task. Experimental design can allow the number of experiments to be drastically reduced. Box-Behnken design (16–17) is one kind of method in the response surface methodology. Optimal separation condition could be obtained using relatively fewer experiments by this design.

On the other hand, the use of artificial neural networks (ANNs) has become a very powerful and practical method for solving various problems in chemistry, especially in separation science. ANNs are nonparametric nonlinear modeling techniques that have attracted increasing interest (18-20). The strength of modeling with layered, feed-forward ANNs lies in the flexibility of the distributed soft model defined by the weight of the network. The theory of different networks has been reviewed by Zupan and Gasteiger (21). Radial basis function neural network (RBFNN) can be applied to establish models about the relationship of physical, chemical, biological property with compound structures. It has also been used for the prediction of peak resolution of bioactive components in traditional Chinese medicinal preparations (22). In reference 22, orthogonal design was used for optimization of CE separation conditions, and a feed-forward type neural network with an extended delta-bar-delta algorithm was developed based on 28 separation results given by orthogonal design and uniform design and was applied to the prediction of peak resolution (*Rs*) of the two analytes given by uniform design.

In this paper, a simple CZE method was used for the separation and determination of magnolol and honokiol in *Magnolia officinalis* and Huoxiang Zhengqi Liquid. Response surface methodology was used for experimental design, and a polynomial equation was generated based on only 13 experimental results. The optimum separation conditions were obtained. In addition a radial basis function neural network was also developed and was used to predict *Rs*/*t*, and satisfactory results were obtained.

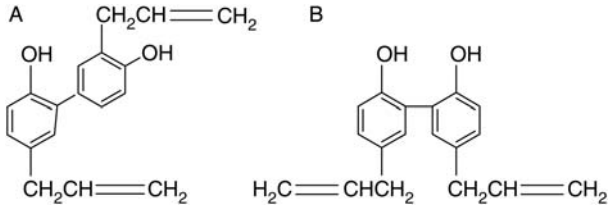

Figure 1. The stuctures of the analytes: (A) Honokiol and (B) Magnolol.

Experimental

Chemicals

The standard of magnolol and honokiol were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The *Magnolia officinalis* bark and Huoxiang Zhengqi Liquid were purchased from the local supermarket. Mili-Q water was used throughout (Billerica, MA). Sodium tetraborate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Methanol was purchased from Tianjin Kermel Chemical Reagents Co. Ltd. (Tianjin City, China). All chemicals used in the study were of analytical grade and used without further purification.

Apparatus, software, and electrophoretic condition

The CZE experiments with UV detection were carried out on a Lumex Capel 105 Capillary Electrophoresis System (Lumex Ltd., 19 Moskovsky Pr. St., Peterburg, 198005 Russia). A 50-cm (40.5 cm to the detector)×75-μm i.d. fused silica capillary (Yongnian Ruifeng Chromatographic Devices Limited Company, Hebei, China) was used. An Ion 510 pH meter (Ayer Rajah Crescent, Singapore) was used. The new capillary was pre-conditioned prior to use by flushing for 30 min with 0.5 mol/L NaOH, followed by 10 min with Milli-Q water and 10 min with running buffer. The capillary was conditioned each day by flushing with Milli-Q water for 2 min, 0.2 mol/L NaOH for 10 min, Milli-Q water for 5 min, and running buffer for 5 min, sequentially. The capillary was washed between runs for 2 min with 0.5 mol/L NaOH, for 2 min with Milli-Q water, and for 3 min with running buffer, sequentially.

The Matlab Version 7.0.1 24704 (R14) Service Pack was implemented in a Matlab language and ran on a personal computer. The LINGO Version 6.1 (Lindo System Company, Chicago, IL) was used to find the optimal value of the variables.

The background electrolyte (BGE) solution consisted of 16 mmol/L sodium tetraborate and 11% methanol and was adjusted to pH 10.0 with 0.5 mol/L NaOH. Operation conditions for CZE were set as follows: applied voltage, 25 kV; injection time, 3 s (hydrodynamic, 30 mbar); UV detection wavelength, 210 nm. All of the experiments were run at room temperature (25 ± 1°C). All the solutions were filtered through a 0.45-μm membrane (Shanghai Xinya Purification Apparatus Factory, Shanghai, China) before use.

Preparation of standard solutions

One milligram per milliliter standard solutions of honokiol and magnolol were separately prepared in ethanol. These stock solutions were diluted with ethanol to give less concentrated standard solutions.

Table I
Factors and Levels of Box-Behnken Design

Factors	Levels		
	−1	0	1
C_1 (mmol/L)	10	20	30
C_2 (%)	5	10	15
V (kV)	15	20	25

Sample preparation

First, 0.2 g ground powder of *Magnolia officinalis* bark was soaked in 10.0 mL methanol for 24 h and then extracted for 30 min in an ultrasonic bath at room temperature. Afterwards, the sample was filtered, and the filtrate was filtered through 0.45 μm membrane prior to injection into the CE system. Then 0.5 mL Huoxiang Zhengqi Liquid was diluted to 10 mL with 50% ethanol and filtered through 0.45 μm membrane prior to CE analysis.

Experimental design and RBFNN

Many parameters have to be optimized to develop a CE method. In this system, it could be seen from the preliminary results that the concentration of borate (C_1), the content of methanol (C_2), buffer pH (pH), and the applied voltage (V) were the primary factors that influenced the value: R_s/t. The pK_{a1} and pK_{a2} values are 7.01, 10.64 for magnolol and 9.92, 10.71 for honokiol (24). Thus, a higher buffer pH should be used with CZE mode. However, at high pH (greater than pH 11.2) the elution order of the honokiol and magnolol changed, which could also be seen in the previous publication (11). So the pH was set at a constant value of 10.0, and the other three parameters were selected as variables to be optimized. A conventional "step-by-step" approach was used, which involves a large number of independent analyses but reveals nothing about the interactions among the variables. It could be replaced by statistically designed experimental protocols in which several factors are simultaneously varied. Hence, a Box-Behnken statistical design with 3 factors, 3 levels, and 13 runs was selected for the optimization study. The experimental design consists of a set of points lying at the midpoint of each edge and the center point of the multidimensional cube. The ranges and intervals of the three variables were determined by preliminary experiments. That is, the optimization ranges for the content of methanol, voltage and borate concentration are 5–15%, 15–25 kV, and 10–30 mmol/L, respectively. The design selected three levels (low, medium, and high) signed as −1, 0, and 1 for the three factors (C_1, C_2, V) as shown in Table I. The independent variables, dependent variables, and 13 runs are listed in Table II. The polynomial equation generated by this experimental design [using Matlab Version 7.0.1 24704(R14) Service Pack] is as follows:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + B_{12}X_1X_3 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad \text{(Eq. 1)}$$

Table II
The Experimental, Predicted Values, and the Format of Box-Behnken Design

No.	C_1 (mmol/L)	C_2 (%)	V (kV)	R_s/t (/min) Experimental	Predicted
1	20	5	15	1.23	1.37
2	20	5	25	3.41	3.14
3	20	15	15	1.64	1.86
4	20	15	25	3.40	3.35
5	10	5	20	1.91	1.99
6	10	10	15	2.19	1.91
7	10	15	20	2.65	3.35
8	10	10	25	3.56	3.74
9	30	5	20	1.95	1.99
10	30	10	15	1.72	1.71
11	30	10	25	3.02	3.14
12	30	15	20	2.27	2.16
13	20	10	20	2.86	2.89
14	16	11	25	3.75	3.86

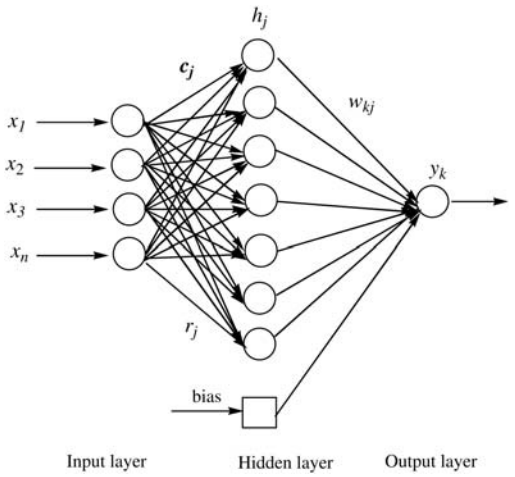

Figure 2. The architecture of RBFNN. x_j (j = 1, 2, . . ., n), the input vector; c_j, the radial basis function center; r_j, the radial basis function width; h_j, the notation for the output of the jth radial basis function unit; w_{kj}, the weight connection between the kth output unit and the jth hiddenlayer unit; y_k, the kth output unit for the input vector x.

Where Y_i is the dependent variable (R_s/t); b_0 is the intercept; b_1 to b_{33} are the regression coefficients; and X_1, X_2, and X_3 are the independent variables that were selected from the preliminary experiments (C_1, C_2, V).

Based on equation 1, a mathematic software Lingo 6.1 was used to obtain the optimal values of C_1, C_2, V, which gave the maximal value of R_s/t. Then the 13 CE results from the Box-Behnken statistical design were used as the training set to construct the RBFNN model, and the model was used to predict the maximum R_s/t value given by Lingo 6.1. The network consisted of three layers, namely: an input layer with three input nodes C_1, C_2 and V; one hidden layer in which the number of nodes would be determined during the training step; and one output layer with a single output node R_s/t. The theory of the RBFNN was demonstrated by an earlier publication (23). The design of the RBFNN is shown in Figure 2.

Results and Discussion

Box-Behnken design for separation

The Box-Behnken design chart with the three variables (C_1, C_2, and V) at three levels and the corresponding values of R_s/t are shown in Table II. The R_s of honokiol and magnolol is calculated from the well-known equation:

$$\mathrm{Rs} = \frac{1.18[t_{(2)} - t_{(1)}]}{w_{1/2(1)} + w_{1/2(2)}} \qquad \text{(Eq. 2)}$$

Where $t_{(1)}$ and $t_{(2)}$ refer to the migration time of honokiol and magnolol, respectively, and $w_{1/2(1)}$ and $w_{1/2(2)}$ refer to the peak widths at half peak height.

The Box-Behnken experimental design has the advantage of requiring fewer experiments than a full factorial design. The values of the three variables in all the runs along with their experimental results are shown in Table II. The R_s/t (dependent variable) obtained at various levels of the 3 independent variables (C_1, C_2, V) was subjected to multiple regression using MATLAB 7.0.1 to yield a second-order polynomial equation (full model):

$$\begin{aligned} \mathrm{R_S/t} = &-4 + 1022\mathrm{C_1} + 0.5089\mathrm{C_2} + 0.2185\mathrm{V} - 0.0021\mathrm{C_1C_2} \\ &- 0.0003\mathrm{C_1V} - 0.0043\mathrm{C_2V} - 0.0023\mathrm{C_1^2} \\ &- 0.017\mathrm{C_2^2} - 0.001\mathrm{V^2} \end{aligned} \qquad \text{(Eq. 3)}$$

The value of the correlation coefficient (r^2) of equation 3 was found to be 0.956, indicating that a good fitness was obtained. The optimal values for the three independent variables were found by the Lingo 6.1 software, and the optimal point must be within the experimental domain investigated. Therefore, the optimum separation conditions were as follows: 16 mmol/L borate, 11% methanol, 25 kV applied voltage with constant buffer pH at 10.0. According to the described conditions, a typical electropherogram is shown in Figure 3A. From Figure 3A it can be seen that honokiol and magnolol are separated in less than 6 min with good peak shape and satisfactory R_s. The peak shape and peak resolution are better in a shorter analysis time compared with the references (11–14), where optimum separation conditions were obtained from monovariate investigation.

Method validation

The method was validated for the repeatability (intra-day) and the time-different intermediate precision (inter-day) of migration time and peak area of the analytes. The inter-day precision was obtained as the relative standard deviation values of migration time and peak area for six consecutive day's results. Maximum relative standard deviation values of migration time and peak area for five replicate injections are 1.73% and 2.86% (intra-day) and 4.66% and 6.64% (inter-day), respectively.

Calibration curves based on peak areas and peak heights were linear for each analyte in the range tested (5.0–1000 μg/mL). Linear relationships between the concentration of honokiol and magnolol (x, μg/mL) and the corresponding peak area (y)

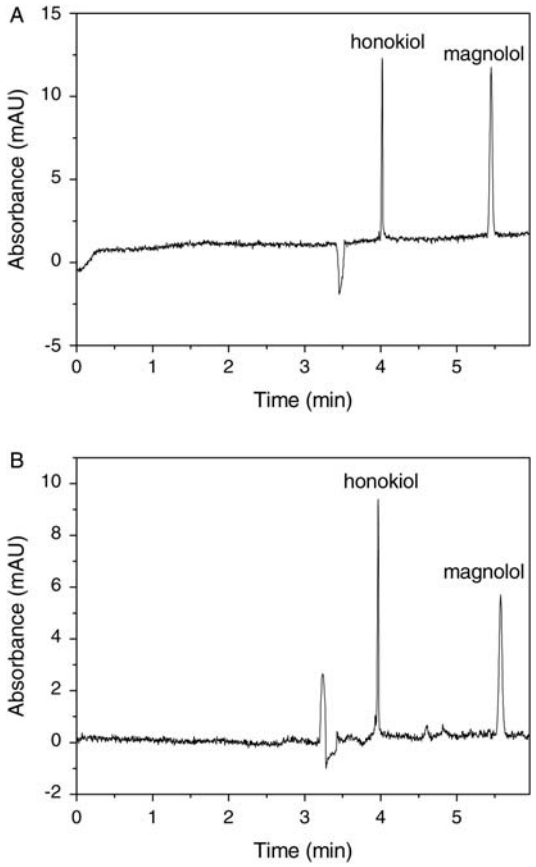

Figure 3. Typical electropherograms, (A) standard mixture of honokiol and magnolol, (B) Magnolia officinalis bark. Conditions: 16 mmol/L borate, 11% methanol, pH 10.0, 25 kV applied voltage.

are $y = 0.3523x - 4.3915$ ($r = 0.9983$, $F = 3.11 < F_{0.05(5,\ 7)} = 3.97$) for honokiol, and $y = 0.4538x + 2.2135$ ($r = 0.9988$, $F = 2.19 < F_{0.05(5,\ 7)} = 3.97$) for magnolol. The limits of detection (LOD), were estimated from these calibration curves and based on the concentration necessary to yield a net height equal to three times the standard deviation of the background. The LODs calculated were 1.67 μg/mL for honokiol and 0.83 μg/mL for magnolol.

Real sample analysis

The optimized CE method was successfully applied to the separation and determination of honokiol and magnolol in *Magnolia officinalis* bark and Huoxiang Zhengqi Liquid. Honokiol and magnolol in the two samples were successfully separated within 6 min as shown in Figure 3B. Peaks were identified by the addition of standard substances of honokiol and magnolol to the samples. Contents of honokiol and magnolol are shown in Table III. The recoveries of the two components were determined by spiking the two standards into the samples with the results of 89.76–110.96% as shown in Table IV.

RBFNN structure optimization

The RBFNN with three input nodes (C_1, C_2, and V), one hidden layer with x nodes, and a single output node (R_s/t) may be designed as a "3-x-1" net. The selection of the optimal width value for RBFNN was performed by systemically changing its value in the training step. The value that gives the best leave-one-out cross-validation result was used in the model. For this data set, the optimal value was determined as 3.1. The corresponding number of centers (hidden layer nodes) of RBFNN was 7. Therefore, a RBFNN with "3-7-1" structure was developed. The overall performance of RBFNN was evaluated in terms of a root-mean-squared (RMS) error. The obtained model had a correlation coefficient $r = 0.9787$ and RMS = 0.1479. The predicted results of the nonlinear model are shown in Table II.

Table III
Contents of the Analytes in Real Samples (n = 5)

	Results			
	Content		RSD (%)	
Sample	Honokiol	Magnolol	Honokiol	Magnolol
Magnolia officinalis	8.761 (mg/g)	7.823 (mg/g)	4.62	2.38
Huoxiang Zhengqi Liquid	0.518 (mg/mL)	0.257 (mg/mL)	5.47	3.29

Table IV
Recoveries for the Determination of the Two Components in Samples ($n = 3$)

Sample	Component	Original Content (μg/mL)	Concentration spiked (μg/mL)	Found (μg/mL)	Recovery (%)	Average (%)	RSD (%)
Magnolia officinalis	Honokiol	26.66	10	36.35	96.92	103.12	7.30
			20	46.56	99.49		
			30	59.95	110.96		
	Magnolol	23.79	10	33.68	98.89	99.71	4.51
			20	42.92	95.67		
			30	55.16	104.56		
Huoxiang Zhengqi Liquid	Honokiol	25.89	10	34.87	89.76	96.67	6.73
			20	46.43	102.68		
			30	55.16	97.56		
	Magnolol	12.85	10	22.81	99.62	101.92	2.58
			20	33.12	101.35		
			30	44.28	104.78		

Predicted results with RBFNN

In order to test the predictive ability of the RBFNN model developed here, the target value (R_s/t) under the optimum separation conditions obtained from LINGO 6.1 based on the results of Box-Behnken design was predicted. The predicted R_s/t by RBFNN was 3.87/min, the optimal R_s/t value calculated by the LINGO software was 3.75/min, and the experimental value of R_s/t was 3.75/min. Obviously, the two former values obtained from different models were consistent with the experimental value, indicating that RBFNN and Box-Behnken design could be a potential way for the selection of separation conditions in CE.

Conclusion

The chemometric approach proposed in this study combined experimental design with RBFNN. The results obtained demonstrated that Box-Behnken design combined with Lingo 6.1 is an efficient approach for the optimization of separating conditions in CE. The proposed method is a good alternative for

simultaneous analysis of active components in Chinese traditional medicinal preparations. The combination of experimental design and RBFNN was found to be a powerful tool in predicting separation results from a small number of experiments.

Acknowledgements

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