Article

Optimization Extraction Process of Aroma Components in Tobacco

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This paper concerns the optimization of the simultaneous distillation extraction process of volatile aromatic components in flue-cured tobacco leaves by single-factor experiments and response surface methodology (RSM). The qualitative and quantitative analysis of the aroma components was performed by gas chromatography−mass spectrometry. The single-factor experiments were adopted to investigate the effects of five independent variables (including material/liquid ratio, distillation time, dosage of NaCl, volume of CH_2Cl_2 and water-bath temperature) on the extraction of aroma components in tobacco. Furthermore, RSM was employed to study the relationship among the five independent variables and their effects on the extraction of aroma components. The results showed that the optimal extraction conditions were as follows: 1:12 ratio of material to solvent, 3.20 h distillation time, 1:1 ratio of NaCl and tobacco, 2:1 ratio of CH2Cl2 and tobacco, 60°C water-bath temperature. Under the optimized conditions, the maximum extraction amount of aroma components reached 2.27 mg/g.

Introduction

Volatile components in cut tobacco are the primary contributors of cigarette flavor. Thus, these compounds are very important factors to appraise the quality and commercial values of tobacco. However, they are very complex and the content of many important components in tobacco are at trace levels. Therefore, suitable sample-preparing methods and a sensitive analyzer are indispensable.

Many techniques have been developed for sample preparing volatile components in tobacco. The different sampling techniques offer various individual advantages, but also suffer from some specific limitations. Here, simultaneous distillation extraction (SDE) was employed to extract the volatile components from flue-cured tobacco leaves by one-factor-at-a-time method and response surface methodology (RSM).

Previously, methods involving solvent extraction and SDE, followed by analysis using gas chromatography−mass spectrometry (GC−MS), have widely been used to study the aroma constituents of fruits and wine. As a sample preparation technique, SDE has been developed to extract and concentrate volatile compounds from food, tobacco and essential oil (1−11). SDE is suitable for the chemical extractions with appropriate volatility and distribution coefficient between water and an organic solvent. SDE has two major advantages: it has only two primary operations (extraction and concentration), and offers a relatively wide spectrum of detected chemical compounds (12). At present, this method has widely been used

to extract aroma components of tobaccos (1, 12−14). However, the available literature includes no information on the statistical optimization of extraction conditions of flue-cured tobacco.

The RSM is a collection of statistical and mathematical techniques that has been successfully used to determine the effects of several variables and to optimize processes (15). RSM uses the quadratic regression equation to fit the functional relation between factors and response value (16−17). RSM has been successfully applied to optimize conditions in food and pharmaceutical research (18−19). Usually, it applies an experimental design such as central composite design (CCD) to fit a second-order polynomial equation by regression analysis of the experimental data. The equation was used to describe how the test variables affect the response and determine the interrelationship among the variables. RSM provides many potential advantages; for instance, more advanced results with less process variability, closer confirmation, less labor and development time than other approaches requiring an optimized process (20).

The objective of the present work was to optimize the extraction conditions of volatile aromatic components in flue-cured tobacco for SDE. In this paper, a Box-Behnken design (BBD) of RSM was used to optimize the extraction conditions for SDE.

Material and Methods

Material and reagents

The flue-cured tobacco-Hongda leaf sample was provided from the raw material production base of the Hongyunhonghe Tobacco Group (Yunnan Province, P.R. China). The tobacco sample was ground to pass through a 60-mesh sieve and stored in brown glass jars at 4°C for further analysis.

Dichloromethane (99.8%) was used as extraction solvent and purchased from Dikma Company. Phenylethyl acetate (98.0%) was used as internal standard in GC−MS and purchased from Fluka Company (Buchs, Switzerland). Other reagents were purchased from Beijing Chemical Factory (Beijing, China) and were of analytical grade.

Simultaneous distillation and extraction

The SDE experiment was performed with a Likens-Nickerson apparatus (21). For each extract, 30.0 g of tobacco leaf sample, various amounts of sodium chloride and various volumes of distilled water, according to the experiment design, were placed

in a 1,000 mL round-bottom flask, and varying volumes of dichloromethane in a 100 mL round-bottom flask was placed in a varying water-bath temperature, and they were both distilled for different times at atmospheric pressure. When the extraction was performed, chilled water was circulated through the cold finger condenser. Finally, approximately 40 mL extract was obtained. One milliliter of phenylethyl acetate dichloromethane solution (0.42 mg/mL) was added to the extract as internal standard. The samples were concentrated to 1.0 mL for further GC−MS analysis.

GC−MS analysis

GC analysis was performed with an Agilent 6890 Series (Agilent, Santa Clara, CA) with a flame ionization detector (FID). For the separation of volatile aromatic constituents, a fused-silica column (HP-5) was used (60 m × 0.25 mm i.d., d.f. = 0.25 μm). The injector temperature was 280°C. A 1 μL autosampler and split ratio of 10:1 were used. Ultra-high purity helium (99.999%) was used as carrier gas at a constant flow of 1.0 mL/min. The column oven was programmed from 50°C (after 2 min) to 270°C at 4°C/min and the final temperature was held for 20 min.

GC−MS analysis of volatile aromatic compounds was conducted on an Agilent 5973 mass select detector (Agilent) directly coupled to an HP 5980 gas chromatograph. The temperature of the GC−MS transfer line was 280°C in the electron impact (EI) mode (70 eV), scanning from m/z 35 ∼ 550 in one scan. The voltage of the electronic multiplier tube (EMT) was 230 V above tuning.

The mass spectral identification of aromatic compounds was conducted by comparing to the NIST 02 (Agilent). Qualitative analysis (mass spectral data) was verified by comparing the retention indices and mass spectra of identified compounds.

The relative quantity of each compound was determined using phenylethyl acetate as the internal standard; without considering the recovery of aroma compounds and response factors (14), many aroma components were analyzed quantitatively as follows:

extraction of aroma components (mg/g) = peak area of aroma components × quality of internal standard / internal standard peak area / quality of tobacco sample / (1 − moisture content).

Results and Discussion

Qualitative and quantitative analysis of target volatile components

The total ionic current (TIC) chromatogram of the volatile components of cut tobacco is shown in Figure 1. As shown in Figure 1, there were hundreds of different compounds and their contents varied greatly. According to the literature (22), ketones, acids, alcohols and esters have great influences on the tobacco quality, especially in a sensitive evaluation of tobacco. Therefore, 150 kinds of these components, which are easy to separate to meet the requirements of qualitative and quantitative analysis in the chromatogram, are selected as the target indicators for the evaluation of simultaneous distillation and extraction.

Single-factor experiments

Effect of material/liquid ratio on extraction of aroma components

The effect of material/liquid ratio (g/mL) on the extraction of aroma components is shown in Figure 2. It was found that the extract volume of aroma components had no significant difference as the material/liquid ratio was increased from 1:8 to 1:12. However, the extract volume of aroma compounds decreased dramatically with the continued increase of the material/liquid ratio up to 1:18. It was easy to cause a gelatinization reaction when the material/liquid ratio was too low. Thus, 1:12 was selected as the most favorable material/liquid ratio.

Effect of distillation time on extraction of aroma components

The effect of distillation time on the extraction of aroma components was studied at different distillation times (1.5−3.5 h). As shown in Figure 3, the extraction volume of the aroma substances was evidently enhanced corresponding to an increase in the distillation time from 1.5 to 3 h, and was decreased when the extraction time exceeded 3 h. This suggested that a distillation time of 3 h was optimal.

Effect of NaCl dosage on extraction of aroma components

The ratio of NaCl and tobacco (g/g) was one of factors that could influence the extraction of aroma substances. The activity coefficient and the relative volatility of the components
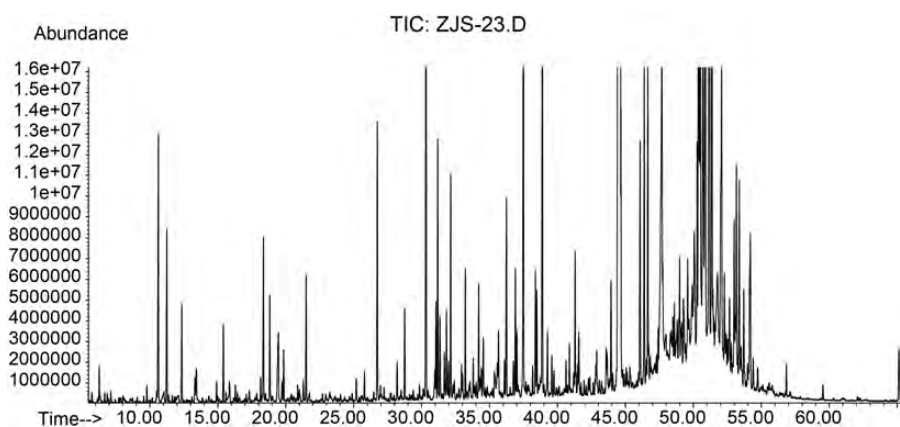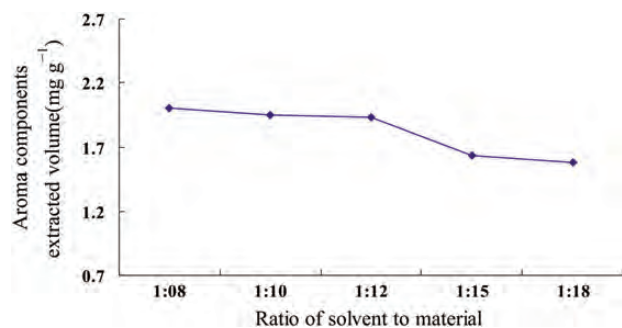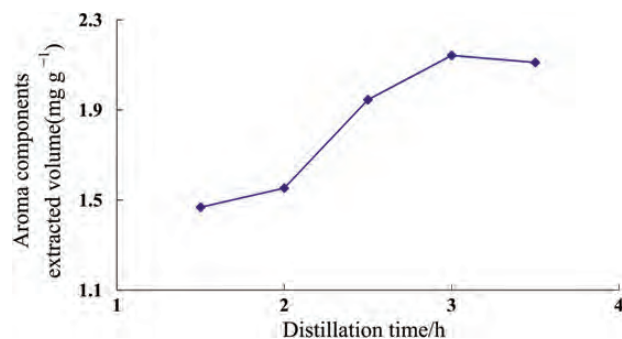

Figure 1. The total ion chromatogram of the free and semi-volatile components in cut tobacco from Yunnan province.

Figure 2. Effect of ratio of material to solvent on aroma components extraction. Experiments were carried out for distillation time 2.5 h, ratio of NaCl and tobacco 1:1, ratio of CH_2Cl_2 and tobacco 2 :1, water-bath temperature 60°C.

Figure 3. Effect of distillation time on aroma components extraction. Experiments were carried out for material/solvent ratio 1:12, ratio of NaCl and tobacco 1:1, ratio of CH_2Cl_2 and tobacco 2 :1, water-bath temperature 60°C.
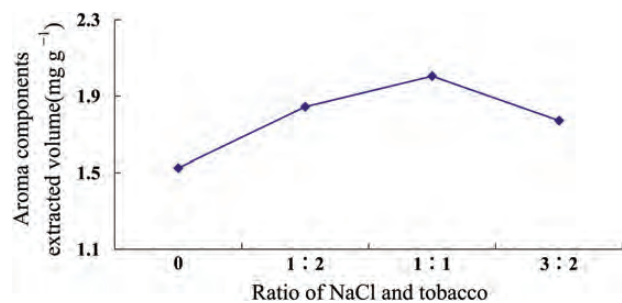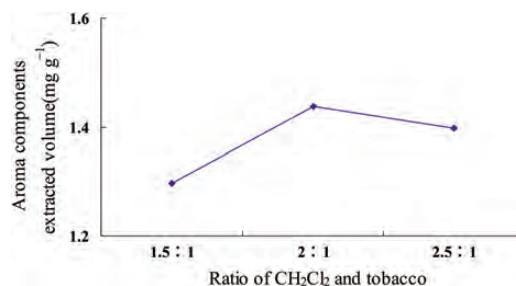

Figure 4. Effect of NaCl dosage on aroma components extraction. Experiments were carried out for material/solvent ratio 1:12, distillation time of 2.5 h, ratio of CH_2Cl_2 and tobacco 2 :1, water-bath temperature 60°C.

in a separation reacting system can be increased by adding a salt amount (23). In Figure 4, a set of ratios of NaCl and tobacco (0, 1:2, 1:1 and 3:2) was investigated. The extraction volume of aroma substances significantly increased with an increase in the ratio of NaCl and tobacco from 0 to 1:1, and declined with a further increase in NaCl dosage, which indicated that a 1:1 ratio of NaCl and tobacco was the optimized condition.

Effect of volume of CH2Cl2 on extraction of aroma components

The effect of the ratio of CH_2Cl_2 and tobacco (mL/g) on the extraction of aroma components is shown in Figure 5. The

Figure 5. Effect of volume of CH_2Cl_2 on aroma components extraction. Experiments were carried out for material/solvent ratio 1:12, distillation time of 2.5 h, ratio of NaCl and tobacco 1:1, water-bath temperature 60°C.
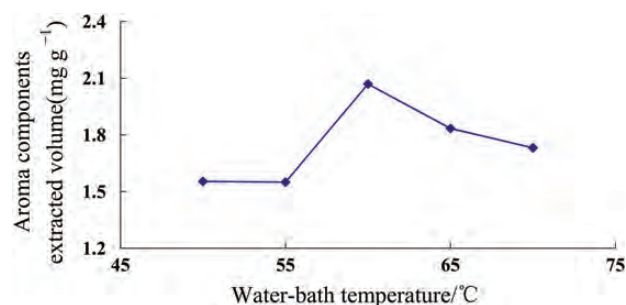
Figure 6. Effect of water-bath temperature on aroma components extraction. Experiments were carried out for material/solvent ratio 1:12, distillation time of 2.5 h, ratio of NaCl and tobacco 1:1, ratio of CH_2Cl_2 and tobacco 2 :1.

Table I
Variables and Experimental Design Levels for RSM

Levels	Factors				
	Material/liquid ratio (g/mL) (A)	Distillation time (h) (B)	Ratio of NaCl and tobacco (g/g) (C)	Ratio of CH2Cl2 and tobacco (mL/g) (D)	Water-bath temperature (°C) (E)
−1	1:10	2.5	0.8:1	1.8:1	55
0	1:12	3	1:1	2:1	60
1	1:14	3.5	1.2:1	2.2:1	65

ratios of CH_2Cl_2 and tobacco were set at 1.5:1, 2:1 and 2.5:1, respectively. The extraction volume of the aroma components increased as the ratio of CH_2Cl_2 and tobacco ascended from 1.5:1 to 2:1, and declined when the ratio rose continually from 2:1 to 2.5:1. Therefore, the optimal ratio of CH_2Cl_2 and tobacco was considered to be 2:1.

Effect of water-bath temperature on extraction of aroma components

A set of water-bath temperatures was investigated from 50 to 70°C for the effects on the extraction of aroma components. As shown in Figure 6, the volume of aroma components had no significant difference with an increase in temperature from 50 to 55°C. However, the extraction of aroma components evidently started to decrease after the water-bath temperature exceeded 60°C. Therefore, 60°C was selected as the optimal temperature.

Table II

BBD Matrix and Responses of Dependent Variables on Aroma Extraction Yield

Run	Factors					The extraction (mg/g)
	A	B	C	D	E	
1	0	1	0	0	1	2.03
2	0	0	−1	0	−1	1.82
3	0	−1	0	−1	0	1.82
4	0	1	1	0	0	2.14
5	1	0	0	1	0	1.93
6	0	0	0	0	0	2.25
7	1	0	0	−1	0	2.24
8	0	0	1	1	0	1.84
9	0	0	0	0	0	2.30
10	0	0	0	1	1	2.03
11	1	−1	0	0	0	1.83
12	0	0	0	−1	−1	2.02
13	1	0	−1	0	0	1.94
14	0	0	−1	0	1	1.92
15	0	−1	−1	0	0	1.71
16	0	0	0	0	0	2.41
17	−1	0	1	0	0	1.94
18	0	0	1	0	−1	1.96
19	−1	−1	0	0	0	1.80
20	0	−1	0	0	1	1.91
21	0	−1	0	0	−1	1.73
22	0	0	−1	−1	0	1.87
23	0	0	−1	1	0	1.96
24	1	0	0	0	−1	1.96
25	0	0	0	0	0	2.39
26	−1	0	0	0	1	1.80
27	−1	0	0	1	0	2.01
28	0	0	0	0	0	2.38
29	0	0	0	1	−1	1.99
30	0	−1	0	1	0	1.86
31	0	0	0	0	0	2.27
32	0	1	−1	0	0	2.09
33	−1	1	0	0	0	2.21
34	−1	0	0	0	−1	2.18
35	1	0	1	0	0	2.06
36	0	0	1	−1	0	2.18
37	−1	0	0	−1	0	1.95
38	0	0	0	−1	1	2.03
39	0	−1	1	0	0	1.93
40	−1	0	−1	0	0	2.00
41	0	0	1	0	1	2.22

RSM optimization of experimental conditions

Design of experiments and results

To investigate the relationships between variables of factors and to optimize their conditions for the extraction of aroma components, RSM was employed to optimize their individual conditions. Design-Expert software (version 7.0) was used to generate the response surfaces and contour plots. According to the results of one-factor-at-a-time method experiments, the range and center point values of five independent variables are presented in Table I. The coded and uncoded values of the independent variables and the experimental conditions for each project are listed in Table II, and the experimental results with the extraction of tobacco aroma components (mg/g) as response value (R) were also included in the last column of this table.

Variance analysis and second-order regression equation fitting

The regression analysis was performed to fit the response. As suggested by the software, no transformation was chosen and the quadratic process order was selected to analyze data. The final regression function for aroma components extraction volume in terms of coded factors used in making of statistical model is as follows:

$$R = 2.33 + 0.033A + 0.16B + 0.060C - 0.044D + 0.019E$$
$$- 9.685 \times 10^{-3}AB + 0.045AC - 0.092AD + 0.17AE$$
$$- 0.042BC - 0.064BD - 0.097BE - 0.11CD + 0.040CE$$
$$+ 7.500 \times 10^{-3}DE - 0.14A^2 - 0.18B^2 - 0.20C^2 - 0.16D^2$$
$$- 0.16E^2 \quad \text{Equation 1}$$

where R is the response value (mg/g, extraction of aroma components in tobacco), A, B, C, D and E are the independent

Table III

ANOVA for the Fitted Quadratic Polynomial Model for Optimization of Aroma Extraction Yield

| Source	Sum of squares	Degree of freedom (DF)	Mean square	F value	p-value (probability > F)	Significance
Model	1.25	20	0.063	12.85	<0.0001	Significant
Material/liquid ratio (A)	0.013	1	0.013	2.70	0.1157	
Distillation time (B)	0.23	1	0.23	47.11	<0.0001	Very significant
Ratio of NaCl and tobacco (C)	0.058	1	0.058	11.81	0.0026	Very significant
Ratio of CH2Cl2 and tobacco (D)	0.023	1	0.023	4.80	0.0405	Significant
Water-bath temperature (E)	4.402×10^{-3}	1	4.402×10^{-3}	0.90	0.3534	
AB	2.332×10^{-4}	1	2.332×10^{-4}	0.048	0.8291	
AC	8.100×10^{-3}	1	8.100×10^{-3}	1.66	0.2122	
AD	0.034	1	0.034	7.02	0.0154	Significant
AE	0.076	1	0.076	15.50	0.0008	Very significant
BC	7.225×10^{-3}	1	7.225×10^{-3}	1.48	0.2377	
BD	7.058×10^{-3}	1	7.058×10^{-3}	1.45	0.2430	
BE	0.024	1	0.024	4.86	0.0393	Significant
CD	0.046	1	0.046	9.48	0.0059	Very significant
CE	6.400×10^{-3}	1	6.400×10^{-3}	1.31	0.2655	
DE	2.250×10^{-4}	1	2.250×10^{-4}	0.046	0.8321	
A^2	0.14	1	0.14	29.38	<0.0001	Very significant
B^2	0.19	1	0.19	39.40	<0.0001	Very significant
C^2	0.33	1	0.33	67.33	<0.0001	Very significant
D^2	0.20	1	0.20	41.55	<0.0001	Very significant
E^2	0.19	1	0.19	38.75	<0.0001	Very significant
Residual	0.098	20	4.877×10^{-3}			
Lack of fit	0.074	15	4.947×10^{-3}	1.06	0.5184	Not significant
Pure error	0.023	5	4.667×10^{-3}			
Total	1.35	40				

variables (*A*: ratio of material to solvent, *B*: distillation time, *C*: ratio of NaCl and tobacco, *D*: ratio of CH_2Cl_2 and tobacco and *E*: water-bath temperature).

To test the goodness-of-fit of the regression equation, R^2 was evaluated. The coefficient of determination, $R^2 = 0.9278$, indicated that the model could explain approximately 92.78% of the total variability in the response.

The analysis of variance (ANOVA) procedure was used to analyze the model for significance and suitability, and a statistical summary is given in Table III. The *p* value was used as a tool to check the significance of each coefficient, and also indicated the interaction strength between each independent variable. ANOVA of the quadratic regression model demonstrated that

the model was highly significant, as evident from the *F*-test with a very low probability value ($p < 0.0001$). The model *F*-value of 12.85 implied that the model was significant. The lack of fit *F*-value of 1.06 implied that the lack of fit was not significant relative to the pure error at 0.05 levels. The model was found to be adequate for prediction within the range of variables employed. The regression coefficients and the corresponding *p* values are also shown in Table III. The *p* values of each model, confirmed that the seven coefficients, including *B* (distillation time), *C* (ratio of NaCl and tobacco), *D* (ratio of CH_2Cl_2 and tobacco), *AE* (interactions between ratio of material to solvent and water-bath temperature), *CD* (interactions between NaCl dosage and CH_2Cl_2 dosage), *AD* and *BE* were significant.
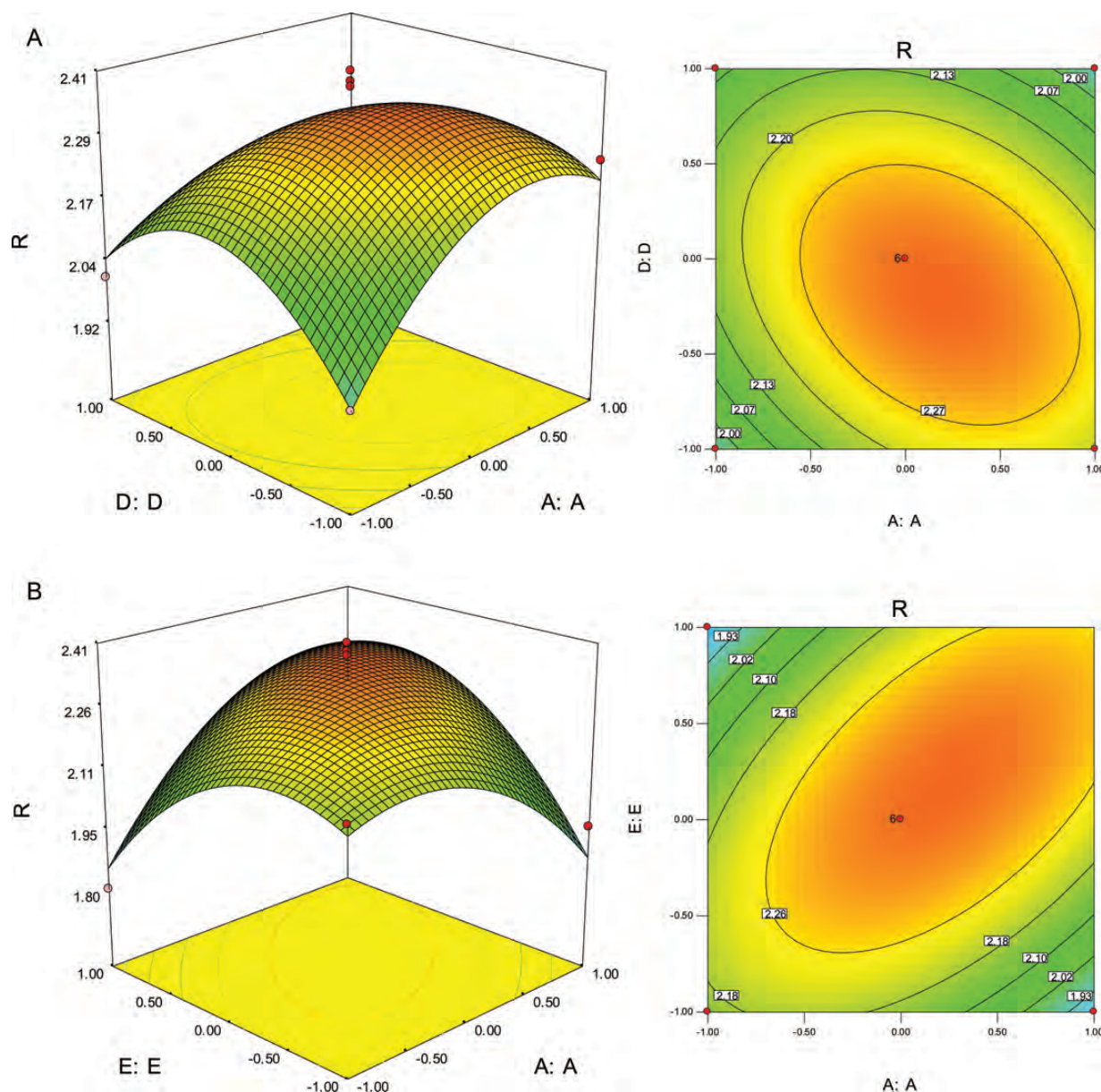
Figure 7. Response surface plots showing the interaction between variables in the aroma extraction yield: (A) interaction between material/liquid ratio and volume of CH_2Cl_2; (B) interaction between material/liquid ratio and water-bath temperature; (C) interaction between distillation time and water-bath temperature; (D) interaction between NaCl dosage and volume of CH_2Cl_2.
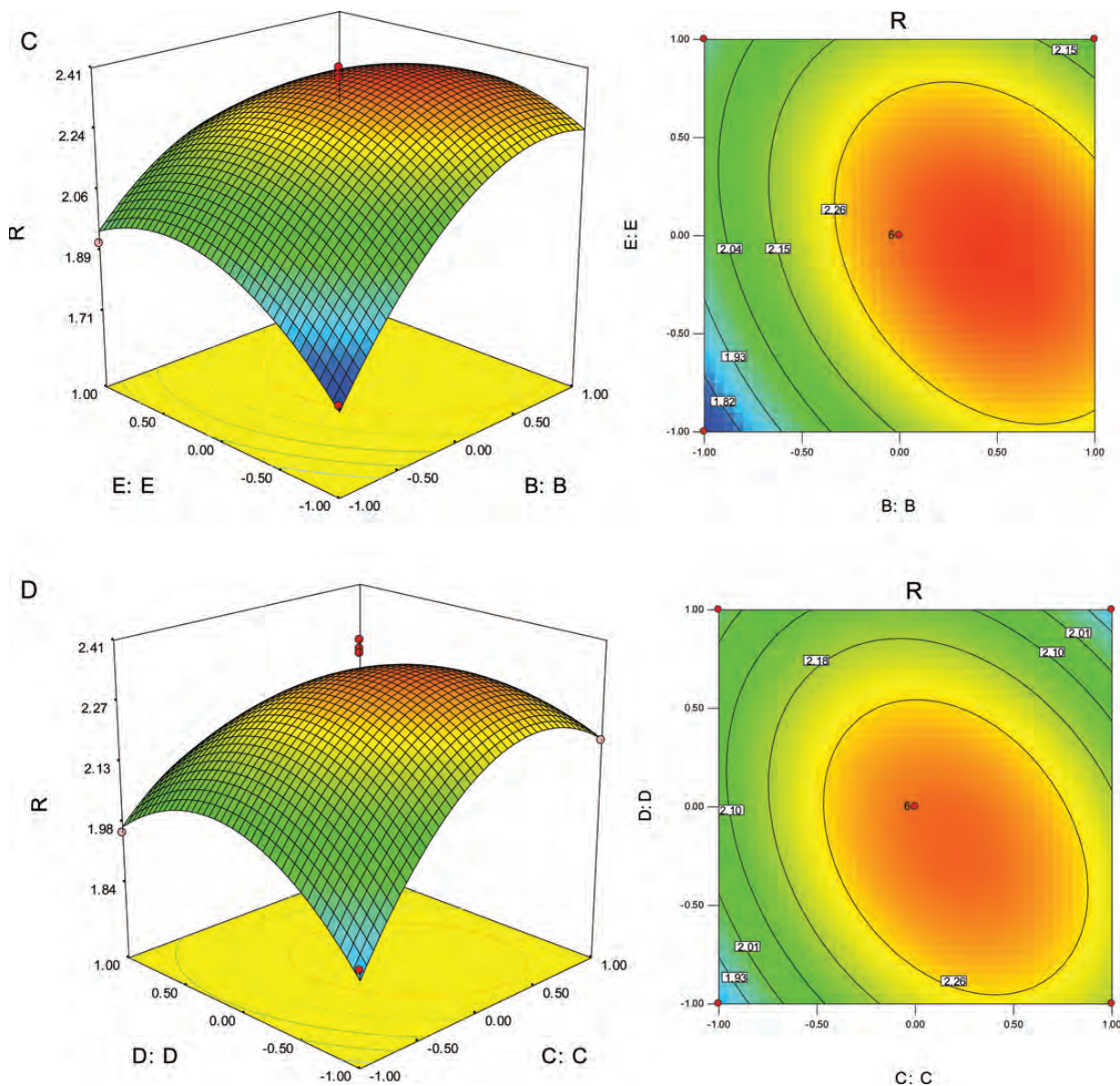
Figure 7. *Continued*

Interaction analysis

To determine the simultaneous interaction between variables (*A* and *D*, *A* and *E*, *B* and *E*, and *C* and *D*) on aroma extraction, three-dimensional response surface plots and two-dimensional contour plots were used, as shown in Figure 6.

Figure 7A shows the changes of extraction yield with varying material/liquid ratio (*A*) and volume of CH_2Cl_2 (*D*). When the material/liquid ratio increased up to 1:12, the volume of CH_2Cl_2 became the critical factor for improving the aroma extraction yield. Fluctuations in the volume of CH_2Cl_2 could lead to larger differences in aroma extraction yield. The optimal material/liquid ratio and ratio of CH_2Cl_2 and tobacco for aroma extraction yield were 1:12 and 1.92:1, respectively. The effect of material/liquid ratio (*A*) and water-bath temperature (*E*) on aroma extraction yield are shown in Figure 7B. When the material/liquid ratio was 1:12, temperature became the critical

factor for improving the aroma extraction yield. Figure 6B shows that the optimal material/liquid ratio and water-bath temperature were 1:12 and 60.65°C, respectively. The response surface plot and contour plot of interactions between distillation time (*B*) and water-bath temperature (*E*) are shown in Figure 7C. The extraction yield of aroma components increased with an was increase in distillation time and water-bath temperature. The maximum extraction yield was achieved at a distillation time of 3.23 h and water-bath temperature of 60.65°C. The response surface plot and contour plot of interactions between NaCl dosage (*C*) and volume of CH_2Cl_2 (*D*) are shown in Figure 7D. The results revealed that the extraction yield increases with an increase in NaCl dosage and volume of CH_2Cl_2. The highest extraction yield was obtained at a 1.05:1 ratio of NaCl and tobacco and a 1.92:1ratio of CH_2Cl_2 and tobacco.

The optimal conditions for the simultaneous distillation extraction were evaluated as follows: ratio of material to solvent, 1:12 (g/mL); distillation time, 3.23 h; ratio of NaCl and tobacco, 1.05:1 (1.05 g/g); ratio of CH_2Cl_2 and tobacco, 1.92:1 (1.92 mL/g); water-bath temperature 60.65°C.

Validation experiment of optimal conditions

Within the scope of the variables investigated in the BBD, additional experiments with different conditions for aroma extraction were conducted to assess the validity of the model. For the convenience of operation in the practice of production, the extraction conditions of tobacco aroma components were adjusted as follows: 1:12 ratio of material to solvent, 3.20 h distillation time, 1:1 ratio of NaCl and tobacco, 2:1 ratio of CH_2Cl_2 and tobacco, 60°C water-bath temperature. A verification experiment was performed using the adjusted-predicted optimum extraction conditions, and the extraction amount of aroma components was determined. The experimental values (mean of five measurements) and predicted values were presented as 2.27 (± 0.027) and 2.31 mg/g, respectively. Therefore, the verification experiment satisfactorily demonstrates the goodness-of-fit for the curve and the reproducibility of the results for an extraction performed with the optimum parameters.

Comparison of samples

The extraction of volatile components from tobacco is a matrix-dependent and method-dependent process. Although the analysis of volatile components in tobacco has also been undertaken through the use of traditional steam distillation (SD) and headspace co-distillation (HCD) techniques, they are not effective for the extraction of volatile tobacco components according the approximately quantitative analysis (12). The available literature has determined that flue-cured tobacco samples always contain similar kinds of flavor compounds with varying quantities, although they are from different sources and grades (22).

Only few reports have employed SDE for the extraction of volatile tobacco components. Peng *et al.* reported that 377 components were extracted in SDE and the total amount of extracted volatile components was 0.445 mg/g (12). Huang *et al.* showed that only 102 volatile components with a very low quality level were detected (13). Although the qualitative comparison showed that fewer compounds were detected in this study (150 compounds), the approximately quantitative analysis showed that the total amount of volatile components in the optimal SDE extract in this study (2.27 mg/g) was much higher than the previous reports. The difference in quantity indicated that the different operation parameters (such as temperature, material/liquid ratio and distillation time) can play an important role in SDE (1, 11, 12, 14, 21). The optimization techniques used in this work can be widely applied to other processes for the optimization of extraction conditions.

Conclusion

Using the single-factor experiments and RSM, it was possible to determine optimal operating conditions to obtain a high aroma extraction yield. Based on the single-factor experiments, RSM

was used to estimate and optimize the experimental variables: material/liquid ratio, distillation time, dosage of NaCl, volume of CH_2Cl_2 and water-bath temperature. The optimal conditions for tobacco aroma components extraction were determined as follows: 1:12 ratio of material to solvent, 3.20 h distillation time, 1:1 ratio of NaCl and tobacco, 2:1 ratio of CH_2Cl_2 and tobacco, 60°C water-bath temperature. The study provided the possibility for the tobacco industry to extract aroma components from tobacco with simultaneous distillation extraction.

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