Optimization for Preparation of Lemon-catalyzed Ginsenoside Rg_3 by Response Surface Method

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Abstract: **Objective** Ginsenoside Rg_3 was prepared by converting protopanaxadiol (PPD)-type saponins using lemon juice as the catalyst. **Methods** Preparation of ginsenoside Rg3 was optimized by response surface method (RSM) based on a four-factor and five-level central composite design. **Results** The optimal yield of ginsenoside Rg_3 was predicted to be 75.98% in the combination of the factors (PPD-type saponins concentration of 23.6 mg/mL, lemon juice concentration of 97.6% at 85.7 ℃ for 130.0 min) through the canonical analysis and ridge analysis with maximum responses. Under the optimum conditions, the actual yield of ginsenoside Rg_3 was 75.57%. **Conclusion** RSM is effective to optimize the preparation of ginsenoside Rg_3 by lemon-catalyzed PPD-type saponins. The achievement for the preparation of ginsenoside Rg_3 would promote the development of health-care production.

Key words: conversion; ginsenoside Rg_3; lemon; protopanaxadiol type saponins; response surface method
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Introduction

Ginseng (*Panax ginseng* C. A. Meyer, Araliaceae) has been used as Chinese materia medica for about 4000 years (Sun, Liu, and Zhang, 2005). Ginseng contains many active components, such as ginsenoside, ginseng polysaccharides, volatile oil, and so on. Among them, ginenoside was considered as the symbolic component. Recently, ginsenoside, especially ginsenoside Rg_3 and Rh_2, showed many importantly pharmacological activities (Kim *et al*, 2003; Karikura *et al*, 1991; Leung *et al*, 2007; Yue *et al*, 2007) in the studies.

Ginsenoside Rg_3 was one of the secondary metabolite of protopanaxadiol (PPD)-type saponins that was existent in the red ginseng and was short of 0.03%. The modern pharmacological studies indicated that ginsenoside Rg_3 showed the cancer cell inhibition (Pan *et al*, 2012), anti-oxidative (Wei, Xu, and Che, 2012), anticarcinogenic (Liu *et al*, 2000), neuroprotective (Kim *et al*, 2007), anti-apoptosis (Tian *et al*, 2005), immunopotentiation (Min *et al*, 2006), and antitumor activities (Park *et al*, 2011). In addition, the combination therapy of ginsenoside Rg_3 and cyclophosphamide (CTX) or paclitaxel may cooperatively enhance antitumor effect or antiangiogenesis (Xu *et al*, 2007; Yuan and Ye, 2011). Recently, ginsenoside Rg3 is commonly prepared by acid hydrolysis, such as HCl, H_2SO_4, and HAc (Bae *et al*, 2004; Han *et al*, 1982; Chen *et al*, 2004), enzymatic hydrolysis (Cheng *et al*, 2008; Yan *et al*, 2008), and steam heating (Wang *et al*, 2007).

Response surface method (RSM) is a useful statistical technique to investigate the relationships between several explanatory variables and one or more response variables, serving the optimizations of complex reaction conditions and predicting the maximum response. RSM could be used in biology, medicine, and engineering, especially food process (Mo and Sun, 2007; Puri, Beg, and Gupta, 2002) as well as the excellent abilities of analysis and prediction.

Lemon (*Citrus Fructus*) is rich in vitamin C and citric acid and shows strong anti-oxidant (Yoshiaki, Kanefumi, and Toshihiko, 1997) and anti-aging (Thapa, 2005) effects. In this work, central composition design

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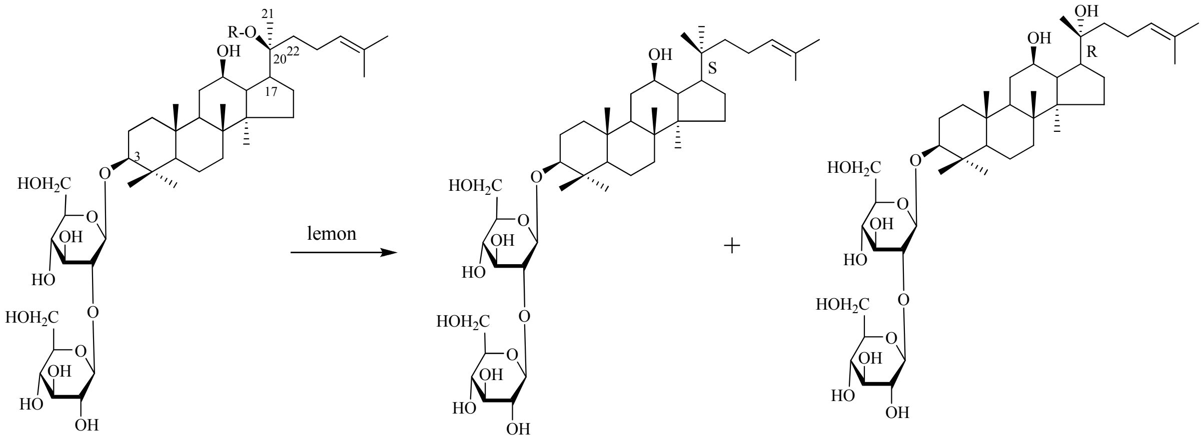
of RSM was used for the preparation of ginsenoside Rg_3 (Fig. 1) by converting PPD-type saponins using lemon. The aim of this study is to optimize the efficient method of green preparation for ginsenoside Rg_3.



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PPD ginsenoside Rg_3

R: -Glc(6-1)glc, ginsenoside Rb_1; -Glc(6-1)arap, ginsenoside Rb_2; -Glc(6-1)araf, ginsenoside Re; -Glc, ginsenoside Rd

Fig. 1 Conversion of PPD-type saponins to ginsenoside Rg_3 by lemon

Materials and methods

Materials

Total saponins of ginseng root were purchased from Fusong, China. Reference substances of ginsenoside Rb_1, Rb_2, Rb_3, Rc, Rd, 20(*S*)-Rg_3, and 20(*R*)-Rg_3 were purchased from Mansite Cooperation (Chengdu, China). Acetonitrile (HPLC grade) was purchased from J. T. Baker, USA. Other solvents were purchased from Damao Chemical Reagent Factory (Tianjin, China).

Preparation of ginsenoside Rg_3

The preparation of ginsenoside Rg_3 was carried out in water bath. PPD-type saponins were treated with lemon juice, neutralized with saturated Na_2CO_3, and extracted with water-saturated *n*-BuOH, successively. The combined *n*-BuOH was evaporated in vacuo at 45 ℃. The residue was filtered through a syringe filter (0.45 μm) and ready for HPLC analysis. Based on the central composite design, the PPD-type saponins concentration ranged from 10 to 30 mg/mL, the lemon juice concentration ranged from 60% to 100%, the temperature ranged from 60 to 100 ℃, and the time ranged from 20 to 220 min.

Model verification

Ginsenoside Rg_3 was prepared under the optimum conditions predicted by the ridge analysis of SAS program. Then the reaction products were neutralized with saturated Na_2CO_3, extracted with water-saturated *n*-BuOH, and evaporated in vacuo at 45 ℃.

Experimental design

A four-factor, five-level central composite design was utilized in this study including 24 noncentral point experiments and six central point experiments. The four factors were PPD-type saponins concentration, lemon juice concentration, temperature, and time. The settings for the independent variables were listed in Table 1. The second-order polynomial Eq. 1 was applied to determine the predicted response:

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_j^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response, β_0, β_i, β_{ii}, and β_{ij} are respectively the intercept, the linear coefficients, the squared coefficients, and the interaction coefficients

Experimental data were applied to the analysis of variance (ANOVA) to fit the response surface quadratic model using Design Expert 8.0.5 (Stat-Ease, Inc., Minneapolis, MN), and the response contour plots and predicted plots were depicted by Design Expert 8.0.5. Further, the canonical analysis was used to determine whether the stationary point was the maximum, minimum, or saddle point (Montgomery, 1991) in the response surface regression procedure of the SAS program (SAS, 9.1). Then, the maximum was predicted by the ridge analysis for the response surfaces.

HPLC analysis

The quantitative analysis of the reaction products (Sun *et al*, 2013) was performed by HPLC (Dalian Elite Analytical Instruments Co., Ltd., China) equipped with a P230P system, a UV detector, and two gradient pumps. The detection wavelength was 203 nm, and the column temperature was at 30 ℃. The column was the YMC J'sphere ODS-H80 (250 mm× 4.6 mm, 4 μm), a 20 μL sample was injected, and the mobile phase utilized the gradient conditions with distilled H_2O (A) and CH_3CN (B). The gradient elution was as follows: 0—30 min, 30%—40% B; 30—37 min, 40%—50% B; 37—45 min, 50%—51% B; 45—60 min, 51%—55% B; 60—60.10 min, 55%—90% B; 60.10—75 min, 90% B; and 75—75.10 min, 90%—30% B with a 1.0 mL/min flow rate.

Results and discussion

Analysis of model

In the central composite design of RSM, four reaction factors, including PPD-type saponins concentration, lemon juice concentration, temperature, and time, were selected for the reaction optimization.

The experimental variable settings were based on the central composite design with the four-factor, five-level system. The experiment design and data were listed in Table 1. Among them, response *Y* is the yield of ginsenoside Rg_3 that is the sum of yields of *R*- and *S*-ginsenoside Rg_3 (Sun *et al*, 2013). From Table 1, the highest yield of ginsenoside Rg_3 was 72.18% in the combination of variables PPD-type saponins concentration

Table 1 Experiment design and experimental data in central composite design

Treatments	PPD-type saponins concentration (X_1)	Lemon juice concentration (X_2)	Temperature (X_3)	Time (X_4)	Response (Y)
1	15 (−1)	70 (−1)	70 (−1)	70 (−1)	45.10
2	25 (1)	70 (−1)	70 (−1)	70 (−1)	34.18
3	15 (−1)	90 (1)	70 (−1)	70 (−1)	46.40
4	25 (1)	90 (1)	70 (−1)	70 (−1)	41.12
5	15 (−1)	70 (−1)	90 (1)	70 (−1)	64.44
6	25 (1)	70 (−1)	90 (1)	70 (−1)	66.64
7	15 (−1)	90 (1)	90 (1)	70 (−1)	64.09
8	25 (1)	90 (1)	90 (1)	70 (−1)	67.24
9	15 (−1)	70 (−1)	70 (−1)	170 (1)	65.97
10	25 (1)	70 (−1)	70 (−1)	170 (1)	42.97
11	15 (−1)	90 (1)	70 (−1)	170 (1)	61.85
12	25 (1)	90 (1)	70 (−1)	170 (1)	59.00
13	15 (−1)	70 (−1)	90 (1)	170 (1)	63.13
14	25 (1)	70 (−1)	90 (1)	170 (1)	64.94
15	15 (−1)	90 (1)	90 (1)	170 (1)	62.14
16	25 (1)	90 (1)	90 (1)	170 (1)	66.88
17	10 (−2)	80 (0)	80 (0)	120 (0)	60.39
18	30 (2)	80 (0)	80 (0)	120 (0)	62.54
19	20 (0)	60 (−2)	80 (0)	120 (0)	70.98
20	20 (0)	100 (2)	80 (0)	120 (0)	70.42
21	20 (0)	80 (0)	60 (−2)	120 (0)	22.80
22	20 (0)	80 (0)	100 (2)	120 (0)	64.78
23	20 (0)	80 (0)	80 (0)	20 (−2)	39.34
24	20 (0)	80 (0)	80 (0)	220 (2)	63.63
25	20 (0)	80 (0)	80 (0)	120 (0)	64.01
26	20 (0)	80 (0)	80 (0)	120 (0)	70.20
27	20 (0)	80 (0)	80 (0)	120 (0)	66.81
28	20 (0)	80 (0)	80 (0)	120 (0)	69.65
29	20 (0)	80 (0)	80 (0)	120 (0)	67.26
30	20 (0)	80 (0)	80 (0)	120 (0)	72.18

of 20 mg/mL, lemon juice concentration of 80%, reaction temperature of 80 ℃, and reaction time of 120 min, while the lowest was 33.48% in the combination of variables PPD-type saponins concentration of 20 mg/mL, lemon juice concentration of 80%, at 60 ℃, and for 120 min.

The ANOVA for the response surface of the quadratic model was preformed to fit the second-order polynomial Eq. 1 by Design Expert. Table 2 showed the regression coefficients of quadratic polynomial model that were determined by employing the least-squares technique. Among the coefficients, some variables (β_3, β_4, β_{13}, β_{34}, $\beta_{11,}\beta_{33}$, and β_{44}) were selected since they were significant ($P < 0.05$), and others (β_1, β_2, β_{12}, β_{14},β_{23}, β_{24}, and β_{22}) were removed from the model since those with big P-values ($P > 0.05$) were of no significance. The fitted second-order polynomial Eq.2 was expressed in the following regression equation:

$$Y = 68.35 + 8.62X_3 + 4.43X_4 + 3.37X_{13} - 4.27X_{34} - 1.66X_{11} - 6.07X_{33} - 4.15X_{44} \quad (2)$$

Table 2 Regression coefficients of predicted second-order polynomial model for response variables

Variable	Coefficients (β)	Standard error	P^a
intercept	68.35	1.58	0.0001
X_1	−1.08	0.79	0.1935
X_2	0.84	0.79	0.3035
X_3	8.62	0.79	0.0001
X_4	4.43	0.79	0.0001
X_{12}	1.85	0.97	0.0749
X_{13}	3.37	0.97	0.0034
X_{14}	−0.53	0.97	0.5937
X_{23}	−1.18	0.97	0.2404
X_{24}	0.27	0.97	0.7819
X_{34}	−4.27	0.97	0.0005
X_{11}	−1.66	0.74	0.0409
X_{22}	0.65	0.74	0.3915
X_{33}	−6.07	0.74	0.0001
X_{44}	−4.15	0.74	0.0001

$^a P < 0.01$, high significance; $0.01 < P < 0.05$, significance; $P > 0.05$, no significance

From the ANOVA (not shown), total model was highly significant as the small P-value ($P = 0.0001$). Lack of fit with P-value > 0.05 ($P = 0.2029$) was of no significance, which showed that the model fitted well for the experimental data. The value of R^2 (0.95) indicated a high degree of correlationship between the actual yields of ginsenoside Rg_3 and predicted ones (Fig. 2). And the fitting degree of the model was good since the value of the determination coefficient Adj R^2 was 0.9034. Therefore, this experimental model was adequate and reproducible for predicting the yield of ginsenoside Rg_3.

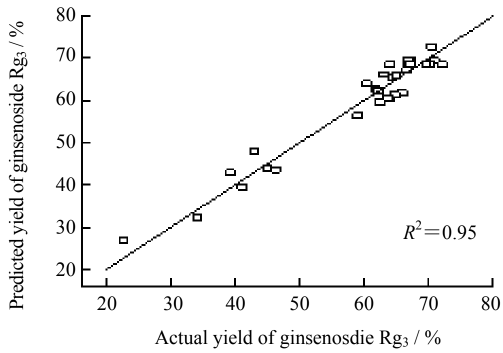

Fig. 2 Relationship between actual yields and predicted values of ginsenoside Rg_3

Effect of variables on yield of ginsenoside Rg_3

According to the result of regression coefficients of predicted second-order polynomial model, the yield of ginsenoside Rg_3 was significantly affected by the first-order linear variables temperature (X_3) and time (X_4), second-order variables PPD-type saponins concentration (X_{11}), temperature (X_{33}), and time (X_{44}), the interaction variable between PPD-type saponins concentration and temperature (X_{13}) and the interaction variable between temperature and time (X_{34}), which could be better understood by Fig. 3. It showed that the first-order variables had a significantly positive effect on the yield of ginsenoside Rg_3 except PPD-type saponins concentration, and the two variables temperature and time were most significant among them. Among the second-order variables, the significant variables, PPD-type saponins concentration (X_{11}), temperature (X_{33}), and time (X_{44}), showed the negative effects on the yield of ginsenoside Rg_3 since their P-values were smaller than 0.05. The interaction between variables, X_{13} (the interaction parameter of PPD-type saponins concentration and temperature) and X_{34} (the interaction parameter of temperature and time), had significantly negative effects on the yield of ginsenoside Rg_3 since the P-values were smaller than 0.05 among them.

Fig. 4 shows the relationship between the variables and the yield generated from the predicted model (Eq. 2) by holding PPD-type saponins concentration (15, 20, and 25 mg/mL) and lemon juice

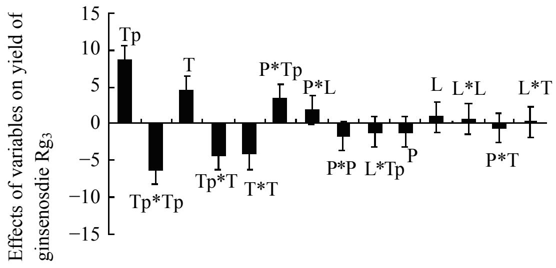

Fig. 3 Main effects of variables on ginsenoside Rg_3 yield

P: PPD saponins concentration; L: lemon juice concentration; Tp: temperature; T: time

concentration (70%, 80%, and 90%) constant. The yield of ginsenoside Rg_3 could be better predicted by temperature and time since their P-values were smaller than 0.01. It quickly increased with temperature and time increasing, then slightly decreased in each of contour plots (Fig. 4). Compared with nine contour plots, it decreased with lemon juice concentration increasing when PPD-type saponins concentration was lower, while the contrary condition was observed (more than 20 mg/mL). The above-mentioned results showed that there was a saddle point in this study expressed by Fig. 5 generated from the predicted model (Eq. 2) by holding temperature (80 ℃) and time (120 min) constant.

Optimization for yield of ginsenoside Rg_3

The optimum yield of ginseoside Rg_3 was determined by the ridge analysis and canonical analysis whose function was to determine whether the stationary point was a maximum, minimum, or saddle point (Montgomery, 1991). The result of canonical analysis depended upon the stationary point could be depicted as the Eq. 3.

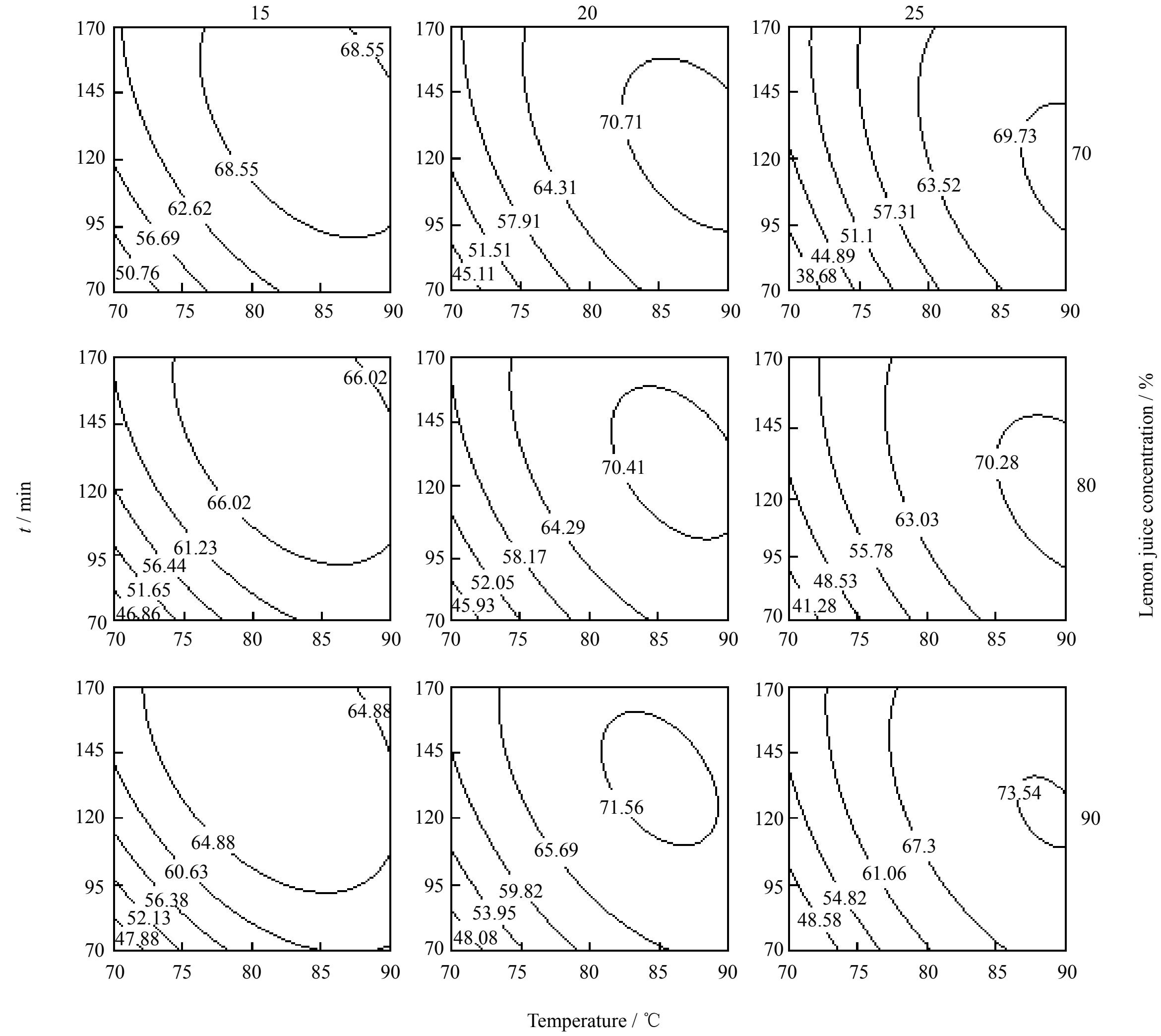

Fig. 4 Contour plots showing effects of temperature and time on yield of ginsenoside Rg_3 by holding constants PPD-type saponins concentration (15, 20, and 25 mg/mL) and lemon juice concentration (70%, 80%, and 90%)

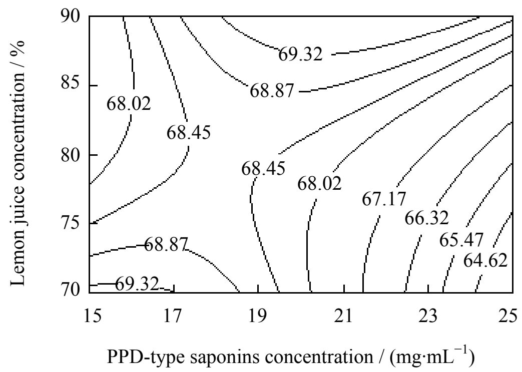

Fig. 5 Contour plot showing the saddle point by holding constant temperature (80 ℃) and time (120 min)

$$Y = 71.66 + 3.92W_1^2 - 3.64W_2^2 - 14.06W_3^2 - 31.12W_4^2 \quad (3)$$

Where Y is the yield of ginsenoside Rg_3, W_1, W_2, and W_3 are the eigenvalues based on coded data

The predicted response surface of the stationary point was a saddle point because the eigenvalues were mixed positive and negative (Montgomery, 1991), which was identical with the above-mentioned analysis (Fig.5). Therefore, the ridge analysis was performed to predict the maximum yield. Based on the ridge analysis, it was predicted to be 75.98% in the combination of variables PPD-type saponins concentration of 23.6 mg/mL, lemon juice concentration of 97.6%, reaction temperature of 85.7 ℃, and reaction time of 130.0 min.

Model verification

The confirmatory experiment was carried out to confirm the adequacy of the model in the predicted maximum reaction conditions. The three-time average value was 75.57% under the optimum reaction conditions (Fig. 6), which turned out that RSM was effective to optimize the preparation of ginsenoside Rg_3 catalyzed by lemon juice in this study.

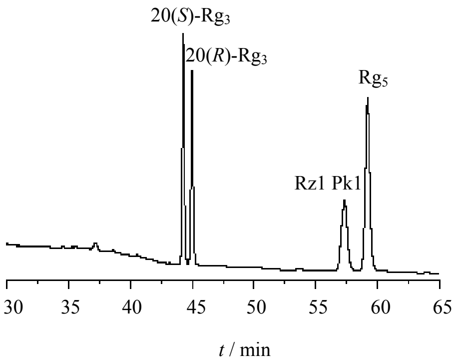

Fig. 6 HPLC of reaction products

Conclusion

In this study, ginsenoside Rg_3 has been successfully prepared by hydrolyzing PPD-type saponins in lemon juice, and the reaction conditions were optimized by RSM. The experimental results showed that the yield of ginsenoside Rg_3 was 75.57% when the PPD-type saponin concentration, lemon juice concentration, reaction temperature, and reaction time were 23.6 mg/mL, 97.6%, 85.7 ℃, and 130.0 min, respectively. Compared with the previous report (Sun *et al*, 2012), the yield of ginsenoside Rg_3 increased from 59.6% to 75.57%. Therefore, RSM in this study was effective to optimize the preparation of ginsenoside Rg_3 catalyzed by lemon juice. This achievement would promote the development of pharmacological studies and health-care production.

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