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Extract optimization of apple flavonoids by RSM and study on •OH scavenging effect

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ABSTRACT

Ethanol extraction of total flavonoids from Zhao tong Golden Delicious Apples were researched in the paper. The response surface methodology (RSM) and Box-Benhnken Design (BBD) were employed to optimize the extraction process. Based on the single-factor experimental results, ethanol concentration of 65~85%, solid-liquid ratio of 15:1~25:1mL·g^{-1}, extraction temperature of 55 ~ 75°C and time of 1.5 ~ 2.5h were selected as the independent variables and scope for BBD. Hydroxyl radicals scavenging effect of Apple extracts were also measured. The optimum extract conditions were ethanol concentration of 72%, solid-liquid ratio of 20:1mL·g^{-1}, extraction temperature of 65°C and extraction time of 2.3h with responding extraction ratio of 5.63% for pericarp, 4.30% for fruit and 3.89% for Pyrenes. The experimental extraction ratios matched well with the theoretical values by solving the multiple regression equation, which confirms that RSM could be successfully used to optimize the extraction process and the fitted quadratic model had a predictive effect on target extracts. The scavenging effect of apple extracts on hydroxyl radicals displayed a significant dose-effect relationship, however, it showed a weaker scavenging effect compare to BHT with the same concentration.

KEYWORDS

Response surface methodology;
Box-benhnken design;
Ethanol extraction;
Zhao tong golden delicious apples;
Scavenging effect.

INTRODUCTION

Flavonoids compounds, refers a series of compounds including two benzene rings connected by the middle three carbon atoms. Flavonoids have significant efficacy as antioxidant, anti-cancer, anti-inflammatory, bactericidal, anti-virus and regulating body immunity, and etc. It is a kind of potential natural medicine with great prospects[1-4]. The reported extraction technology of flavonoids includes organic solvent extraction, ultrasonic extraction, microwave extraction, supercritical fluid extraction and Enzyme-assisted extraction in the literature[5-6].

Apples were flavonoids-riched. Tsao etal measured the content of flavonoids in eight kinds of apples by using liquid chromatography, the experimental results showed that the content of flavonoids in pericarp ranged from 834.2 to 2300.3mg·kg^{-1}, while the content of flavonoids in fruit only contained 15-605.6 mg ·kg^{-1}. The content distributions of flavonoids in apple were mainly concentrated in pericarp, while the content of flavonoids in fruit and

Pyrenees were very limited[7]. As there were no standards for most flavonoids, the reported contents of apple total flavonoids were measured by spectrophotometry[8-9]. JY Nie reported the extraction optimization of total flavonoids from apple fruit and the determination of total flavonoids content by spectrophotometry[10]. The ultrasonic assisted extraction of total flavonoids from apples and related research work were also reported in the literature[10]. Research data showed that total flavonoids concentrated in the pericarp, but related research on the extraction and content determination of total flavonoids from pericarp were very limited[11-13]. There were no report on the extraction and content determination of total flavonoids from Zhao tong golden delicious apples in the literature.

Response Surface Methodology (RSM) was demonstrated an effective statistic technique for optimizing complex processes, which has been successfully used to optimize the extraction process of total flavonoids compounds from many medicine plants[14-15]. The extraction ratios of total flavonoids were greatly influenced by extraction conditions, Box-Behnken Designs (BBD) were performed to predict the optimal extraction conditions and analyze the sensitivity of extraction rate to corresponding factors[16]. Here, Ethanol extraction flavonoids from Zhao tong golden delicious apples and determination of flavonoids content by spectrophotometry were reported, RSM and BBD were employed to optimize the extraction process. The hydroxyl radicals scavenging effect of apples extracts were also studied. This research would provide valuable data of flavonoids content for the identification and utilization of apple nutritional value.

MATERIALS AND METHODS

Materials

Zhao tong golden delicious apples → separation → dry → crush → Spare.

Experimental methods

Optimization ethanol extraction of total flavonoids from Zhao tong golden delicious apples by Response Surface Methodology and their scavenging effect on hydroxyl radicals were illustrated in. Extraction of total flavonoids, Qualitative experiments of apples extracts, and obtains of linear equations from Rutin standard curve were performed according to the literature[17-19].

Optimization of the extraction process by RSM

Optimization of extraction process by Response surface methodology was operated as References[19]. Box-Benhnken Design combining with quadratic response model of four factors and three levels were performed to optimize the extraction process. The independent variables of four- variables were firstly determined, the level of variables were coded by -1, 0, 1 based on the results of single-factor experiment (as shown in). A total of 27 points were designed, including points 16 factorial, 8 star points and 3 central points to ensure the precision of experiment.

Study on hydroxyl radical inhibition activity

Total flavonoids were extracted from apples under the preferred conditions by RSM. The extracts were centrifuged, purification by macroporous resin, ethanol elution (ethanol volume fraction 72%), solvent evaporation, freeze-dried to obtain the total flavonoids powder. Apple total flavonoids solutions with different concentrations were prepared. Hydroxyl radical scavenging activity were operated according to the references[17-20], apple total flavonoids and BHT solutions with different concentrations were added, The absorbance were measured under 510nm, the average values of absorbance were collected by parallel experiments. The scavenging ratios were calculated as follows:

The scavenging ratio of hydroxyl radical (%)

$$= [A_0 - (A_x - A_{x0})] / A_0 \times 100$$

A_0 is the absorbance of control solution, A_x was the absorbance of apple extracts or BHT; A_{x0} was background absorbance of the extract without H_2O_2.

RESULTS AND DISCUSSION

Chromogenic reactions of apple extracts were listed in, which were consistent with Rutin. It confirms that delicious apples contained total flavonoids. The absorption spectra of Rutin were shown in, so 510nm was determined as the maximum absorption wavelength of flavonoids. The linear regression equation was formulated as A= -0.00853 + 12.6C, R^2 = 0.9991 by the standard curve, as shown in.

Results of single-factor experiments

Influences of each single factor on extraction ratio were shown in. The optimum ethanol concentration was 75% with the responding extraction ratio 6.30% for pericarp, 4.31% for fruit and 4.05% for pyrenes. The content of flavonoid glycosides with moderately polar were high in apples. The polarity of the dissolution system was reduced with increasing ethanol concentration, which reduced the solubility of flavonoid glycosides and increased the dissolution of fat-soluble impurities. The presences of impurities were not conducive to post-separation and purification of total flavonoids, so the optimum ethanol concentration was selected as 75%. The impacts of solid-liquid ratio on extraction ratio of apples flavonoids were also shown in, Extraction ratio was significantly increased with the increasing of solid-liquid ratio, when the solid-liquid ratio was higher than 1:20 g·mL^{-1}, extraction ratio began to decrease. The preferred solid-liquid ratio was determined as 1:20 g·mL^{-1} with the responding extraction ratio of 5.70% for pericarp, 4.31% for fruit and 4.05% for pyrenes. Lower than 1:20 g·mL^{-1}, resulted in a waste of materials; Higher than 1:20 g·mL^{-1}, the concentration gradient of solid-liquid phase was two small, which was not conducive to the dissolution of total flavonoids. As shown in, with the increasing of temperature, extraction ratio increased significantly, the maximum of extraction ratio was achieved at 65 !. The higher temperature would lead to ethanol evaporation and oxidative degeneration of total flavonoids. The lower temperature decreases the dissolution rate of flavonoids. The optimum extraction temperature was determined as 65 ! for extraction of total flavonoids from apple. The optimum extraction time was 2.0h with the responding extraction ratio of 5.23% for pericarp, 3.73% for fruit and 3.48% for pyrenes. Less than 2.0h, the dissolution balances were not achieved, more than 2.0h, the dissolution of other fat-soluble impurities complicated the post-separation and purification of total flavonoids. According to the results of single factor experiments, ethanol concentration of 65~85%; solid-liquid ratio of 1:15~1:25 g·mL^{-1}, extraction temperature of 55~75! and extraction time of 1.5 ~ 2.5h were determined as the factors and levels for response surface analysis.

Response surface optimization of extraction process

Multiple regression model and analysis of variance (ANOVA)

The extraction process of total flavonoids from apple was further optimized by RSM. According to the single-factor experimental results of 3.1, ethanol concentration of 65~85%; solid-liquid ratio of 1:15~1:25 g·mL^{-1}, extraction temperature of 55~75! and extraction time of 1.5 ~ 2.5h were selected as the actual levels of factors to maximize the extraction ratio of total flavonoids by Box-Benhnken design, as listed in. A total of 27 experiments were designed, including 16 factorial experiments, 8 star experiments and 3 central experiments to estimate the errors.

The RSM experimental design and results of extraction ratio from apple were shown in. Extraction ratio ranged from 2.07 to 5.33% for pericarp, from 1.58 to 4.07% for fruit, and from 1.43 to 3.68% for pyrenes. The maximum of extraction ratio was recorded under the experimental conditions of ethanol concentration of 75%; solid-liquid ratio of 1:20 g·mL^{-1}, extraction temperature of 65! and extraction time of 2.5h. The experimental data was analyzed by RSM using Design-Expert8.0 software, the response variable of total flavonoids extract ratio and the four factors were related by the following multiple regression equation:

Extract ratio =-111.689+1.025*A+1.317*B+1.702*C+10.385*D-0.00238*A*B-0.00228*A*C+0.0441*A*D+0.00754*B*C+0.0273*B*D-0.00547*C*D-0.00646*A^2-0.0421*B^2-0.0130*C^2-2.984*D^2 (Pericarp)

Extract ratio =-85.304+0.783*A+1.006*B+1.300*C+7.932*D-0.00182*A*B-0.00174*A*C+0.0337*A*D +0.00576 *B*C+0.0209*B*D-0.00418*C*D-0.00493*

A^2-0.0322*B^2-0.00989*C^2-2.279*D^2 (Fruit)

Extraction ratio =-77.171+0.708*A+0.910*B+1.176*C+7.175*D-0.00165*A*B-0.00158*A*C+0.0305*A *D+0.00521*B*C+0.0189*B*D-0.00378*C* D-0.00446*A^2-0.0291*B^2-0.00895*C^2-2.0619*D^2 (Pyrenes)

Showed the analysis of variance (ANOVA) for the multiple regression equation, the linear terms were all significant for response variables. The quadratic terms were extremely significant for the response variables. The interaction terms of ethanol concentration and extraction temperature, ethanol concentration and extraction time were significant for the responding values of extraction ratio; the interaction term of solid-liquid ratio and extraction temperature were extremely significant for the responding values. The analysis result indicated the response variable (the extraction ratio of total flavonoids) and the four test factor were not a simply linear relationship. The adequate precision value of 27.797 was greatly higher than the desirable value of 4.00, which presented a higher "signal (response) to noise (deviation)" and indicated that the model was significant for the total flavonoids extraction process of from apple. The value of R^2 (0.988) and R_{Adj}^2 (0.974) for the multiple regression equation was approaching and closed to 1, indicated a high degree of correlation between the experimental and predicted values and suitable of the model. The lower value of coefficient of the variance (C.V. =4.777%) also indicated a good reproducibility of the model.

The result of analysis of variance (ANOVA) showed that significant levels of the four factors were sorted by ethanol concentration > extraction time> extraction temperature> solid-liquid ratio. The linear terms of ethanol concentration and extraction time, interaction term of solid-liquid ratio and extraction temperature, and all of quadratic terms were extremely significant for response variable. The linear terms of solid-liquid ratio and extraction temperature, and interaction terms of ethanol concentration and extraction temperature, ethanol concentration and extraction time were significant for the response variable.

RSM analysis and research on the optimum extract process for total flavonoid

The multiple regression models could be vividly reflected by the 3D response surface and Contour lines plots, as shown in. The 3D response surface plots reflected the effects of multiple independent variables on the response value, the sensitivity of response value to different factors could also be analyzed. In the Contour lines plots, the closer the curve to the center, the greater of the value corresponding response variable; contour lines with circular indicated weak interactions between independent variables, contour lines with oval indicated strong interaction between independent variables. An increase of ethanol concentration (A) and extraction time (D) resulted in a monotonous increase of response variable to a maximum at a certain levels; while an increase of solid-liquid ratio (B) and extraction temperature (C) resulted in an initial increase and then decrease of response variable.

The interaction effects of ethanol concentration and solid-liquid ratio on the respond value were illustrated in the 3D response surface plots of, the corresponding surfaces of ethanol concentration were more steeper, indicated its extremely significant impact on extraction ratios. Contour lines were far away from the center, indicated the interaction of the two terms were not significant for the responding values. the maximum of extraction ratios were achieved as ethanol concentration of 72% and solid-liquid ratio of 20: 1 mL·g^{-1}. The liner terms of ethanol concentration and extraction temperature on the responding values were displayed in the 3D response surface plots of, the impact of the former term on extraction was more significant than the latter. Contour lines plots were close to Oval, indicated the interaction effects of the above two terms on the response values were a little significant P = 0.017. the maximum of extraction ratios were obtained as the ethanol concentration of 72% and extraction temperature of 65!. The liner terms of ethanol concentration and extraction time both displayed extremely significant impact on the responding value of extraction ratio, as shown from the steeper surface with large curvature in the 3D response surface plots in.

Contour lines plots were close to Oval, indicated the interaction effects of the above two terms on the response values were a little significant P = 0.0203. The maximum of extraction ratios were obtained as ethanol concentration of 72% and extraction time of 2.3 h.

From the 3D response surface plots of, the impact of solid-liquid ratio and extraction temperature terms on the extraction ratios were both a little significant for the responding value of extraction ratio. Contour lines plots were Oval-like, indicated interaction effects of the above two terms on the response values were extremely significant P = 0.0006. the maximum of extraction ratios were achieved as the solid-liquid ratio of 20: 1 mL· g^{-1} and extraction temperature of 65!. The 3D response surface and Contour lines plots of the interaction effect of solid-liquid ratio and extraction time on the response values of extraction ratio were shown in, the corresponding surface of extraction time was steeper, indicated its impacts on the response value were more significant than solid-liquid ratio. the interaction effects of the above two terms on the extraction ratios were not obvious from the Contour lines plots with circle-like. The interaction effects of extraction temperature and time on the extraction ratio were shown in. The impact of extraction time on the responding value were more significant than that of extraction temperature, as the corresponding surfaces of latter were steeper in the 3D response surface plots. From Contour lines plots, Contour lines were circle-like and far away from the centre, indicated the weaker interaction impact of the terms on the extration ratio.

The optimum values of the selected variables were obtained by solving the multiple regression equation. The values obtained were A=72.24%, B=21.19:1mL·g^{-1}, C=64.74! and D=2.30 h, with the corresponding extraction ratio of 5.62% for pericarp, 4.30% for fruit and 3.89% for pyrenes, calculated by Design-Expert 8.0 software. In the experiment, the preferred extract conditions were selected as ethanol concentration of 72%; solid-liquid ratio of 21.19:1mL·g^{-1}, extraction temperature of 65 ! and time for 2.30 h. Three triplicate experiments were performed under the preferred extract conditions to confirm the experimental data, the average values of extraction ratio were 5.63% for pericarp, 4.30% for fruit and 3.89% for pyrenes. The obtained experimental results were listed in, the experimental and calculated values of extraction ratios matched with each other very well, which indicated that the model were reliable for extraction of flavonoids from apples.

Study on hydroxyl radical scavenging activity

The Flavonoids compounds had a scavenging effect on hydroxyl radical, superoxide radicals and •DPPH radicals as the o-dihydroxy from the structural benzene ring. As operated in the literature, Hydroxyl radical scavenging activities of apple extracts and BHT with different concentrations were measured, as listed in. With the concentration increasing of apple extracts and BHT, the scavenging ratio for hydroxyl radicals increased, which showed a significant degree of dose-effect relationship. But the apple extracts showed weaker scavenging effect compare to BHT with the same concentration. The reasons were analyzed as follows: first, o-dihydroxy from benzene rings were partly methylated, leading to the reduction of scavenging activity on hydroxyl radical[19-21]. Second, the lack of necessary separation and identification for apple extracts, and the presence of impurities also affected its scavenging effect.

CONCLUSIONS

The RSM and BBD were successfully employed to optimize the extraction conditions of flavonoids from apples. The impacts of ethanol concentration and extraction time terms on the extraction ratio were extremely significant. While the effects of other two terms on extraction ratio were not as obvious. According to the results of single-factor experiments, ethanol concentration of 65~85%, solid-liquid ratio of 15:1~25:1 mL·g^{-1}, extraction temperature of 55 ~ 75! and time of 1.5 ~ 2.5h were selected as the independent variables and scope for response surface analysis. The preferred extract conditions optimized by RSM and Box-Benhnken design were ethanol concentration of 72%, solid-liquid ratio of 20:1

mL·g^{-1}, extraction temperature of 65! and time of 2.3h with responding extraction ratio of 5.63% for pericarp, 4.30% for fruit, 3.89% for pyrenes. The experimental values of extraction ratio matched well with the calculated ones by solving the multiple regression equation, which indicated the predictive effect of the fitted quadratic model on target extracts. The scavenging effects of apple extracts on hydroxyl radicals displayed a significant dose-effect relationship, but showed a weaker scavenging effect compare to BHT with the same concentration. The isolation, purification and structure identification of apple total flavonoids, relationship between antioxidant activity and structure of flavonoids, and related research works are underway.

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