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Optimization of purification conditions of radish (*Raphanus sativus* L.) anthocyanin-rich extracts using chitosan

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ABSTRACT

This study developed a chitosan-treatment procedure to remove impurities from radish anthocyanin extracts to promote their utilization as natural food colorants. Effects of purification conditions (independent variables) including pH (3.7, 4.0, and 4.3), chitosan concentration (1.5, 2.0, and 2.5 g/100 mL), and treatment duration (2, 2.5, and 3 h) on anthocyanin content, glucosinolate content, and clarity of radish anthocyanin-rich extracts (dependent variables) were investigated via a Box–Behnken experimental design. A set of preferred chitosan treatment conditions was determined to be 2.74 h at pH 3.92 with an initial chitosan concentration of 1.59 g/100 mL, according to the desirability function analyses. Under these purification conditions, glucosinolate reduction was ~61%, clarity was enhanced from 45.5% to 86.9%, and anthocyanin retention was ~95%. This chitosan-treatment procedure uses a biodegradable agent, and is safe, economic and efficient for solving the off-flavor and clarification problems of radish anthocyanin extracts. The procedure requires no special equipment and has potential to be scaled up for commercial production.

1. Introduction

Anthocyanins are a group of water-soluble natural pigments found primarily in red-toned fruits and vegetables, including berries, grapes, red cabbage, and radishes. Novel anthocyanin preparations are in high demand for a number of reasons and have the potential to replace synthetic food colorants in certain applications. Consumer-demand for natural food products is driving manufacturers to find new, natural sources of colors and flavors. In addition to their distinct red color, a wide body of research demonstrates anthocyanins' potential health properties, including reducing the risk of chronic diseases. However, anthocyanins have a limitation due to their relative instability, which causes a loss of color, particularly under food processing conditions. A successful anthocyanin food coloring product would therefore need enhanced stability. Radish anthocyanins are considered superior to most other natural anthocyanins because they have demonstrated outstanding stability, in addition to an intense red color with high tinctorial strength (Giusti & Wrolstad, 1996). For these reasons, radish anthocyanin extracts have been identified as a potential natural food coloring alternative to synthetic red pigments.

The uses of radish anthocyanin pigments as food colorants are currently limited due to the distinct undesirable flavors associated with these preparations. The off-flavors result from endogenous enzymatic hydrolysis of glucosinolates (sulfur-containing compounds in cruciferous vegetables) during mastication or processing (Bones & Rossiter, 2006). Several approaches have been evaluated in the literature for their potential to eliminate the off-flavors from the radish anthocyanin preparations. These approaches included resin absorption and membrane processes. Resin absorption improves the quality of radish anthocyanin preparations, but the US Food and Drug Administration would likely require an extensive, long, and expensive approval process before the procedure could be used due to lingering debates on its safety. Membrane processes such as ultrafiltration and osmosis have been found to alleviate undesirable aroma compounds, but do not completely solve the off-flavor problem

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(Rodriguez-Saona, Giusti, Durst, & Wrolstad, 2001). These approaches also generally cost more for removing the sulfur-containing components from radish anthocyanins.

Chitosan, a copolymer of N-acetyl-D-glucosamine and D-glucosamine residues, carries a positive charge in acidic environments due to the presence of amine groups (Peter, 2005). This specific chemical property allows chitosan to attract negatively-charged or electron-rich chemicals and to coagulate anionic polymers. To date, the utilization of chitosan treatment to remove the electron-rich, sulfur-containing glucosinolates from radish anthocyanins has not yet been investigated.

In the present study, chitosan was tested for its ability to remove glucosinolates from radish anthocyanin extracts using a multiple-response surface methodology. Anthocyanin content, glucosinolate content, and clarity were evaluated as the dependent variables. Meanwhile, purification conditions (pH, chitosan concentration, and treatment duration) were evaluated as independent variables in a Box–Behnken model design and optimized for desirability based on the best compromise among the above three responses. The Box–Behnken design is suggested to be a more efficient and economical method than the central composite design for fitting a response surface for a large number of variables (Ferreira et al., 2007). It has been utilized by a number of researchers for optimization of analytical methods (Preu, Guyot, & Petz, 1998; Ragonese, Macka, Hughes, & Petocz, 2002), and several processing procedures (Sharma, Singh, & Dilbaghi, 2009; Tiwari, Muthukumarappan, Donnell, & Cullen, 2008; Vega, Balaban, Sims, O'Keefe, & Cornell, 1996). This method allows for the combination of multiple responses into one measurement of desirability function for overall multiple response optimization (Myers & Montgomery, 2002). The results presented in this paper provide a plausible method for improving the flavor of anthocyanin-type pigments from cruciferous vegetables through minimal additional processing.

2. Materials and methods

2.1. Materials and chemicals

Radishes (*Raphanus sativus* L. cv. Jiangsu green) were harvested locally in Jiangsu province, China. All chemicals were of ACS or HPLC grade from Sinopharm chemical reagent (Shanghai, China). Sinigrin and arylsulphatase were purchased from Sigma–Aldrich (Shanghai, China).

2.2. Preparation of radish anthocyanin extracts and chitosan treatment

Peeled radish roots (2 kg) were squeezed for juice using a juice maker and the puree was removed using six layers of cheesecloth. The juice yield was 60–80 mL/100 g raw radish roots. About 700 mL of radish extracts were heated at a stove to 100 °C and held for 5 min, then immediately cooled in an ice bath to a room temperature (23–25 °C) and stored at 4 °C until chitosan treatment. Radish extract (30 mL) was added with chitosan (95% deacetylation, Zhejiang Golden-shell Biochemical Company, Yuhuan, China) to reach the percentage of 1.5, 2.0, or 2.5 g/100 mL of chitosan in radish extracts. Then the mixture was adjusted to pH 3.7, 4.0 or 4.3 with 6 mol/L hydrochloric acid (approximately 0.3–0.7 mL). The mixtures were held for 2, 2.5, and 3 h at room temperature, after which time the supernatants were collected for further analyses.

2.3. Clarity

The clarification of radish anthocyanin extract was determined by the transmittance, which was read at 660 nm by using a Rayleigh UV–visible spectrophotometer (Beijing, China) immediately after samples were homogenized for 30 s on a Vortex and placed in cuvettes with a 1 cm path length. Analyses of each treatment were performed in triplicate.

2.4. Monomeric anthocyanins

The total monomeric anthocyanin content was measured by the pH differential method (Giusti & Wrolstad, 2001). The Rayleigh UV–visible spectrophotometer was used to read absorbance at the maximum visible wavelength of absorption of each extract (ranging from 490 to 535 nm) and at 700 nm. Monomeric anthocyanins were calculated as equivalents of pelargonidin–3–glucoside, using the extinction coefficient of 31600 L cm^{-1} mg^{-1} and a molecular weight of 433.2 g L^{-1} (Giusti & Wrolstad, 1996). Cuvettes with a 1 cm path length were used. Analyses of each treatment were performed in triplicate.

2.5. Glucosinolates

A modified HPLC method of Schreiner et al. (2002) was used to determine the total glucosinolates. Briefly, 5 mL of radish anthocyanin extract was immersed in a water bath at 75 °C and mixed with 0.5 mL of 5 mg/mL sinigrin as an internal standard and then 25 mL hot methanol ($T = 70$ °C) for 10 min. The mixture was cooled down rapidly in an ice bath, and then centrifuged at 1431 g for 10 min with the addition of 5 mL 0.4 moL/L barium acetate. Residual methanol in the supernatant portion was removed in a rotary evaporator at 40 °C under vacuum. Distilled, deionized water was added to the remaining aqueous extract to bring the total volume to 25 mL. A 5 mL aliquot of extract was applied to a DEAE–Sephadex A–25 anion exchanger (Sigma–Aldrich, Shanghai, China) and washed with 10 mL of dd water. After application of 100 μL of 1 mg/mL arylsulphatase solution and an overnight incubation period, the desulphonated glucosinolates were eluted with 3 mL of dd water and refrigerated at 4 °C until analysis.

The analysis of the desulphoglucosinolates was carried out on a reverse–phase HPLC system (Shimadzu Corporation, Tokyo, Japan) with an LC–20AB prominence liquid chromatograph, an SPD–M20A prominence diode array detector, and a SIL–20AC prominence autosampler at 4 °C.

A 4.6 μm Shim–pack VP–ODS column (4.6 × 250 mm, Shimadzu Corp., Tokyo, Japan) fitted with a 4.6 × 10 mm Shim–pack GVP–ODS guard column (Shimadzu Corp., Tokyo, Japan) was used. Solvent and sample were filtered though 0.45 μm hydrophobic/hydrophilic membranes (Shanghai Yaxing Corp., China) and 0.45 μm MS nylon membrane filters (Shanghai Mosu Scientific Instruments and Materials, China), respectively. Solvents used were A: acidified water (water/acetic acid, 99.5:0.5 v/v) and B: HPLC-grade acetonitrile. Separation was achieved through a gradient elution, as following: 10% B, 0–10 min; a linear gradient from 10 to 25% B, 10–15 min; 25% B, 15–35 min; followed by a linear gradient from 25 to 10% B from 35 to 40 min to return to initial conditions. An injection volume of 30 μL with a 1 mL/min flow rate was used. Spectral information was recorded at 229 nm. Total amount of glucosinolates was calculated as sinigrin equivalent based on a sinigrin standard curve. Analyses for each treatment were performed in triplicate.

2.6. Experimental design and statistical analysis

In order to achieve the minimum glucosinolate residue and the maximum clarity and anthocyanin content values in the processed radish pigment extracts, response surface methodology (RSM) was employed to determine the optimal conditions for chitosan