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Analytical Methods

Simultaneous separation and purification of chlorogenic acid, epicatechin, hyperoside and phlorizin from thinned young *Qinguan* apples by successive use of polyethylene and polyamide resins

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ABSTRACT

The method for separating and purifying chlorogenic acid (CA), epicatechin (EC), hyperoside (HY) and phlorizin (PH) simutaneously from young *Qinguan* apples by successive use of X-5 and polyamide resins has been developed in this study. The order of adsorption capacities of X-5 for the four phenolics was PH > HY > EC > CA, and the adsorption equilibriums of the four phenolics onto X-5 resin conformed to Langmuir isotherms preferentially. The adsorption kinetics of EC and CA onto X-5 conformed to the pseudo-first-order model, while that of HY and PH accorded with the pseudo-second-order model. Interestingly, the values of equilibrium adsorption capacities (Q_e) calculated in the preferential kinetics models were closer to that of theoretical maximum adsorption capacities (Q_0) calculated by Langmuir isotherms. Through dynamic adsorption and desorption using X-5 and polyamide resins with ethanol solution as strippant, CA, EC, HY and PH were obtained with purities of 96.21%, 95.34%, 95.36% and 97.36%, respectively.

1. Introduction

Apple is the fruit of malus that is classified as rosaceae plant. It is an important component in people's diet as it contains plentiful polyphenols including procyanidins, flavonoids, etc. (Vrhovsek, Rigo, Tonon, & Mattivi, 2004). The types and contents of polyphenols in apples vary with the cultivars and growth cycles (Zheng, Kim, & Chung, 2012). Usually, the content of total polyphenols in unripe apples is approximately ten times as that in ripe apples (Akiyama et al., 2005). In order to guarantee the output and increase the apple quality, it is important and necessary to thin flowers and fruits every year (Link, 2000). Hence, there are a lot of thinned young apples (~1.6 million tonnes) produced annually in China, and these apples (~around one month after blossom) are usually discarded in grove (Dou et al., 2015). However, this is a waste of agricultural and food resources, due to the relatively high content of phenolic compounds in young apples (Sun, Guo, Fu, Li, & Li, 2013). Besides, the discarded apples rot in the grove soil, increasing the soil acidity and thus disturbing the microbial community in the grove soil. This, in turn, affects the growth of fruit trees (Mazzola, 1998). Therefore, it is valuable and necessary to collect these young apples to develop their application values.

Apple polyphenols have been reported to have some healthy benefits, like anticancer (Liu, Liu, & Chen, 2005), inhibition of cariogenic bacterial glucosyltransferases (Yanagida, Kanda, Tanabe, Matsudaira, & Cordeiro, 2000), inhibition of bacterial toxin (Rasooly, Do, & Friedman, 2010) and inhibition of α-amylase activity (Sun et al., 2016). The main constituents in apple polyphenols have been determined to be chlorogenic acid, phlorizin, procyanidins, epicatechin, anthocyanins and flavonoid glycosides (Vrhovsek et al., 2004). Because of the bioactive functions and commercial values (Li et al., 2005; Martinez, Ugartondo, Vinardell, Torres, & Mitjans, 2012), the methods for separation and purification of these phenolic compounds have been developed in recent years. To obtain single phenolic compounds with high purities, some precise purification approaches have been established, such as polyamide resin followed by semi-preparative high performance liquid chromatography for hyperoside (Li, Sun, Liu, Zhang, & Cui, 2014),

Abbreviations: YAP, young apple polyphenols; F-YAP, *Fuji* young apple polyphenols; *R*-YAP, *Royal Gala* young apple polyphenols; *Q*-YAP, *Qinguan* young apple polyphenols; CA, chlorogenic acid; EC, epicatechin; HY, hyperoside; PH, phlorizin; BV, bed volume; 20E, 20% (v/v) ethanol aqueous solution; 30E, 30% (v/v) ethanol aqueous solution; 30E, 70% (v/v) ethanol aqueous solution.

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molecular imprinting technique for chlorogenic acid (Gu et al., 2010), thin layer chromatography for epicatechin (Amarowicz & Shahidi, 1996) and high-speed counter-current chromatography for phloretin (Xu, Lu, Qu, Shan, & Song, 2010). Although the purities of these products are above 90%, relatively low yield and high cost are two restraining factors that should be considered further for industrial large-scale production. In addition, macroporous resins have been widely applied in enrichment of a certain phenolic compound because of the good adsorption and desorption properties, low cost and easy regeneration (Sun et al., 2013, 2015; Zhang, Yang, Zhao, & Liu, 2008). However, the purity of target phenolic compound has not been significantly improved (e.g. chlorogenic acid), which may be caused by the non-specific adsorption properties of the resins (mainly polystyrene) for both the target and unexpected phenolic compounds (Yao, Zhang, Wang, & Wang, 2015). In addition, after one usual run treatment of separation by macroporous resin, only one substance with unsatisfied purity could be obtained because of the limit of raw materials and the influence of other polyphenols.

Polyamide resin (applied in this study), known as Nylon-6, is synthetized by polycondensation of caprolactam, forming a linear polymer containing alternative 5 units of -CH_2- chains and 1 unit of amide group. Therefore, this structure allows stronger both hydrophobic interaction and hydrogen bonding between adsorbent and adsorbate (Gao et al., 2011). By this way, individual phenolic compounds can be separated and purified according to the difference in molecular polarity (Sun et al., 2013). Here, we established a hypothesis that target phenolic compounds may be separated initially by a macroporous resin, and then purified further by a polyamide resin. Through this successive resins method, more than one purified phenolic compounds may be obtained with high purities and yields. Furthermore, according to our finding that the solubilities of chlorogenic acid and epicatechin in 5% cold ethyl acetate solution are totally different, thus this solution is applied assistantly in the successive resins to develop an efficient method to simultaneously separate and purify chlorogenic acid, epicatechin, hyperoside and phlorizin with high purities and yields from thinned young apples, which provides a potential for pilot- or large-scale production of single phenolic compounds from agricultural and food resources (Kammerer, Carle, Stanley, & Saleh, 2010).

2. Materials and methods

2.1. Reagents

Gallic acid, procyanidin B2, chlorogenic acid, caffeic acid, 4-hydroxybenzoic acid, epicatechin, hyperoside, rutin, phlorizin and quercitrin were purchased from Chengdu Must Biological Technology Co., Ltd. (Chengdu, China). Analytical grade sodium carbonate and ethanol were obtained from Tianli Chemical Reagent Co., Ltd. (Tianjin, China). Folin-Ciocalteu reagent, HPLC grade methanol and trifluoroacetic acid were obtained from Sigma Chemical Co. (St. Louis, USA). All the solutions and eluents were prepared with distilled water. All the solutions before loaded onto HPLC were filtered through 0.45 μm membranes (Fisher Scientific).

2.2. Materials

Thinned young apples of *Fuji*, *Royal Gala* and *Qinguan* cultivars were collected 30 days after blossom in Liquan Country, Shaanxi Province, China, and then transported to the lab followed by storage at −80 °C before use.

X-5 resin (polystyrene) and polyamide resin (Nylon-6, 60–80 mesh) were obtained from Xi'an Lanshen special resin Ltd. Co., (Xi'an, China). To remove the residual material monomers and porogenic agent inside the pores during the synthesis process, the resins were pretreated in 1 M HCl and NaOH solution successively, followed by a washing step with distilled water, and then dried at 60 °C under vacuum. The dried resins were soaked in 95% ethanol for 12 h, followed by a washing step using distilled water thoroughly before use.

2.3. Separation of young apple polyphenols (YAP)

Young apples were ground into 3–4 mm particles by a grinder with the protection of 1% $NaHSO_3$ (w/w, $NaHSO_3$/Young apples). Then, the apple particles were steamed for 30 s to inactivate endogenous polyphenol oxidase, which is the first key step in the whole separation process, because it can protect polyphenols from enzymatic oxidation (Kazandjian & Klibanov, 1985). After that, young apple particles were extracted with 10 times volumes of 60% (v/v) ethanol aqueous solution at 65 °C for 3 h (Sun et al., 2013). The extracting solution was filtered using a Buchner funnel and concentrated to remove ethanol using a rotary evaporator (RE 52-99, Shanghai Yarong Biochemistry Instrument Factory, China) at 65 °C, followed by centrifugation using a centrifuge (LXJ-IIB, Shanghai Anting Scientific Instrument Factory, China) at 3500*g* for 20 min to obtain clear supernatant with 2.75 mg/mL total polyphenols determined by Folin-Ciocalteu method. Then, the raw extract was loaded onto a glass column (45 × 600 mm) filled with X-5 resin at a feeding speed 1.0 bed volume (BV)/h. In the next step, 2 BV of distilled water was used to rinse the resin column at the same speed to wash out the impurities that were not adsorbed onto the resin, such as some polysaccharides, proteins and pigment (Kammerer et al., 2010). To desorb total polyphenols out of X-5 resin, 70% (v/v) ethanol aqueous solution (70E) was applied to elute the resin column at an eluting speed of 2.0 BV/h until the desorption solution became clear. The desorption solution was collected and concentrated to remove ethanol using a rotary evaporator at 65 °C, followed by lyophilization to obtain *Fuji*, *Royal Gala* and *Qinguan* young apple polyphenols (*F*-YAP, *R*-YAP and *Q*-YAP), respectively.

2.4. Determination of phenolic compounds in YAP

The content of total polyphenols was determined using the Folin-Ciocalteu method and expressed as gallic acid equivalents (mg GAE/g) (Xu et al., 2010). The contents of individual phenolic compounds in YAP were determined using a Dionex® HPLC system (P680, Japan) equipped with a Dionex® UV–VIS detector (UVD170U, Japan) and an Agilent® RP-C18 column (250 × 4.6 mm I.D., 5 μm, USA). An elution with solvent A (methanol) and solvent B (3‰ trifluoroacetic acid) in a step gradient way at a flow rate of 1.0 mL/min was carried out as follows: 0–30 min, 90–75% B; 30–50 min, 75–62% B; 50–70 min, 62–55% B; 70–90 min, 55–90% B. During the run, the detection wavelength was set at 280 nm, and the injection volume was 20 μL. The determination of phenolics in respective desorption solutions in the following experiment were conducted using the same method as above.

2.5. Details of adsorption of phenolic compounds on X-5 resin

2.5.1. Adsorption isotherms of phenolic compounds on X-5 resin

Adsorption isotherms indicate the specific effect of equilibrium concentration of adsorbate on its degree of accumulation on the surface of adsorbent at a certain temperature, which can reflect the interaction between adsorbent and adsorbate, as well as the process and mechanisms of adsorption (Kammerer et al., 2010). Langmuir and Freundlich equations are two most popular formulas widely applied in description of the mechanisms of adsorption as