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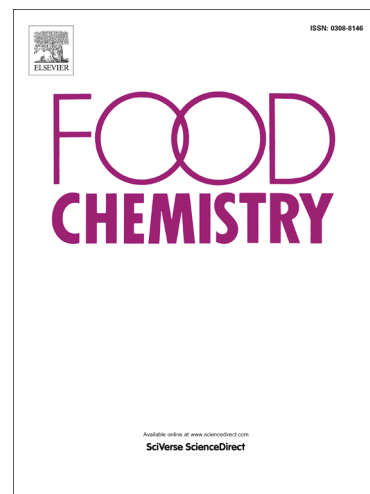
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Simultaneous separation and purification of chlorogenic acid, epicatechin, hyperoside and phlorizin from thinned young *Qinguan* apples by successive use of polyethylene and polyamide resins

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Abstract

The method for separating and purifying chlorogenic acid (CA), epicatechin (EC), hyperoside (HY) and phlorizin (PH) simultaneously 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.

Keywords: chlorogenic acid; epicatechin; hyperoside; phlorizin; successive resins; separation and purification

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, Meng, Liu, Li, Ren, & Guo, 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, Rigo, Tonon, & Mattivi, 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), 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, Guo, Fu, Li, & Li, 2013; Sun et al., 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 synthesized by polycondensation of caprolactam, forming a linear polymer containing alternative 5 units of -CH₂- 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, Guo, Fu, Li, & Li, 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 $-80\text{ }^{\circ}\text{C}$ 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 1M HCl and NaOH solution successively, followed by a washing step with distilled water, and then dried at $60\text{ }^{\circ}\text{C}$ under vacuum. The dried resins were soaked in 95% ethanol for 12h, 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% NaHSO₃ (w/w, NaHSO₃/Young apples). Then, the apple particles were steamed for 30s 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 3h (Sun, Guo, Fu, Li, & Li, 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-IIIB, Shanghai Anting Scientific Instrument Factory, China) at 3500g 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, Carle, Stanley, & Saleh, 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, Lu, Qu, Shan, & Song, 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 (250×4.6mm 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, Carle, Stanley, & Saleh, 2010). Langmuir and Freundlich equations are two most popular formulas widely applied in description of the mechanisms of adsorption as they are relatively simple and reasonably accurate (Baskaralingam, Pulikesi, Elango, Ramamurthi, & Sivanesan, 2006).

Langmuir equation is applied under two ideal assumptions that the interaction force between adsorbed molecules is negligible, as well as the adsorption on a homogenous surface is monolayer (Saeed, Iqbal, & Höll, 2009). Langmuir adsorption model is described by the following equation:

$$\frac{Q}{Q_0} = \frac{K_{ad}C}{1+K_{ad}C} \quad (1)$$

where Q is the amount of phenolic compounds adsorbed on per unit mass of X-5 resin (mg/g), C is the equilibrium concentration of phenolic compounds in solution (mg/mL), Q_0 is the theoretical maximum adsorption capacity (mg/g), and K_{ad} is a constant related to the free energy of adsorption.

Freundlich equation is applied under the condition of nonideal adsorption on heterogeneous surface that is caused by different functional groups on the surface of adsorbent. Therefore, this model is based on the assumption that there are several available acting sites on the surface of adsorbent, on which there occur adsorptions with different free energies (García-Zubiri, González-Gaitano, & Isasi, 2009). This adsorption model is described by the following equation:

$$Q = K_f C^{1/n} \quad (2)$$

where Q is the amount of phenolic compounds adsorbed on per unit mass of X-5 resin (mg/g), K_f is the Freundlich constant indicating adsorption capacity, $1/n$ is an empirical constant demonstrating adsorption intensity of the system, and C is the equilibrium concentration of phenolic compounds in solution (mg/mL).

The adsorption thermodynamics parameters, including Gibbs energy, enthalpy and entropy are calculated using the following equations (Abrams & Prausnitz, 1975):

$$K = MK_{ad} \quad (3)$$

$$\Delta G^0 = -RT \ln K \quad (4)$$

$$\ln K = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (5)$$

where K is the equilibrium constant (L/mol), M is the molecular weight of phenolic compounds (g/mol), R is the universal gas constant (8.134 J/(mol·K)) and T is the absolute temperature (K).

2.5.2 Adsorption kinetics of phenolic compounds on X-5 resin

To better describe the adsorption behavior, the adsorption kinetics is usually analysed using three models: pseudo-first-order, pseudo-second-order and intra-particle diffusion kinetics models (Noroozi, Sorial, Bahrami, & Arami, 2007).

The equation for pseudo-first-order model is as follows:

$$\frac{dQ_t}{dt} = K_1(Q_e - Q_t) \quad (6)$$

The equation for pseudo-second-order model is as follows:

$$\frac{dQ_t}{dt} = K_2(Q_e - Q_t)^2 \quad (7)$$

The equation for intra-particle diffusion model is as follows:

$$Q_t = K_i t^{0.5} + I \quad (8)$$

Where Q_e and Q_t are the adsorption capacities at equilibrium and at time t (mg/g), respectively; K_1 (min^{-1}), K_2 ($\text{g} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$) and K_i ($\text{mg} \cdot \text{g}^{-1} \cdot \text{min}^{-0.5}$) are the constants for pseudo-first-order, pseudo-second-order and intra-particle diffusion models, respectively; I is the boundary layer thickness (mg/g). Then equation (6) and (7) can be integrated to equation (9) and (10), respectively as follows:

$$\ln(Q_e - Q_t) = \ln Q_e - K_1 t \quad (9)$$

$$\frac{t}{Q_t} = \frac{1}{K_2 Q_e^2} + \frac{t}{Q_e} \quad (10)$$

Further, equation (9) and (10) can be transformed to equation (11) and (12), respectively as follows:

$$Q_t = Q_e(1 - e^{-K_1 t}) \quad (11)$$

$$\frac{1}{Q_t} = \frac{1}{K_2 Q_e^2} \cdot \frac{1}{t} + \frac{1}{Q_e} \quad (12)$$

All the kinetics parameters can be calculated from the fitting equation (8), (11) and (12).

2.6 Six key steps in separation and purification of CA, EC, HY and PH dynamically by successive use of X-5 and polyamide resins.

The whole process for separation and purification of CA, EC, HY and PH is shown in Fig.S1, in which there are six key steps totally. The first one is the steaming treatment for the ground young apples in order to inactivate polyphenol oxidase (1st key step). Then, 30% ethanol solution (30E) and 70% ethanol solution (70E) were successively used to elute X-5 resin column (45×600 mm) that adsorbed YAP to obtain component A and B, respectively (2nd key step). After that, the finding that 5% cold ethyl acetate could dissolve CA but only 10% EC was applied to initially separate the two phenolics in component A preliminarily (3rd key step). To obtain purified CA and EC, 20% ethanol solution (20E) was selected to elute the polyamide resin column (30×600 mm) that adsorbed component C (mainly CA) and D (mainly EC), respectively (4th and 5th key steps). Meanwhile, the polyamide resin column (30×600 mm) that adsorbed component B was eluted by 30E, and the desorption solutions that were enriched with HY and PH were collected, respectively (6th key step).

2.7 Statistical analysis

The data in this study are expressed as the means of duplicates and analyzed through one-way analysis of variance (ANOVA) using SPSS 18.0 Statistics (Chicago, USA). The mean values were evaluated by Dunnett's *t* Test at the 95% significance level ($P < 0.05$). The results of details of adsorption were fitted and analyzed using Origin 8.0 Statistics.

3. Results and discussion

3.1 Determination of phenolic compounds in three YAP

In our study, the quantity of total polyphenols in the three fresh apples (*Fuji*, *Royal Gala* and *Qinguan*) was determined as 1.64, 1.52 and 1.70 g of GAE per 100 g of fresh apples, respectively. Then, the contents of total polyphenols in the three lyophilized YAP were determined as 85.36, 84.27 and 86.66 mg of GAE per 100 mg of lyophilized YAP,

respectively, and the contents of individual phenolic compounds in the three YAP were determined by HPLC (Fig.1) and summarized in Table S1. *Q*-YAP was screened as the excellent extract to separate and purify CA, EC, HY and PH further, because the four target phenolic compounds totally accounted for higher content in *Q*-YAP than in the other two YAP (Table S1), and the contents of unexpected compounds in it were relatively low (Fig.1D and Table S1).

3.2 Details of adsorption of phenolic compounds on X-5 resin

3.2.1 Adsorption isotherms of phenolic compounds on X-5 resin

The adsorption of polyphenols onto X-5 resin is one important step in the separation and purification process, and the adsorbing effects can reflect the interaction between resin and polyphenols. Since the temperature may affect the adsorption capacity of resin, and the maximum adsorption capacity may decide the sequence in which the different phenolic compounds are eluted out of the resin column (Sun et al., 2015), it is necessary to determine the isothermal adsorption properties of X-5 resin. The adsorption isotherms, equations and parameters of phenolic compounds on X-5 resin at four temperatures were suggested in Fig.2A, Fig.S2 and Table 1A. For all the four phenolic compounds, Langmuir equations characterized the adsorption behaviours of X-5 resin better than Freundlich ones because the regression coefficients of Langmuir equations were higher than that of Freundlich ones (Table 1A). Also, the adsorption process more likely exhibited monolayer one according to the characteristics of Langmuir adsorption (Saeed, Iqbal, & Höll, 2009; Zhang, Yang, Zhao, & Liu, 2008). On the other hand, for the Freundlich model, it means that the sorbents adsorb the solute molecules easily if the value of $1/n$ is less than 1 (Mohanty, Jha, Meikap & Biswas, 2006). In Table 1A, all the values of $1/n$ are less than 1, indicating that X-5 resin were favourable for the adsorption of the four phenolic compounds. It is found that both Q_0 and K_f

of X-5 resin for the four phenolics decreased with the temperature increasing (Table 1A), indicating that the adsorption was an exothermic process and that relatively low temperature favoured the adsorption of phenolics onto X-5 resin. K_{ad} , a constant related to the free energy of adsorption, also decreased with the temperature increasing, which is similar to one previous observation that AB-8 macroporous resin showed a decreasing K_{ad} for chlorogenic acid as the temperature increased (Sun, Liu, Yi, Li, Fan, & Xia, 2015). This may arise from the swelling of resin and the increased kinetic energy of phenolic molecules at a higher temperature, both of which may decrease the free energy required for adsorption (Xu, Zhang, & Fang, 2010; Hagedorn, 1965).

Notably, the order of Q_0 values for X-5 resin at a constant temperature was PH>HY>EC>CA, and the order of K_f values showed the same tendency, indicating that the adsorption capacities of X-5 resin for PH and HY were higher than that for EC and CA (Table 1A). Therefore, higher concentration of strippant solution (ethanol) may be required to elute PH and HY out of the resin column, as it requires higher desorption force to overcome the interactions between phenolics and resins (Yao, Zhang, Wang, & Wang, 2015). In addition, X-5 resin, as a non-polar adsorbent, adsorbs the solute molecules that are non- or weak-polar preferentially (Jia & Lu, 2008). Normally, the substance that has a longer retention time on RP-C₁₈ HPLC column offers weaker molecular polarity (Chinnici, Gaiani, Natali, Riponi, & Galassi, 2004; Vrhovsek, Rigo, Tonon, & Mattivi, 2004), indicating that PH and HY had weaker polarity than CA and EC according to the performance of the four phenolics on HPLC chromatography in Fig.1D. Therefore, X-5 showed a higher adsorption capacity for PH and HY than CA and EC.

By plotting $\ln K$ against $1/T$ according to equation (5), the adsorption thermodynamics parameters (ΔG^0 and ΔH^0) of the four phenolic compounds can be estimated from the slopes and intercepts of linear correlation (Fig.2B). Because both the values of ΔG^0 and ΔH^0 were

negative (Table 1B), the adsorption of the four phenolic compounds on X-5 resin were exothermic, consistent with the results of the equilibrium adsorption study (Table 1A). As the absolute values of ΔH^0 were too much lower than 43 kJ/mol, the adsorption processes of the four phenolics were driven by physical mechanisms, instead of chemical ones (Sun et al., 2015). Besides, the positive values of ΔS^0 indicate a random adsorption of phenolic compounds at the solid-liquid interface. The adsorption of phenolic compounds disturb the equilibrium system where water molecules are adsorbed on the surface of resin previously, producing the 'solvent replacement effect' (Wei, Chen, & Yang, 2010). Due to the random adsorption at the surface of resin, the disorder degree at the solid-liquid interface increases, inducing the increase of entropy, namely positive values of ΔS^0 .

3.2.2 Adsorption kinetics of phenolic compounds on X-5 resin

The adsorption cases of the four phenolic compounds on X-5 resin at 20 °C along with time are described in Fig.2C. The order of practical maximum adsorption capacities of X-5 resin for the four phenolics was PH>HY>EC>CH, in accordance with that of theoretical maximum adsorption capacities (Q_0) obtained in the adsorption isotherms study. The three adsorption kinetics models, including pseudo-first-order, pseudo-second-order and intra-particle diffusion ones were fitted according to equation (11), (12) and (8), and displayed in Fig.2D, E and F, respectively. From the coefficients of equations, the kinetics constants of the three models were calculated and summarized in Table 1C. As shown, pseudo-second-order kinetics model could describe the adsorption of CH and EC on X-5 resin better than pseudo-first-order and intra-particle diffusion models, because the correlation coefficients (R^2) for pseudo-second-order kinetics equations were closer to 1 (0.9838 and 0.9947 for CH and EC, respectively). On the other hand, pseudo-first-order model was more suitable for describing the adsorption processes of HY and PH on X-5 resin (R^2 were 0.9860 and 0.9800 for HY and PH, respectively). Although the intra-particle diffusion kinetics models could not well-

describe the whole adsorption process of the phenolics in this study (R^2 ranges from 0.6567 to 0.7578), the process could be divided into two stages, where there was a respective linear correlation without passing through the origin. This indicates that intra-particle diffusion is not the only rate-limiting step in the adsorption processes of the four phenolics on X-5 resin. The adsorption may be driven by multi-diffusion steps, including film diffusion (transport of adsorbates from boundary film to resin surface) and intra-particle diffusion (transport of adsorbates within resin pores) (Gao, Yu, Yue, & Quek, 2013). Interestingly, for the four phenolic compounds, the values of adsorption capacities at equilibrium (Q_e) calculated in the respective preferential kinetics models (pseudo-second-order for CH and EC, pseudo-first-order for HY and PH) were closer to that of theoretical maximum adsorption capacities (Q_0) obtained through the respective preferential adsorption isotherms (Langmuir isotherms for all the four phenolic compounds). Therefore, the combination of adsorption isotherms and kinetics is reasonable to analyze the details of adsorption of the phenolic compounds on X-5 resin in this study.

3.3 Initial separation of component A and B

Q-YAP was reloaded onto a X-5 resin column (45×600 mm) at a feeding speed of 1.0 BV/h, so polyphenols were adsorbed onto the macroporous resin through the interaction between them, such as electrostatic force and hydrogen bonding (Gao, Huang, & Liu, 2007). As a strippant, the desorption capacity of ethanol solution increases with its concentration increasing. The adsorbates that are adsorbed on the resin are, usually in turn, desorbed and eluted out of the resin column by gradient strippant, according to the adsorption capacities of resin for the adsorbates. In our study, X-5 resin that adsorbed *Q*-YAP was eluted by 30E at an eluting speed of 2.0 BV/h, followed by being eluted by 70E at the same speed. The desorption solutions were collected at intervals of 0.5 BV and determined for the

concentrations of phenolics (Fig.3A and B). The desorption solutions by 30E and 70E at individual intervals were combined respectively, and then concentrated and dried. Therefore, component A (obtained from 30E) and component B (obtained from 70E) were obtained, respectively. Phenolic compounds in the two components were determined by HPLC. As shown in Fig.3C and D, 30E could well-desorb both CA and EC, but not HY and PH, while 70E could desorb HY and PH. This agrees with the result determined above that X-5 resin had a higher adsorption capacity for HY and PH than CA and EC, and thus, higher concentration of ethanol was required to desorb HY and PH. In addition, most of CA and EC (93.56% and 92.64%, respectively, Table S2) could be eluted out without containing HY and PH using 6.5 BV of 30E (Fig.3A and C); most of HY and PH (90.45% and 91.62%, respectively, Table S2) could be eluted out without containing CA and EC using 2.5 BV of 70E (Fig.3B and D).

3.4 Separation and purification of CA and EC

Component A was loaded onto a polyamide resin column (30×600 mm) at a flow rate of 1.0 BV/h. For selection of strippant concentration, ethanol solution with low concentration could not effectively desorb CA and EC (Fig.S3A), and ethanol solution with high concentration desorbed some more parts of pigment as well (the colour of desorption solution became darkened, Fig.S3B), affecting the purities of CA and EC, and could not separate CA from EC effectively (Fig.S3C). Then, 20E was selected to elute the polyamide resin. In this step, although the constituents of desorption solution were relatively simple from 0 to 600 mL (Fig.4A), EC gradually appeared in the desorption solution from 600mL (Fig.4B). Therefore, although polyamide resin could well-adsorb and thus remove some pigment substances in the extract (Wen et al., 2016), this method was unavailable to obtain high recovery yield of CA. Modified methods should be explored. In our study, one finding was

obtained and described in Fig.S4. Both CA and EC (22 mg) were almost insoluble in cold water (0.8 mL), but after adding a bit of cold ethyl acetate (40 μ L, the final concentration was ~5%, volume fraction), CA was totally soluble, while only 10% of EC became soluble. Therefore, CA and EC may be initially separated by use of this finding.

Then, 80 mL of 5% cold ethyl acetate solution was added to the component A (~5 g) obtained in the initial separation process and mixed thoroughly, followed by centrifugation. After this step, most of CA was extracted in the supernatant, and most of EC was precipitated in the sediment. The supernatant and sediment were collected respectively, and then dried. Therefore, component C (from the supernatant) and D (from the sediment) were obtained.

Component C was dissolved in warm distilled water and loaded onto a polyamide resin column (30 \times 600 mm) that was then eluted by 20E. The desorption solutions were collected at intervals of 150 mL and determined for the concentrations of phenolics. It is indicated that the main constituent of desorption solution of component C was CA with few EC (Fig.4C, D and E), and that most of CA could be desorbed by 1900 mL of 20E (Fig.4G). Taking low energy cost into account, 150-1600 mL of desorption solution that was enriched with CA was collected, concentrated and recrystallized in methanol-water solution (v/v, 1:1; pH 1~2) (Hulme, 1953). Finally, 0.534 g of crystalline CA was obtained with the purity of 96.21% (HPLC, Fig.4F) and a total recovery yield of 60.34%.

Here, the initial separation of component A into component C and D by adding 5% cold ethyl acetate aqueous solution is an essential and novel step for further purifying CA and EC, as it successfully solved the problem that CA and EC could not be separated completely and directly on the polyamide resin column (Fig.4B). More importantly, the step is simple and convenient. Besides, the separating effect is also indicated in Fig.4H where EC accounted for the main constituent in component D. Thus, component D was mixed with warm water and loaded onto a polyamide resin column (30 \times 600 mm) that was then eluted by 20E. The

desorption solutions were collected at intervals of 150 mL and determined for the concentrations of phenolics. The residual CA concentrated mainly in 0-300 mL of desorption solution (Fig.4I), and EC was gradually eluted out of the polyamide resin with few CA (Fig.4J and K). Therefore, 300-1750 mL of desorption solution of component D that was enriched with EC (Fig.4M) was collected, concentrated and recrystallized in methanol-water solution (v/v, 1:1). Finally, 0.382 g of crystalline EC was obtained with the purity of 95.34% (HPLC, Fig.4L) and a recovery yield of 52.54%.

3.5 Separation and purification of HY and PH

As discussed above, component B was primarily composed of HY and PH (Fig.3D), and then it was applied to separate HY and PH. Component B was dissolved in warm distilled water and loaded onto a polyamide resin column (30×600 mm). Since the hydrogen bonding between polyamide resin and flavonoid glycosides (like HY and PH) is relatively strong because of the existence of more hydroxyl groups in the phenolic molecules, higher concentration of ethanol solution is required to elute flavonoid glycosides out of the polyamide resin (Gao et al., 2011; Li, Sun, Liu, Zhang, & Cui, 2014; Shahat, Abdelshafeek, & Hussein, 2011). Therefore, at first, the polyamide resin that adsorbed component B was eluted by 60E, and the desorption solutions were collected and determined for the concentrations of phenolics. As shown in Fig.5A, both HY and PH existed in 100-200mL of desorption solution, indicating that 60E was not suitable for separating HY and PH. As discussed above, PH and HY are two flavonoid glycosides with relatively weak polarity (which could be reflected from the longer retention time in RP-HPLC chromatography in Fig.1D); hence, the strippant with a relatively low polarity may not separate the two compounds outstandingly. Then, it is necessary to increase the polarity of the strippant to obtain the two separated phenolics. Because water possesses higher polarity than ethanol, the

polarity of ethanol-water solution decreases with the concentration of ethanol increases. Therefore, the concentration of ethanol in the strippant should be decreased. By doing this, 30E was selected to elute the polyamide resin that adsorbed component B, and the eluates were collected at intervals of 100 or 125 mL. The concentrations of HY and PH were determined by HPLC. As shown in Fig.5B and G, HY was desorbed mainly in 0-300 mL of eluate in which there was hardly PH, while most of PH existed in 300-1425 mL of eluate that contained little HY (Fig.5D and E). Besides, the recovery yields of HY and PH after desorption by 30E in this step were 83.12% and 86.26% (Table S2), respectively. Therefore, 30E was suitable for separating HY and PH. Then, 0-300 mL of desorption solution that was enriched with HY was collected, concentrated and lyophilized to obtain 39.54 mg of HY powder with the purity of 95.36% (HPLC, Fig.5C) and a recovery yield of 71.50%. 300-1300 mL of desorption solution that was enriched with PH was collected, concentrated and recrystallized in methanol-water solution (v/v, 1:1) to obtain 0.512 g of crystalline PH with the purity of 97.36% (HPLC, Fig.5F) and a recovery yield of 66.51%.

4. Conclusion

Overall, a novel and effective method for simultaneous separation and purification of CA, EC, HY and PH from thinned young *Qinguan* apples by successive use of two kinds of resins, namely X-5 resin followed by polyamide resin, has been developed in this study for the first time. X-5 resin showed good adsorption capacities for the four phenolic compounds, conforming to both Langmuir and Freundlich adsorption isotherms. The pseudo-first-order model was more suitable to characterize the adsorption kinetics of EC and CA on X-5 resin, and the pseudo-second-order model was found to describe that of HY and PH better. During the whole extraction and separation process, in addition to the selection for the suitable apple cultivar, there are six key steps should be considered. After one run treatment by combined

use of X-5 and polyamide resins with ethanol solution as the strippant, four purified CA, EC, HY and PH were obtained with the purities above 95%. Actually, the production of purified phenolic compounds from thinned young apples is a comprehensive utilization of agricultural and food resources with the advantages of convenience and low cost, as well as relief of the replant problem. Besides, the developed method in this study may also provide a reference for pilot-or large-scale production of chlorogenic acid, epicatechin, hyperoside and phlorizin from plant and food extracts.

Acknowledgements

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Abbreviations

Young apple polyphenols, YAP; *Fuji* young apple polyphenols, *F*-YAP; *Royal Gala* young apple polyphenols, *R*-YAP; *Qinguan* young apple polyphenols, *Q*-YAP; Chlorogenic acid, CA; Epicatechin, EC; Hyperoside, HY; Phlorizin, PH; Bed volume, BV; 20% (v/v) ethanol aqueous solution, 20E; 30% (v/v) ethanol aqueous solution, 30E; 70% (v/v) ethanol aqueous solution, 70E.

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Figure Captions

Figure 1. HPLC chromatograms of phenolic standards (A) and polyphenols isolated from young *Fuji* (B), *Royal Gala* (C) and *Qinguan* (D) apples. Individual peaks are 1. Gallic acid, 2. Procyanidin B2, 3. Chlorogenic acid, 4. Caffeic acid, 5. 4-Hydroxybenzoic acid, 6. Epicatechin, 7. Hyperoside, 8. Rutin, 9. Phlorizin, 10. Quercitrin. The chemical structures of the four target phenolic compounds are indicated in (D).

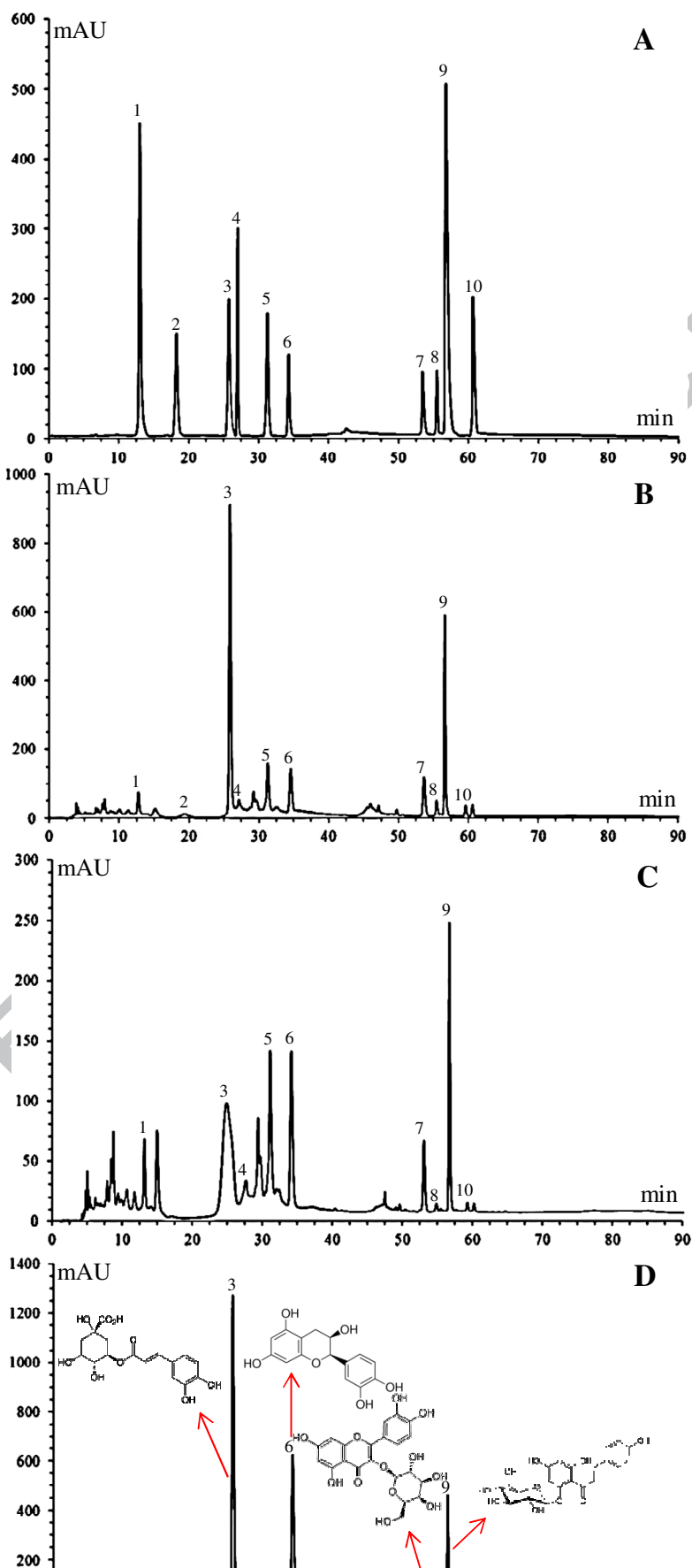
Figure 2. Details of adsorption of phenolic compounds on X-5 resin. (A) Adsorption isotherms of phenolic compounds on X-5 resin at 20 °C; (B) Adsorption of thermodynamic curve of phenolic compounds on X-5 resin; (C) Adsorption kinetics curve of phenolic compounds on X-5 resin; (D) Pseudo-first-order adsorption kinetics model of phenolic compounds on X-5 resin, and the curves were fitted using equation (11); (E) Pseudo-second-order adsorption kinetics model of phenolic compounds on X-5 resin; (F) Intra-particle diffusion adsorption kinetics model of phenolic compounds on X-5 resin.

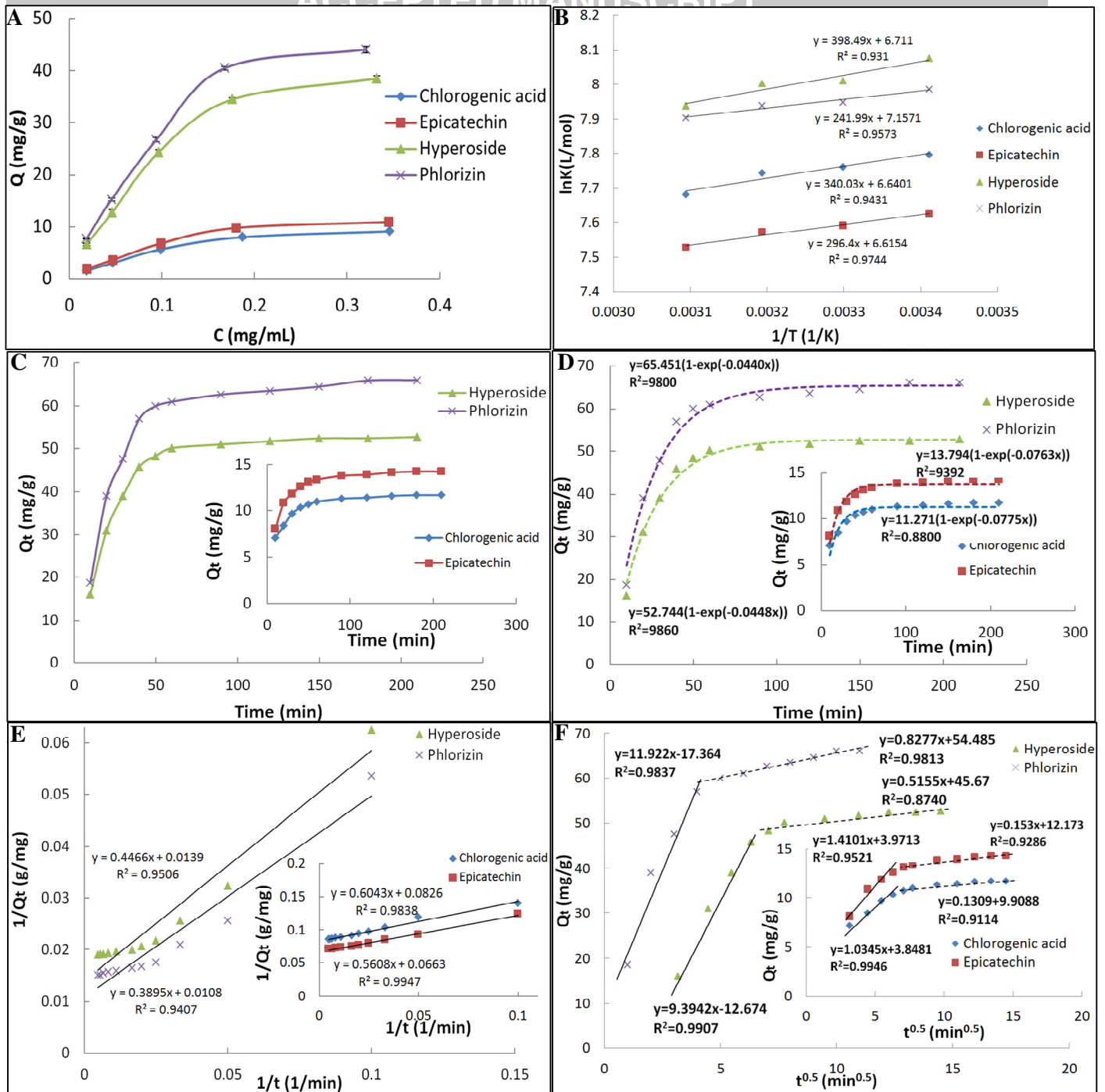
Figure 3. Initial separation of component A (mainly containing chlorogenic acid and epicatechin) and B (mainly containing hyperoside and phlorizin). (A) Concentrations of chlorogenic acid and epicatechin in different volumes of desorption solutions (resin packed in glass column: X-5; strippant: 30% ethanol solution); (B) Concentrations of hyperoside and phlorizin in different volumes of desorption solutions (resin packed in glass column: X-5; strippant: 70% ethanol solution); (C) HPLC chromatograms of component A; (D) HPLC chromatograms of component B.

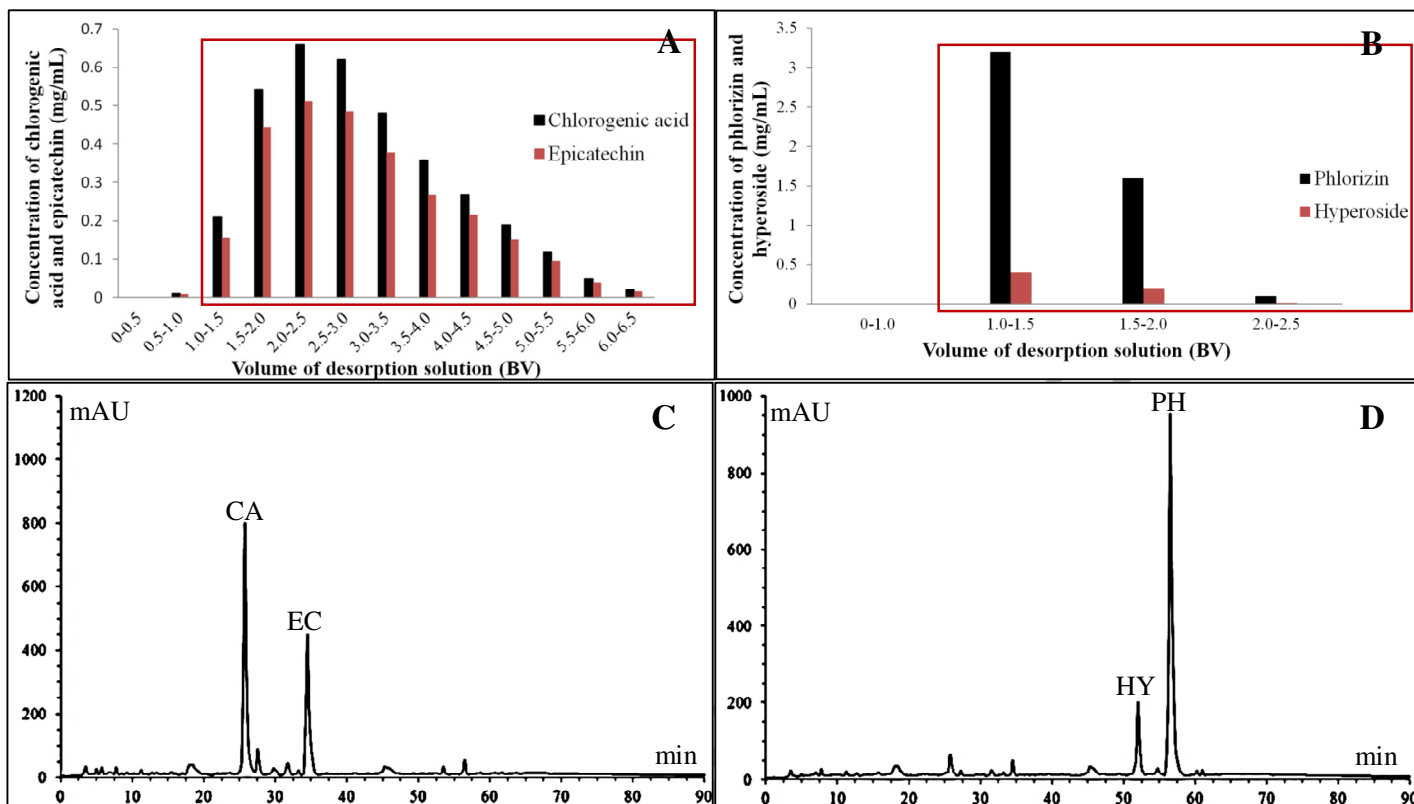
Figure 4. Purification of chlorogenic acid and epicatechin. HPLC chromatograms of 450-600 mL (A) and 600-750 mL (B) desorption solutions of component A; HPLC chromatograms of 450-600 mL (C), 600-750 mL (D) and 750-900 mL (E) desorption solutions of component C; (F) HPLC chromatograms of chlorogenic acid product after separation and purification; (G) Concentrations of chlorogenic acid and epicatechin in different volumes of desorption solutions of component C; For (A-E) and (G), resin packed in glass column: polyamide resin; strippant: 20% ethanol solution; (H) HPLC chromatogram of component D; HPLC chromatograms of 150-300 mL (I), 750-900 mL (J) and 900-1050

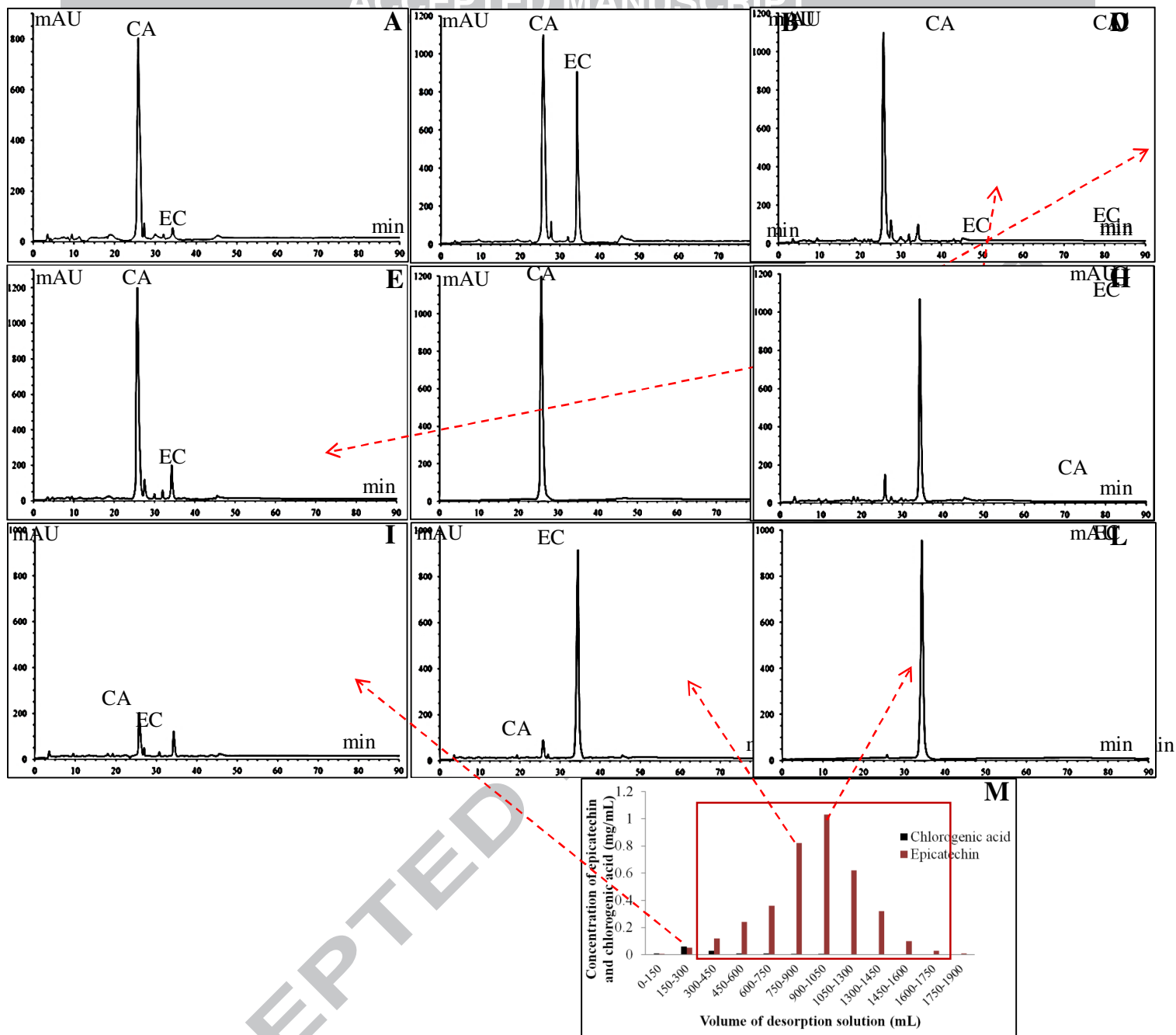
mL (**K**) desorption solutions of component D; (**L**) HPLC chromatogram of epicatechin product after separation and purification; (**M**) Concentrations of epicatechin and chlorogenic acid in different volumes of desorption solutions of component D; For (**H-K**) and (**M**), resin packed in glass column: polyamide resin; strippant: 20% ethanol solution.

Figure 5. Purification of hyperoside and phlorizin. (**A**) HPLC chromatogram of 100-200mL desorption solution of component B by 60% ethanol solution; (**B**) HPLC chromatogram of 100-200 mL desorption solution of component B by 30% ethanol solution; (**C**) HPLC chromatogram of hyperoside product after separation and purification; HPLC chromatograms of 675-800 mL (**D**) and 800-925 mL (**E**) desorption solutions of component B by 30% ethanol solution; (**F**) HPLC chromatogram of phlorizin product after separation and purification; (**G**) Concentrations of hyperoside and phlorizin in different volumes of desorption solutions of component B by 30% ethanol solution. For (**A, B, D, E and G**), resin packed in glass column: polyamide resin.









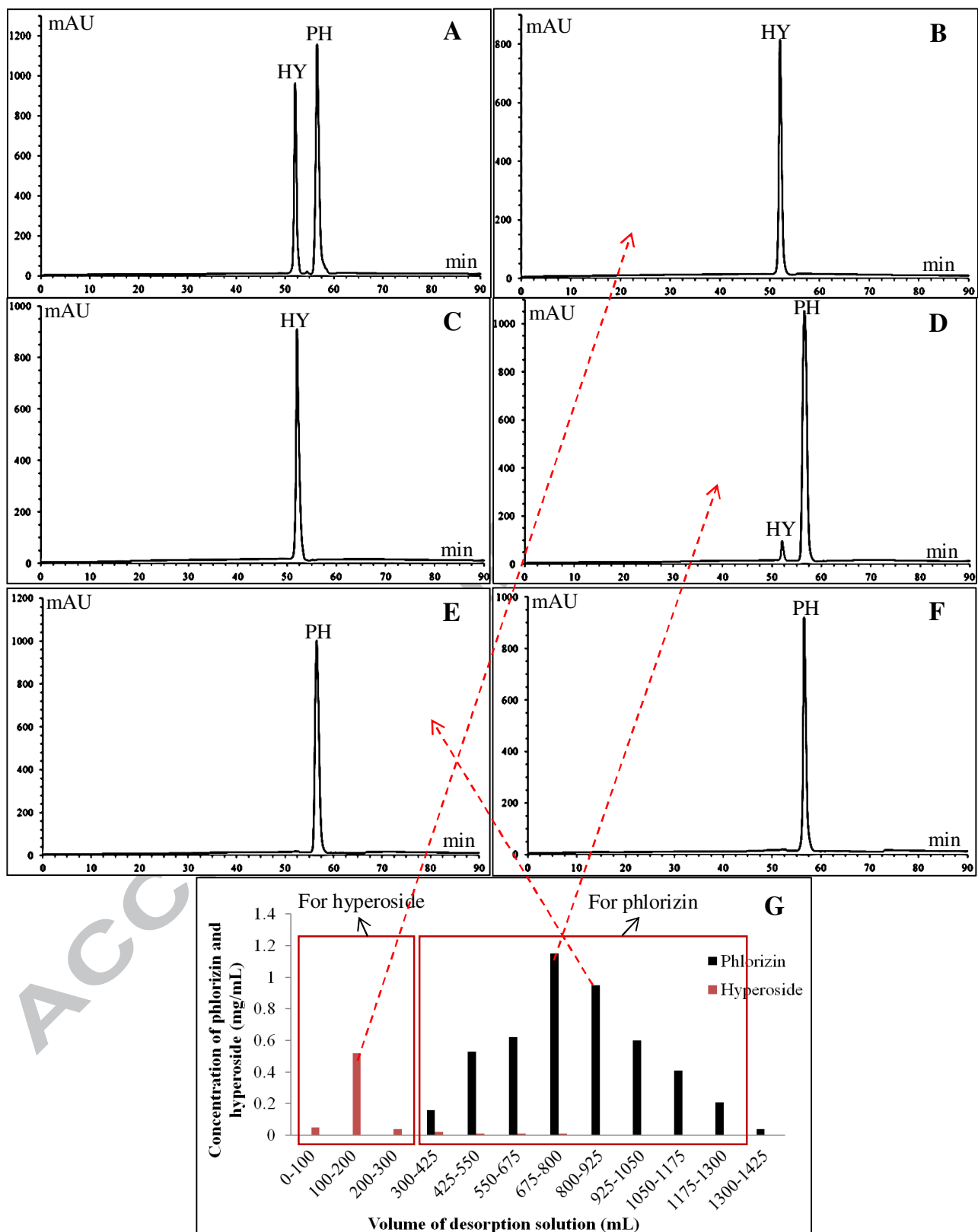


Table 1A. Adsorption isotherm equations and parameters of phenolic compounds on X-5 resin.

Phenolic compound	Equation type	20°C	30°C	40°C	50°C	
Chlorogenic acid	Linear equation	$1/Q=0.0110/C+0.0755$	$1/Q=0.0115/C+0.0761$	$1/Q=0.0121/C+0.0786$	$1/Q=0.0129/C+0.0790$	
	Langmuir	Q_0 (mg/g)	13.24	13.14	12.72	12.66
		K_{ad} (ml/mg)	6.86	6.62	6.50	6.12
		R^2	0.9943	0.9957	0.9968	0.9998
	Freundlich	Linear equation	$\text{Ln}Q=0.6405\text{Ln}C+3.0546$	$\text{Ln}Q=0.6320\text{Ln}C+2.9995$	$\text{Ln}Q=0.6338\text{Ln}C+2.9582$	$\text{Ln}Q=0.6291\text{Ln}C+2.9059$
		K_f (mg/g)	21.21	20.08	19.26	18.28
		$1/n$	0.6405	0.632	0.6338	0.6291
		R^2	0.9683	0.9693	0.9783	0.9812
Epicatechin	Linear equation	$1/Q=0.0090/C+0.0636$	$1/Q=0.0094/C+0.0641$	$1/Q=0.0099/C+0.0662$	$1/Q=0.0104/C+0.0666$	
	Langmuir	Q_0 (mg/g)	15.72	15.60	15.10	15.02
		K_{ad} (ml/mg)	7.06	6.82	6.69	6.40
		R^2	0.9944	0.9965	0.9979	0.9992
	Freundlich	Linear equation	$\text{Ln}Q=0.6393\text{Ln}C+3.2499$	$\text{Ln}Q=0.6312\text{Ln}C+3.1955$	$\text{Ln}Q=0.6324\text{Ln}C+3.1530$	$\text{Ln}Q=0.6278\text{Ln}C+3.1008$
		K_f (mg/g)	25.79	24.42	23.41	22.22
		$1/n$	0.6393	0.6312	0.6324	0.6278
		R^2	0.966	0.9681	0.9762	0.9795
Hyperoside	Linear equation	$1/Q=0.0025/C+0.0173$	$1/Q=0.0027/C+0.0175$	$1/Q=0.0028/C+0.018$	$1/Q=0.0030/C+0.0181$	
	Langmuir	Q_0 (mg/g)	57.80	57.14	55.56	55.25
		K_{ad} (ml/mg)	6.92	6.48	6.43	6.03
		R^2	0.9949	0.9967	0.9983	0.9995
	Freundlich	Linear equation	$\text{Ln}Q=0.6453\text{Ln}C+4.5409$	$\text{Ln}Q=0.6372\text{Ln}C+4.4868$	$\text{Ln}Q=0.6392\text{Ln}C+4.4462$	$\text{Ln}Q=0.6349\text{Ln}C+4.3951$
		K_f (mg/g)	93.77	88.84	85.30	81.05
		$1/n$	0.6453	0.6372	0.6392	0.6349
		R^2	0.9634	0.9651	0.9740	0.9775
Phlorizin	Linear equation	$1/Q=0.0022/C+0.0148$	$1/Q=0.0023/C+0.0149$	$1/Q=0.0024/C+0.0154$	$1/Q=0.0025/C+0.0155$	
	Langmuir	Q_0 (mg/g)	67.57	67.11	64.94	64.51
		K_{ad} (ml/mg)	6.73	6.48	6.42	6.20
		R^2	0.9972	0.9985	0.9987	0.9986
	Freundlich	Linear equation	$\text{Ln}Q=0.6461\text{Ln}C+4.7034$	$\text{Ln}Q=0.6384\text{Ln}C+4.6507$	$\text{Ln}Q=0.6396\text{Ln}C+4.6095$	$\text{Ln}Q=0.6354\text{Ln}C+4.5594$
		K_f (mg/g)	110.32	104.66	100.43	95.53
		$1/n$	0.6461	0.6384	0.6396	0.6354
		R^2	0.9641	0.9665	0.9719	0.9740

Table 1B. Adsorption thermodynamic parameters of phenolic compounds on X-5 resin.

Phenolics	ΔS^0 (J/(mol·K))	ΔH^0 (kJ/mol)	ΔG^0 (kJ/mol)			
			20°C	30°C	40°C	50°C
Chlorogenic acid	55.21	-2.83	-19.00	-19.56	-20.16	-20.64
Epicatechin	55.00	-2.46	-18.58	-19.13	-19.71	-20.22
Hyperoside	55.80	-3.31	-19.68	-20.19	-20.83	-21.33
Phlorizin	59.50	-2.01	-19.46	-20.03	-20.67	-21.23

Table 1C. Adsorption kinetics parameters of phenolic compounds on X-5 resin.

Kinetics model	Parameters	Phenolics			
		Chlorogenic acid	Epicatechin	Hyperoside	Phlorizin
Pseudo-first-order	Q_e (mg·g ⁻¹)	11.27	13.79	52.74	65.45
	K_1 (min ⁻¹)	0.0775	0.0763	0.0448	0.0440
	R^2	0.8800	0.9392	0.9860	0.9800
Pseudo-second-order	Q_e (mg·g ⁻¹)	12.10	15.08	71.94	92.59
	K_2 (g·mg ⁻¹ ·min ⁻¹)	0.0113	0.00784	0.000434	0.000299
	R^2	0.9838	0.9947	0.9506	0.9407
Intra-particle diffusion (whole process)	K_i (mg·g ⁻¹ ·min ^{-0.5})	0.343	0.424	2.473	3.162
	I (mg/g)	7.472	9.076	23.264	27.834
	R^2	0.7578	0.7156	0.6403	0.6567
Intra-particle diffusion (1 st stage: 10-40 min)	K_i (mg·g ⁻¹ ·min ^{-0.5})	1.035	1.410	9.394	11.922
	I (mg/g)	3.848	3.971	-12.674	-17.364
	R^2	0.9946	0.9521	0.9907	0.9837
Intra-particle diffusion (2 nd stage: 40-210 min)	K_i (mg·g ⁻¹ ·min ^{-0.5})	0.1309	0.153	0.516	0.828
	I (mg/g)	9.909	12.173	45.670	54.485
	R^2	0.9114	0.9286	0.8740	0.9813

- The adsorption of the four phenolics on X-5 conformed to Langmuir isotherms better
- The adsorption kinetics of CH and EC conformed to pseudo-first-order model better
- The adsorption kinetics of HY and PH accorded with pseudo-second-order model better
- Q_e in preferential adsorption kinetics model was closer to Q_0 in Langmuir isotherms
- 4 phenolics were isolated simultaneously by successive use of X-5 and polyamide resins