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Synthese and characterization of boronic acid functionalized macroporous uniform poly(4-chloromethylstyrene-co-divinylbenzene) particles and its use in the isolation of antioxidant compounds from plant extracts

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ABSTRACT

Aminophenyl boronic acid (APBA) carrying uniform-macroporous poly(chloromethylstyrene-co-divinylbenzene), poly(CMS-co-DVB) particles were synthesized for selective separation of cis-diol-containing flavonoids from plant extracts. For this purpose, 2.5 μm polystyrene seed particles were first swelled by a mixture of dibutyl phthalate (DBP), toluene and dodecanol, then by a monomer mixture including CMS and DVB. The repolymerization of the monomer phase in the swollen seed particles provided macroporous and uniform particles, approximately 7 μm in size. Chlorine atoms on the surface of these particles were derivatized with APBA to gain affinity properties for flavonoids containing vicinal hydroxyl groups. Model adsorption studies showed that these particles selectively adsorbed quercetin and rutin containing cis-diol groups, but did not adsorb apigenin similar to quercetin and not carrying cis-diol groups. These particles were also tested in adsorption/desorption studies for ethanol and ethyl acetate extracts of the *Hypericum perforatum* (HP) stems to obtain high antioxidant mixtures. With ethanol extract, the antioxidant activity of the desorption solution was a bit higher than that of the post-adsorption solutions. However, the DPPH radical scavenging activity of the desorption solution decreased with respect to the original extract and post-adsorption solutions. A similar result was obtained for the antioxidant activity of the desorption solution using ethyl acetate extract. An interesting result was obtained that DPPH radical scavenging activity of the post-adsorption solution was higher than that of the original ethyl acetate extract and desorption solutions. These results were attributed to selective adsorption of antioxidant characterized cis-diol-containing apolar molecules much more rather than that radical scavenger characterized polar molecules.

1. Introduction

Flavonoids are a large group of phenolic compounds that constitute one of the largest groups of secondary metabolites in plants [1]. Due to their biological and physiological properties and activities, flavonoids have been commonly used in health products, cosmetics and medicines [2–12]. Although the solvent extraction method is the conventional technique [13,14] used in the isolation of these compounds it leads to large amounts of residual solvents and results in environmental pollution.

Because of its selectivity, adsorbent technology emerged as an alternative method. Adsorbent beads containing macroporous pores with high surface area were used to enrich the antioxidant activity of plants [15–19] Adsorbents, such as the cross-linked polystyrene beads used in these studies, generally have a hydrophobic character. Some aliphatic acrylic modifications and functionalizations of these beads give them different adsorption selectivity to flavonoids.

Besides millimeter-sized beads, monosize micron and sub-2-micron organically functionalized silica particles have also been used in liquid chromatography of flavonoids from plant extracts to fractionate and identify flavonoid content and to evaluate the quality of the plant [20,21]. For the purification or identification of flavonoids, hydrophobic interactions and hydrogen bonding can be utilized as two primary forces. However, no research was encountered in the literature with adsorbent-specific target cis-diol-containing flavonoids.

The focus of this study was the production of adsorbent that can be specifically used to isolate cis-diol-containing flavonoids. For this purpose, first an adsorbent that selectively interacts with cis-diol-containing flavonoids was produced in a monosize particle form for an alternative and potential use in liquid chromatography. Then, its adsorption properties and selectivity were evaluated using two model flavonoids, namely quercetin and rutin both

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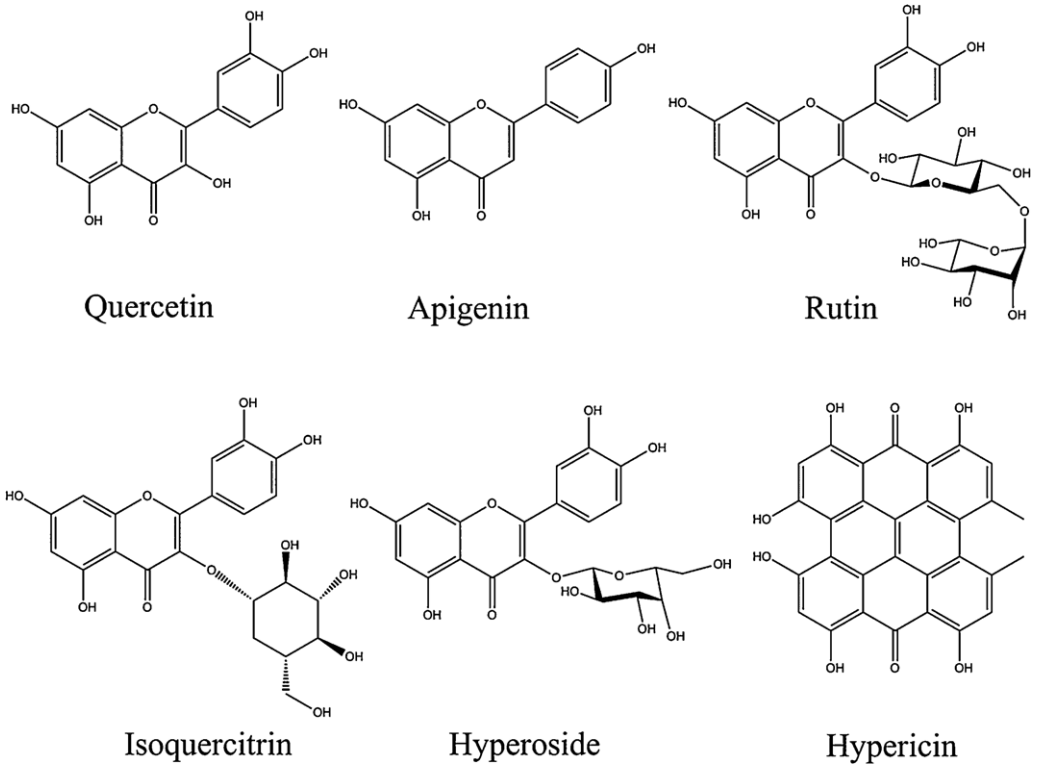

Fig. 1. Molecular structures of some phenolic compounds.

containing vicinal hydroxyl groups. Finally, the efficiency of this adsorbent for isolation of the cis-diol-containing phenolics from the HP extracts was evaluated (Fig. 1). These studies showed that, by using this sensitivity, the adsorbent developed here can be used in the enhancement of antioxidant activity.

2. Experimental

2.1. Materials and instruments

Styrene (99%, Sigma–Aldrich) was used as the main monomer after vacuum distillation at 25 °C. An absolute ethanol (Aldrich Chem. Co.) and 2-methoxyethanol (Merck, Germany) mixture was used as continuous phase to produce seed latex by dispersion polymerization. In this polymerization, 2,2′-Azobis(2-methylpropionitrile) (AIBN, Acros Organics, New Jersey, USA) was crystallized in methyl alcohol crystallization and poly(vinylpyrrolidone) (PVP K-30, M_w: 40,000, Sigma Chem. Co., St. Louis, MO, USA) were used as initiator and stabilizer, respectively. Dibutyl phthalate (DBP, Aldrich Chem. Co.), toluene and 1-dodecanol were selected as the components of diluent mixture. 4-Vinylbenzylchloride (4-chloromethylstyrene, CMS, 90%, Aldrich Chem. Co., USA) and divinylbenzene (DVB, 55%, *para*- and *meta*-divinylbenzene isomers, Aldrich Chem. Co., USA) was extracted with 5% (w/w) aqueous NaOH solution for the removal of the inhibitor. Sodium dodecyl sulfate (SDS, Sigma Chem. Co., St. Louis, MO, USA) was the emulsifier in the preparation of the emulsion medium. Polyvinyl alcohol (PVA, 87–89% hydrolyzed, M_w: 85,000–124,000, Aldrich Chem. Co., USA) was used as a stabilizer in the postpolymerization of monomer-swollen particles. Tetrahydrofuran (THF, HPLC grade) was obtained from Aldrich Chem. Co. and was used for the extraction of unreacted components from particles interior. Distilled–deionized water was used in the preparation of the solutions for polymerizations and in all other adsorption experiments. 3-Aminophenyl boronic acid-hemi sulfate salt (APBA) was supplied by Sigma Chemical Co. and used in the boronic acid functionalization of particles.

Rutin hydrate (95%, Sigma), quercetin (>95%, Aldrich) and apigenin (95%, Sigma) were selected model flavonoids to present the selectivity of the adsorbent. In the preparation of the adsorption and desorption medium, methanol (GC grade, Merck), absolute ethanol (Sigma–Aldrich) and water were used. The buffer solutions for the adsorption experiments were prepared using N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES, Sigma Chem. Co.) and 0.1 N NaOH solution. Adsorption experiments were observed by UV–visible spectrophotometer (SHIMADZU W-1601).

Separation was achieved on a reversed phase HPLC column (ZORBAX, Eclipse SB-C18, 7 μm, 4.6 mm × 250 mm, Agilent, USA) provided with a C18 guard column. A methanol–acetonitrile (99.9%, Carlo Erba)-water (40:15:45, v/v/v, isocratically) mixture containing 1% acetic acid (100%, Riedel-de Haen) was employed as the mobile phase. The flow rate was kept constant at 1.0 ml/min and the peaks were identified using UV absorbance at 254 nm. The temperature of the column during analysis was maintained at 40 °C. The sample injection volume was 10 μl HPLC grade water. In adsorption and desorption experiments with plant extracts, extracts and adsorption–desorption solutions were dried by a vacuum rotary evaporator (Heidolph).

β-Carotene (97%, Fluka), linoleic acid (99%, Aldrich), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (85%, Fluka), Tween-40 (Merck), potassium acetate (>99%, Merck), aluminum nitrate (98.5%, Merck) used in free radical scavenging, antioxidant activity and total flavonoid determination experiments were analytical grade. For the preparation of plant extracts, ethyl acetate (99.5%, Sigma–Aldrich) was used in addition to ethanol.

2.2. Synthese of monodisperse–macroporous particles

The monodisperse polystyrene (PS) seed particles in 2.4 μm particle size were prepared by the dispersion polymerization of styrene

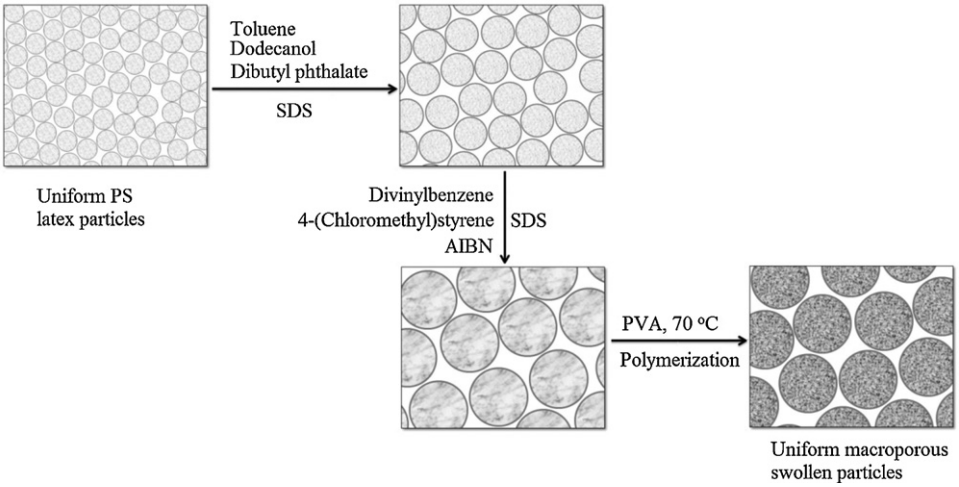

Fig. 2. Schematic representation of the seeded polymerization used for the preparation of the macroporous particles.

[22,23]. The seeded polymerization method used for the synthesis of monodisperse–macroporous particles from seed latex is given in Fig. 2 [22,23]. In a typical synthesis of monodisperse porous particles, a diluent mixture containing 2 ml toluene, 1 ml DBP and 0.25 ml dodecanol was emulsified in the aqueous medium (40 ml) containing 0.25% (w/w) SDS as the emulsifier. For this purpose, the mixture of diluent-aqueous SDS solution was sonicated 4 min with an ultrasonic probe (Bandelin Sonopuls HD 2070, Germany). The latex dispersion (approximately 2.6 ml) including PS seed particles (0.36 g) was added to the emulsion. The new dispersion was stirred magnetically (400 rpm) at room temperature for 24 h for the absorption of toluene by PS seed particles. In the following step, a monomer emulsion including monomer (1.2 ml CMS), the crosslinking agent (1.8 ml DVB), and the initiator (0.08 gr AIBN) were emulsified in the aqueous medium (45 ml) containing 0.25% (w/w) SDS by sonication for 12 min. The monomer emulsion was mixed with the aqueous emulsion containing diluent-swollen seed particles. The resulting emulsion was stirred at room temperature for 24 h at 400 rpm for the absorption of the monomer phase by diluent-swollen seed particles. Following this stage, an aqueous solution (10.0 ml) containing 0.8 g PVA was added to the resulting dispersion and the medium was purged with nitrogen for 10 min. In the next stage, the repolymerization of the monomer phase in swollen seed particles was conducted at 70 °C at a 120-cpm shaking rate and macroporous poly(CMS-co-DVB) particles were obtained. The monodisperse particles isolated by centrifugation were washed with ethanol several times. In these washings, the particles were redispersed in ethanol (150 ml) and the dispersion was magnetically stirred at room temperature for 4 h. The particles were then precipitated by centrifugation at 3000 rpm for 5 min and the solvent (i.e. ethanol) was removed by decantation. The washing was repeated several times. To obtain final porous particles by the complete removal of diluent and polymeric porogen (i.e. seed polymer), the particles were extracted several times with THF (150 ml) at 60 °C for 12 h in a sealed batch system. After the removal of the diluent and seed polymer, the polymer particles with a porous structure were obtained [22,23]. The particles were then dispersed in THF.

2.3. Particle size and morphology

The average size, size distribution and surface morphology of seed and swollen porous particles were determined by a scanning electron microscope (SEM, JSM-7600 F FEG) according to the method given in literature [22–25].

2.4. Determination of surface area and pore size properties of particles

Specific BET surface area, micro–meso pore size distribution, pore volume distribution and average pore dimensions of swollen particles were determined in Quantochrome Autosorp 1 MP device by standard volumetric method containing analysis of nitrogen adsorption/desorption isotherms [26]. Before analysis, particles were put in an oven at 70 °C for 48 h and were degassed at 80 °C for 6 h to completely evaporate the water.

2.5. Ligand attachment to poly(CMS-co-DVB) microparticles

Because of its affinity to cis-diol-containing flavonoids, the APBA ligand was attached to swollen poly(CMS-co-DVB) microparticles [27]. For this, 1 g microparticle containing-solution (dry basis) was taken, centrifuged at 5000 rpm and the supernatant was discarded. APBA (0.4 g) was dissolved in 10 ml water and decanted particles were added to this solution. The pH of the media was adjusted to 12 by 3 N NaOH and the reaction was conducted at 70 °C for 24 h with a shaking rate of 120 cpm. Then, particles were homogenized in a 100 ml ethanol/water (50/50) mixture and a 1 ml sample solution was taken for gravimetric analysis. For FTIR spectroscopy, a 3 ml solution was taken and centrifuged. After supernatant decantation, a 10 ml HCl solution was added to the particles and the particles were washed repeatedly with this solution for neutralization. Before FTIR spectroscopy, the sample was neutralized by washing with distilled water and dried in a vacuum at 40 °C for 24 h. The FTIR spectra of the seed latex, polychloromethyl styrene-containing particles without APBA ligand and APBA-attached poly(CMS-co-DVB) particles were taken with a Perkin Elmer FTIR spectrophotometer.

2.6. Model flavonoid adsorption with APBA-attached poly(CMS-co-DVB) microparticles

The adsorption experiment was planned in two main steps. In the first step, the adsorption of model flavonoids was performed individually in a UV spectrophotometer. In the second step, three model flavonoids were mixed and adsorption from this medium was measured by HPLC device.

First, the effect of the pH of the adsorption medium on flavonoid adsorption was investigated using a methanol/HEPES (85/25, ml/ml) buffer solution at different pHs adjusted with 0.1 N NaOH. The adsorption capacity of quercetin, rutin and apigenin was determined by changing the pH of the adsorption medium in the

range of 6–9.5 [27]. At the end of adsorption period, the adsorption medium was centrifuged and the particles were separated by filtration. The APBA concentration in the solution was determined by measuring absorbance of the solution at different wavelengths for each flavonoid in a UV spectrophotometer.

Equilibrium flavonoid adsorption capacity (Q) (mg flavonoid/g particle) of particles for different flavonoid types was calculated based on the following expression [28],

$$Q = \left(\frac{1}{W_p}\right)\left[\frac{A_o - A_f}{A_o}\right] C_o V \quad (1)$$

where A_o and A_f are the initial and final flavonoid absorbances in the adsorption medium, respectively. C_o (mg/ml) is the initial flavonoid concentration calculated by using calibration curves. V (ml) and W_p (g) are the volume of the adsorption medium and the amount of particles, respectively.

To show the cis-diol selectivity of adsorbent particles in a mixture, the adsorption from the rutin, quercetin and apigenin mixture was performed by HPLC. First, 20 μl solutions of every flavonoid at different concentrations in a methanol/HEPES buffer at pH 8.5 were injected to the HPLC column and their retention time versus concentration and calibration curves were determined. Then, the flavonoid mixture in the methanol/HEPES buffer at pH 8.5 was prepared by adjusting the concentration of each flavonoid in the mixture to 25 μg/ml. Adsorption was repeated with APBA-attached poly(CMS-co-DVB) particles as described before. The adsorption medium was centrifuged and a supernatant was used for HPLC analysis. Twenty μl of this medium was injected in the mobile phase. The mobile phase-containing methanol–acetonitrile–water (40:15:45, v/v/v) solution and 1% acetic acid was passed through the column at a flow rate of 1 ml/min [29].

Equilibrium flavonoid adsorption capacity (Q, mg flavonoid/g particles) of particles was calculated based on the following expression [28],

$$Q = \left(\frac{1}{W_p}\right)\left[\frac{P_o - P_f}{P_o}\right] C_o V \quad (2)$$

where P_o and P_f are the areas under the initial and final peak of each flavonoid obtained from intensity–time curves, respectively. C_o (mg/ml) is the initial flavonoid concentration calculated using calibration curves. V (ml) and W_p (g) are the volume of the adsorption medium and the amount of particles, respectively.

2.7. Using of poly(CMS-co-DVB) microspheres for antioxidant activity increments

HP, a well-known plant in Mugla due to its positive health effects, was chosen for antioxidant activity increment experiments. Ethanol and ethyl acetate extracts of HP were prepared to evaluate the efficiency of APBA attached poly(CMS-co-DVB) microspheres and its activity experiments performed as given below in detail.

2.7.1. Preparation of ethanol and ethyl acetate extracts from HP stems

The stems of HP were obtained from a local bazaar in Mugla, Turkey. Ethanolic extracts of HP stems were prepared according to the procedure given by Çakır et al. [30].

For the preparation of the ethyl acetate extract, the prepared ethanol extract was used. Approximately 2 g of ethanol extract was dissolved in 200 ml ethyl acetate extract 2 times (12 h 2×) at room temperature (25 °C), extracted and dried.

2.7.2. Adsorption from ethanol and ethyl acetate extracts of HP

The changing antioxidant activity of ethanol extract after adsorption with APBA-attached poly(CMS-co-DVB) particles was determined by adsorption/desorption experiments. First, the adsorption solution of ethanol extract was prepared in a concentration of 200 μg/ml. Because of its possible undesired effect on test methods, a HEPES buffer was not used in these experiments. In place of the HEPES buffer, the methanol/water (85/25, v/v) solution was used by adjusting its pH to 8.5 with 0.1 N NH_3 solution. Ethanol extract in a buffer solution (100 ml) and 1 g of APBA-attached poly(CMS-co-DVB) was mixed and divided into two 50 ml plastic centrifuge tube. The tubes were placed on a shaker (50 cpm) and the experiment was conducted for 2 h. The test tubes were centrifuged at 5000 rpm and the supernatant was separated for testing. Settled particles desorbed with 100 ml ethanol/water at pH: 5.5–6.5 (pH adjusted by 0.1 N acetic acid water solution) in a tube at a 100 cpm shaking rate. Particles and the desorption solution were separated by centrifugation at 5000 rpm. Pure ethanol extract (HPEtOH), the unadsorbed adsorption solution (HPA) and the desorbed solution (HPD) were tested for DPPH radical scavenging, β-carotene antioxidant activity and total flavonoid content. The same adsorption and desorption experiments were repeated for the ethyl acetate extract. Similarly, the tests given above were also conducted with the original ethyl acetate extract adsorption solution (EtOAc), adsorption solution remaining after adsorption (EtOAcA), and the desorbed adsorbate solution after adsorption (EtOAcD).

Desorption percent of adsorbed extract from adsorbent particles was calculated according to the following equation [28], where W_o is the total dry amount of extract in the adsorption solution, W_d is the total dry amount of extract in the desorption solution, and W_s is the total dry amount of adsorption solution remaining (unadsorbed) after the adsorption process.

$$\text{Desorption}\,(\%) = \frac{\text{desorbed amount}\,(\text{mg})}{\text{adsorbed amount}\,(\text{mg})} \times 100 \quad (3)$$

2.7.3. Free radical scavenging activity (DPPH method)

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). The free radical scavenging activity of ethanol and ethyl acetate extracts of the stems of HP was determined by the DPPH assay described by Blois [31]. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. The capability to scavenge the DPPH radical (radical inhibition) was calculated using the following equation [31].

$$\text{DPPH scavenging effect}\,(\%) = \left[\frac{A_0 - A_1}{A_1} \times 100\right] \quad (4)$$

where A_0 and A_1 are the absorbance of the control and sample respectively.

2.7.4. Determination of antioxidant activity with β-carotene-linoleic acid assay

In this assay, the antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [32]. The antioxidant activity of chloroform and methanol extracts of HP stems was evaluated using the β-carotene–linoleic acid model system [33].

The color bleaching ratio (R) of β-carotene was calculated according to the equation given below [34],

$$R = \frac{\ln(a/b)}{t} \quad (5)$$

where ln: natural logarithm, a: initial absorbance and, b: absorbance after 360 min of incubation.

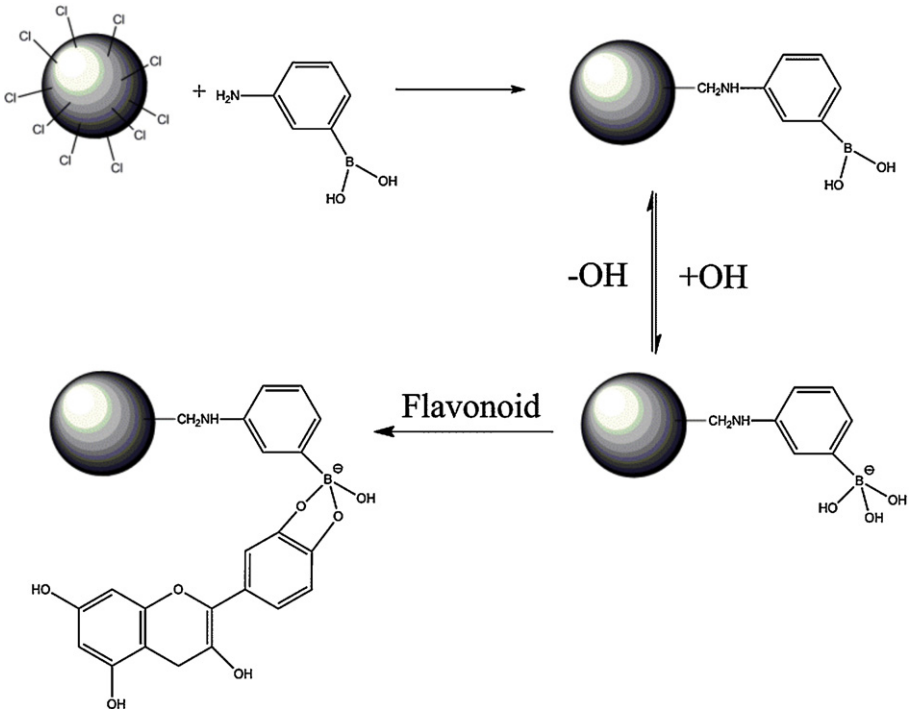

Fig. 3. Representative scheme for the attachment of APBA on chlorine-containing poly(CMS-co-DVB) particles and the adsorption mechanism of cis-diol-containing flavonoids by using them.

Antioxidant activity (AA) was calculated according to following equation [35],

$$AA = \left[\frac{R_{control} - R_{sample}}{R_{control}}\right] \times 100 \quad (6)$$

2.7.5. Determination of total flavonoid concentration

The measurement of flavonoid concentration of the extracts is based on the method described by Moreno et al. [36] with a slight modification and results were expressed as quercetin equivalents. Quercetin was used as a standard [37]. The concentrations of flavonoid compounds were calculated according to following equation that was obtained from the standard quercetin graph:

$$A = 0.0064[\text{quercetin (mg)}] + 0.0637(R^2 : 0.999) \quad (7)$$

3. Results and discussion

3.1. Characterization of particles

SEM photographs of seed latex and APBA-attached poly(CMS-co-DVB) microparticles are given in Fig. 4. Their size and size distribution index is also given in Table 1. Monodispersity of seed latex can be seen from Fig. 4a. Particle diameter of seed particles is approximately 2.4 μm. The diameter of seed latex particles increased to 6.6 μm (Fig. 4c) by seeding protocol. By comparing surface morphologies of seed latex particles (Fig. 4b) and poly(CMS-co-DVB) particles (Fig. 4d), it is clear that there are macropores on the surface of poly(CMS-co-DVB), but not on the surface of seed latex particles. The diameters of the sponge type pores on the surface of the particles are approximately between 50 and 100 nm (Fig. 4e and g). A BET analysis shows that the specific surface area, pore volume and average pore diameter of poly(CMS-co-DVB) particles are 84.35 m^2/g, 0.5603 cm^3/g and 26.57 nm, respectively. The difference between BET and SEM results for pore diameter indicates that the pores inside the particles are smaller than those that are on the surface. The use of toluene-DBP diluent causes sponge-type porosity rather than crater-like porosity [23]. Because of its high viscosity, dodecanol is added to the diluent mixture to increase the pore diameter of poly(CMS-co-DVB) particles [22]. Since data is obtained by BET, the physical adsorption isotherm of particles can be classified as Type II according to IUPAC, and this type can be represented as a macroporous structure [38].

The macroporous character of the obtained poly(CMS-co-DVB) particles produce smaller pore volumes than similar poly(styrene-co-DVB) particles in the literature, having 1 cm^3/g pore volume [23]. From Fig. 4 and Table 1, it is clearly seen that the produced particles can be accepted as monodisperse. In addition to pore size, surface properties and the monodispersity of produced particles, their high specific surface area present the possibility of HPLC applications and also for the separation of valuable molecules from a mixture aimed in this study.

Table 1
Size and size distribution of PS and poly(CMS-co-DVB) particles.

Particle type	D_w (μm)	D_n (μm)	CV (%)
PS	2.487	2.474	3.76
Poly(CMS-co-DVB)	6.666	6.552	7.01

3.2. Characterization of the APBA-functionalized particles by FT-IR

The FT-IR spectra of seed particles (PS), macroporous poly(CMS-co-DVB) particles produced by seeded polymerization and APBA-attached poly(CMS-co-DVB) particles are given in Fig. 5. A chlorine band was detected at 678 cm^{-1} in Fig. 5b, in comparison to Fig. 5a. An increase of the band by 1340 cm^{-1} in Fig. 5c indicated the presence of amine groups and also the attachment of amine groups carrying the APBA ligand. After this attachment the band at 678 cm^{-1} also decreased and can be interpreted as evidence of APBA derivation.

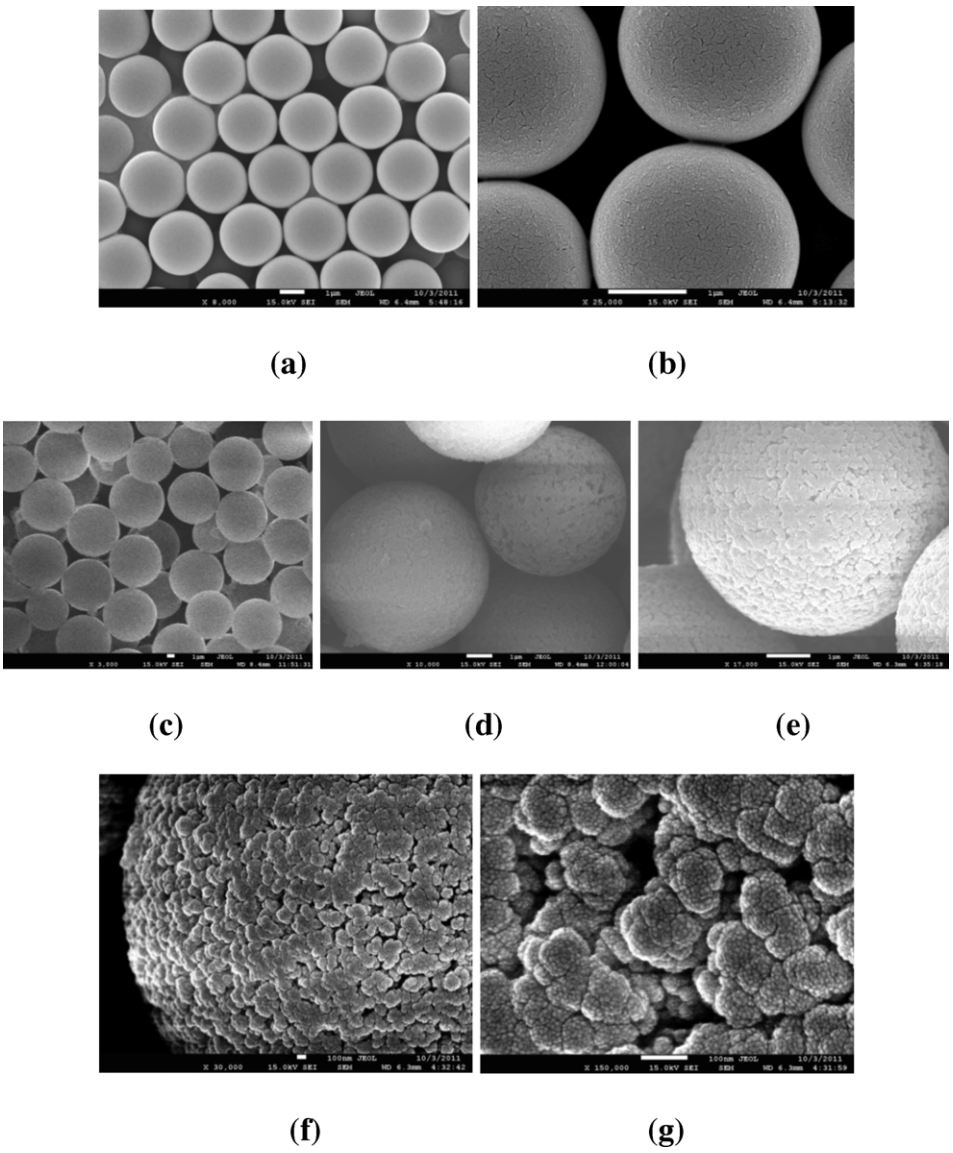

Fig. 4. SEM photographs of PS seed latex particles (magnifications a: 8000× and b: 25,000×), and swollen poly(CMS-co-DVB) particles (magnifications c: 3000×; d: 10,000×; e:17,000×; f: 30,000× and g: 150,000×).

3.3. Model flavonoid adsorption

In adsorption experiments, the selectivity of APBA-attached poly(CMS-co-DVB) particles to cis-diol-containing model flavonoids (rutin and quercetin) was examined. Apigenin, not containing cis-diol, a structural analog of cis-diol-containing quercetin, was used as another model flavonoid. First, the effect of pH on the adsorption capacity for each model flavonoid was determined using a maximum flavonoid concentration value of 0.02 mg/ml, which was determined by taking into account of the solubility of each flavonoid. The pH effect on adsorption capacity determined at this concentration is given in Fig. 6. As seen in Fig. 6 the maximum adsorption capacity was obtained between pH 8.5 and 9.0 for cis-diol-containing quercetin and rutin molecules. Since the pK_a value of APBA ligand on adsorbent particles is 8.9, it converts from the trigonal form to tetragonal form around this pH [27]. This form is the most suitable form for cis-diol capturing (Fig. 3). Thus, the adsorption of cis-diol-containing flavonoids increased as pH increased up to 9. Although maximum adsorption is obtained at approximately pH 9, all other experiments were performed at pH 8.5 to minimize structural deformation risk of flavonoids. On the other hand, the adsorption capacity of apigenin non-containing-cis-diol groups did not increase with pH, and the adsorption of apigenin was lower than that cis-diol-containing rutin and quercetin in the alkaline pH region. A small amount of apigenin adsorption can be attributed to nonspecific adsorption with adsorbent particles. These results clearly show specific selectivity of developed ABPA-attached particles to cis-diol-containing molecules.

The effect of the initial concentration of quercetin on quercetin adsorption capacity is given in Fig. 7. As seen in Fig. 7, the maximum adsorption capacity for quercetin was approximately 2.6 mg/g particle.

The compatibility of quercetin adsorption with two well-known adsorption models [27,28], Langmuir and Freundlich, was tested and their calculated parameters are presented in Table 2.

Comparing two q_o values, one obtained from a model (2.12 mg/g) and the other obtained from Fig. 7, it can be observed that they are close to each other. This result indicates that it may be a monolayer adsorption process. From Table 2, it can be seen that the "R^2" value calculated for each model is high enough and very close to 1. Thus, it may be assumed that the quercetin adsorption

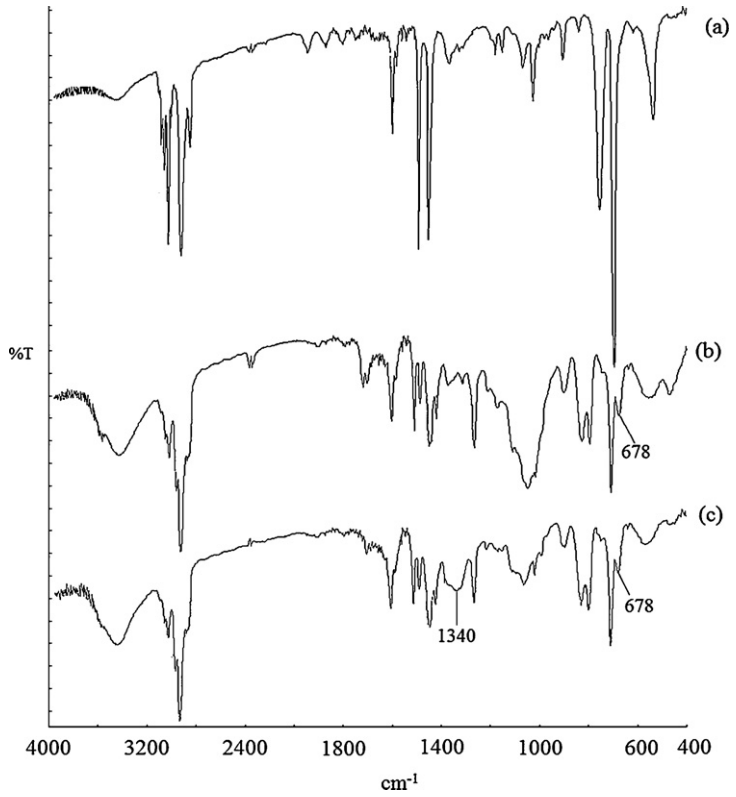

Fig. 5. FTIR spectra of particles: (a) seed PS latex particles; (b) poly(CMS-co-DVB); (c) APBA-attached poly(CMS-co-DVB).

process can be adequately represented by both the Langmuir and Freundlich models.

3.4. Adsorption from model flavonoid mixture

It is expected that the adsorption of model flavonoids from their mixtures should give similar results to those that are obtained from adsorption studies of individual model flavonoids. Selectivity of APBA-attached particles to cis-diol-containing molecules quercetin and rutin can be seen clearly in Fig. 8. Adsorption of apigenin molecules not containing cis-diol groups from the mixture of model flavonoids was similarly low as it was in individual adsorption studies.

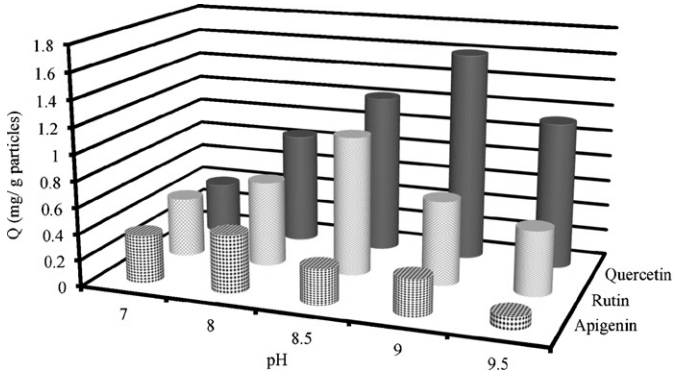

Fig. 6. The variation of adsorption capacity (Q) of quercetin, rutin and apigenin with pH for APBA-attached poly(CMS-co-DVB) particles, temperature: 20 °C, initial flavonoid concentration: 0.02 mg/ml.

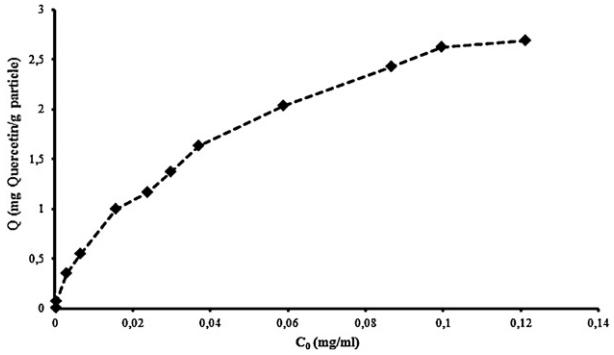

Fig. 7. The variation of equilibrium quercetin concentration with initial quercetin concentration for attached poly(CMS-co-DVB) particles, temperature: 20 °C, initial flavonoid concentration: 0.02 mg/ml, pH: 8.5.

3.5. Adsorption from HP stems extract

The ability of APBA-attached poly(CMS-co-DVB) particles to adsorb flavonoids from ethanol and ethyl acetate extracts of the HP stems were determined by comparing the antioxidant activities of the original extract and the extract treated with the adsorbent.

As seen in the HPLC results given in Fig. 9c, the number of peaks in the desorption medium are less than the number of peaks in

Table 2
Langmuir and Freundlich model parameters calculated for adsorption of quercetin by using the data obtained from Fig. 7.

Langmuir			Freundlich		
q_o (mg/g)	K (mg/ml)	R^2	n	k	R^2
2.12	0.026	0.9989	0.5639	0.2948	0.9942

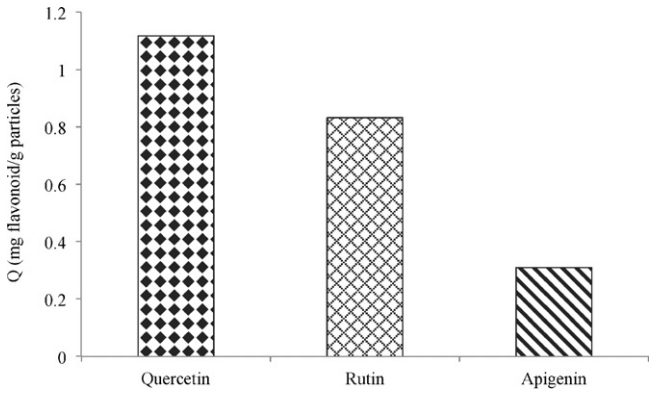

Fig. 8. HPLC results of the adsorption capacity of flavonoids from the mixture of quercetin, rutin and apigenin for APBA-attached poly(CMS-co-DVB) particles, temperature: 20 °C, initial flavonoid concentration: 0.025 mg/ml, pH: 8.5.

the original extract (Fig. 9a) and unadsorbed medium (Fig. 9b). Adsorption of similar molecular structures by the adsorbent may explain this result. As discussed below, it is anticipated that these compounds are mostly cis-diol-containing quercetin molecules.

The total flavonoid contents of HPEtOH, HPA and HPD media were determined and presented in Fig. 10. From this figure, the total flavonoid contents of the HPA and HPD medium are approximately equal. Antioxidant activity results with β-carotene and linoleic system of the ethanol extract of the HP stems is given in Fig. 11. The antioxidant activity of three media was given in order of HPEtOH > HPD > HPA. According to this result the adsorbed molecules produce a bit better antioxidant activity than unadsorbed molecules. It was reported that the number and concentration of flavonoid components in the HP stems were very high [39–44]. Therefore, it is not surprising to obtain high antioxidant activity for each of the three media. But, selectivity of the adsorbent to cis-diol-containing flavonoids could not be clearly seen from these results.

The DPPH radical scavenging activities of three media were determined and results are given in Fig. 12. It is clear that the DPPH activity of each of the three media increased by increasing

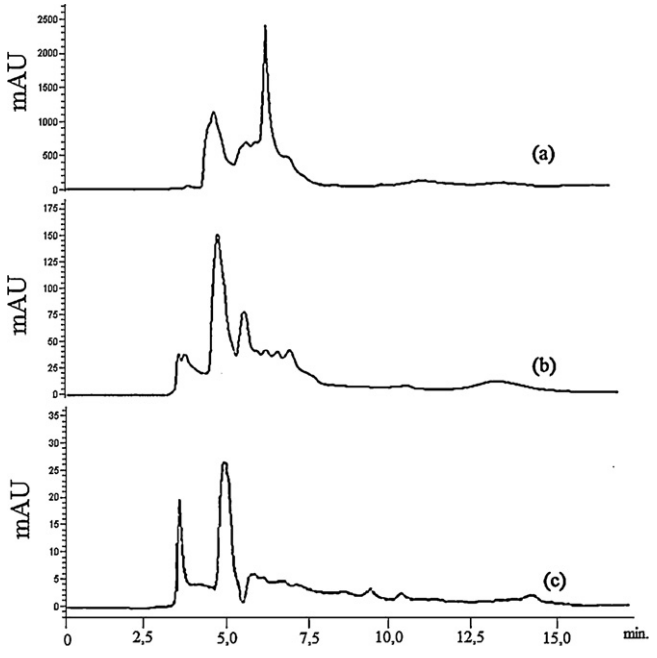

Fig. 9. HPLC chromatograms of (a) HPEtOH, (b) HPA and (c) HPD solutions.

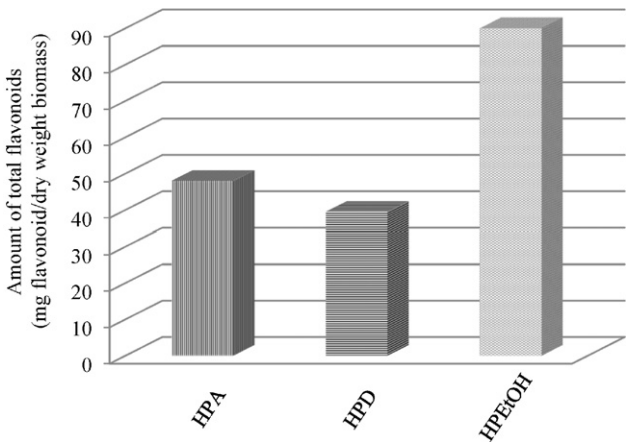

Fig. 10. Total flavonoid content of the original ethanol extract solution of the HP stems before adsorption (HEtOH), adsorption solution after adsorption (HPA) and desorption solution (HPD).

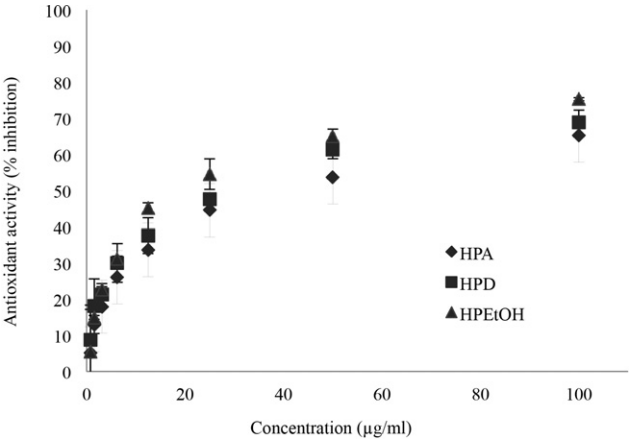

Fig. 11. Antioxidant activity of the original extract solution of the ethanol extract of the HP stems before adsorption (HEtOH), adsorption solution after adsorption (HPA) and desorption solution (HPD).

the concentration of the adsorption solution. To compare DPPH activities, EC_{50} (effective concentration for 50% percent activity, μg dwb/ml) values were calculated for HPEtOH, HPA and HPD as 28, 30 and 110, respectively. These results show that the DPPH activity of the HPD medium was lower than that of the others. Thus, the HPD solution differed molecularly from the other two media (HPEtOH and HPA).

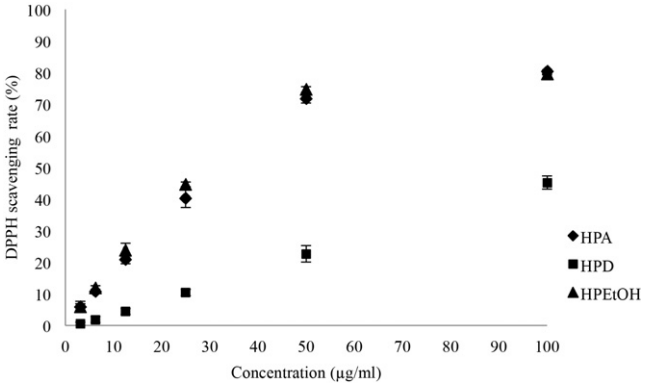

Fig. 12. DPPH radical scavenging of the original solution of the ethanol extract of the HP stems before adsorption (HEtOH), adsorption solution after adsorption (HPA) and desorption solution (HPD).

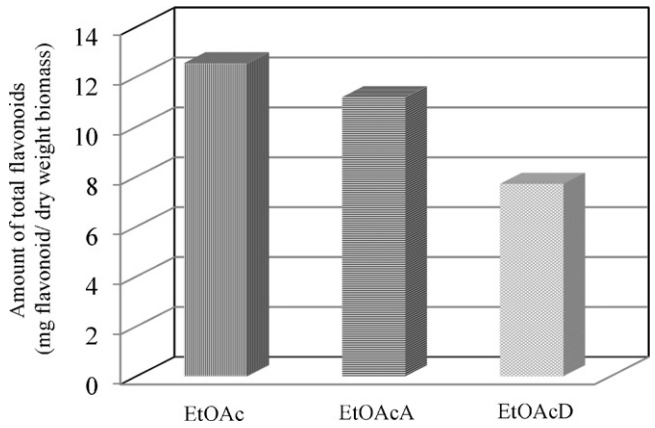

Fig. 13. Total flavonoid content of the original solution of ethyl acetate extract of HP stems before adsorption (EtOAc), adsorption solution after adsorption (EtOAcA) and desorption solution (EtOAcD).

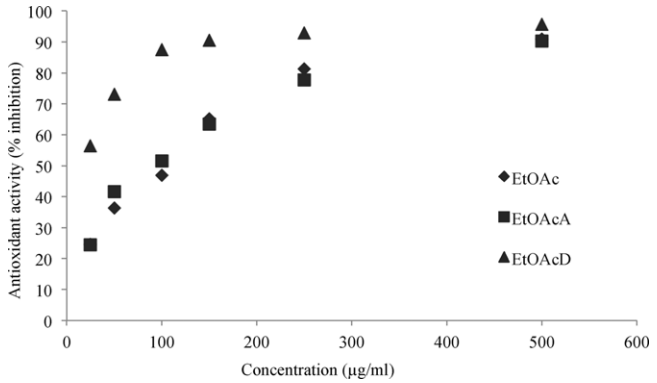

Fig. 14. Antioxidant activities of the original solution of the ethyl acetate extract of the HP stems before adsorption (EtOAc), adsorption solution after adsorption (EtOAcA) and desorption solution (EtOAcD).

As can be seen in Fig. 10, in 1 g dry extract of HPEtOH and HPD, there are 90 mg and 39.5 mg flavonoids respectively. This result displays the adsorption character of APBA-attached poly(CMS-co-DVB) particles for some type of flavonoids. Although HPD media have the least flavonoid content (Fig. 10), it displayed the highest antioxidant activity but lower DPPH radical scavenging activity.

A detailed review of the literature [45–47] for DPPH radical scavenging and antioxidant activity of flavonoid compounds in HP revealed that fractions containing flavonoid aglycones (i.e. quercetin, kaempferol, biapigenin) produced higher antioxidant activity than all others. In addition, flavonol glycoside-containing (i.e. rutin, hyperoside, isoquercitrin and kaempferol) fractions had more antiradical scavenging properties than all others.

The adsorbent in this study has a hydrophobic character and the capability for adsorbing apolar molecules like quercetin more than polar molecules, such as flavonol glycosides. Adsorption probability of quercetin and similar molecules by the hydrophobic adsorbent may have caused a bit more high antioxidant activity of desorption solution than unadsorbed solution. At the other hand, high antiradical scavenging of unadsorbed solution than desorbed solution indicated that the adsorbent could not adsorb molecules carrying high antiradical scavenging capacity. Less adsorption probability of flavonoid glycosides may have caused this result. Since ethanol extract of HP stems contain other molecules different from flavonoids, selectivity of APBA-attached poly(CMS-co-DVB) particles to cis-diol-containing flavonoids may have not been presented clearly. Thus, ethyl acetate extract of ethanol extract of HP stems was prepared to make an extract rich in antioxidant and DPPH scavenger molecules. Adsorption–desorption experiments with poly(CMS-co-DVB) particles and antioxidant tests were repeated similar to procedures for the ethanol extract as given above.

The dry weight of the original ethyl acetate extract (EtOAc), solution after adsorption (EtOAcA) and desorbed solution (EtOAcD) were calculated as 100 mg, 36 mg and 55 mg respectively. By using these three values in Eq. (3) the desorption yield was calculated as 84%.

For EtOAc, EtOAcA and EtOAcD solutions, total flavonoid content, β-carotene antioxidant activity and DPPH radical scavenging measurement results are given in Figs. 13, 14 and 15, respectively.

As seen in Fig. 13, the flavonoid content of the EtOAcD medium (approximately 8 mg/g dry extract) is lower than those of EtOAc and EtOAcA medium. However, the antioxidant activity of EtOAcD is clearly higher than that of EtOAc and EtOAcA (Fig. 14). Although desorption products for both ethanol and ethyl acetate extract have caused antioxidant activity, it is higher with ethyl acetate extract as seen in Figs. 11 and 14. By changing extract type from ethanol to ethyl acetate, antioxidant activity of the desorption medium jumped to an antioxidant activity value beyond the original extract. In the case of DPPH radical scavenging, it is clearly seen that EtOAcD medium showed the least activity. On the contrary, EtOAcA had the highest activity and this activity is higher than both the EtOAcD and EtOAc medium (Fig. 15). These results can be explained by more adsorption of flavonoids carrying high antioxidant activity and less adsorption of flavonoids carrying high DPPH radical scavenging properties [47]. Arguments made for ethanol extracts are also valid for ethyl acetate extract results. Steric effects that prevent the adsorption of big-sized flavonol glycoside molecules should also be considered to explain these results.

Briefly, it was considered that a high concentration cis-diol-containing quercetin and similar molecules in ethyl acetate extract caused higher antioxidant activity (Fig. 14) than ethyl alcohol extract (Fig. 11) in adsorption process. An adsorption medium after adsorption (EtOAcA) showing higher DPPH radical scavenging behavior than the original extract (EtOAc) was not encountered in literature. Generally, adsorbed molecules isolated by adsorbents produce higher DPPH radical scavenging than original extract [17,19]. The results in this study can be clarified by differentiation of the EtOAc medium after adsorption to different compositions (EtOAcA).

The surface of poly(CMS-co-DVB) adsorbents has a nonpolar character and also capability to capture cis-diol-containing molecules with their APBA ligand. Thus, its capability to capture less-polar molecules like cis-diol-containing quercetin is higher than capturing polar glycoside-derived and large flavonoid molecules. As a result, residue in an adsorption solution (EtOAcA) following adsorption may be rich in polar characterized glycoside flavonoids and a desorption solution may be rich in nonpolar characterized small molecules containing cis-diol groups.

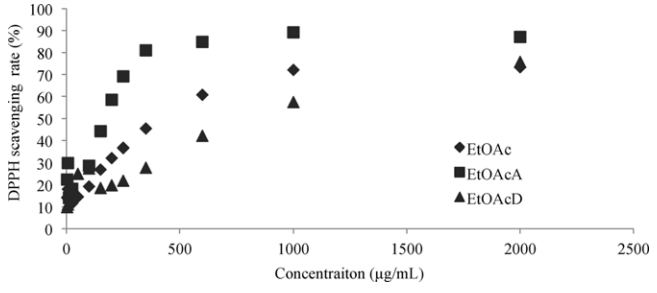

Fig. 15. DPPH radical scavenging of the original solution of the ethyl acetate extract of the HP stems before adsorption (EtOAc), adsorption solution after adsorption (EtOAcA) and desorption solution (EtOAcD).

As seen in Fig. 15, EC_{50} (μg dwb/ml) values were obtained for EtOAc, EtOAcA and EtOAcD as 360, 170 and 800 μg dwb/ml, respectively. Compared to ethanol extract results, these values increased and DPPH scavenging activity decreased. Less amount of DPPH radical scavenger compounds and lacking of phenolic acids (like chlorogenic acid and neochlorogenic acid) in ethyl acetate extract than in ethanol extract may have caused this interesting result [47].

4. Conclusion

Adsorbents in literature have a nonionic character and structures creating physical interactions (especially hydrogen bonding) with target molecules to prepare mixtures containing both antioxidant and radical scavenger molecules. The main difference between adsorbent (APBA-attached poly(CMS-co-DVB) particles) presented in this article and similar adsorbents in the literature is their functionality and interaction mechanism with target molecules. APBA-functionalized particles synthesized in this study have selectivity to cis-diol-containing flavonoids and their interaction mechanism is different. This differentiation causes more interesting fractionation results. While desorption mixtures obtained in similar studies with different adsorbents in the literature exhibited both antioxidant and free radical scavenging activity higher than original extracts, only antioxidant activity increment was obtained with this study's adsorbent and conversely, DPPH radical scavenging capacity decreased.

Although the conventional approach is to investigate the antiradical and antioxidant behavior of desorption solutions, this research primarily notes that these behaviors can also be differentiated in post desorption mixtures by changing the extraction type of plant. Especially in studies with ethyl acetate extracts of HP stems, for the first time, the highest DPPH radical scavenging capacity were observed with post-adsorption medium instead of original extract or desorption solution.

Monosize adsorbent particles were produced in this research and have the potential for HPLC use and affinity chromatography for selective isolation, and the analysis of plant extracts and biomolecules like nucleotides, RNA, glycated proteins and glycoenzymes.

Acknowledgements

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