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Characterization of synthetic adsorbents with fine particle sizes for preparative-scale chromatographic separation

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Abstract

Synthetic adsorbents with fine particle sizes (15–30 μm) were manufactured. These adsorbents are made of poly(styrene–divinylbenzene) and polymethacrylate, and have the same chemical structure as analytical- (5–10 μm) or industrial-(200–600 μm) grade synthetic adsorbents. Both of them have very similar porous structure to those of analytical or industrial sizes, so that they can adsorb compounds of various molecular masses. Chromatographic separation characteristics of newly manufactured fine-particle grades of synthetic adsorbents were evaluated and compared to those of analytical or industrial adsorbents. Reasonable dependency of separation performance on particle size of synthetic adsorbents was obtained. Hydraulic properties of fine-grade adsorbents had also been measured in view of column operations. Furthermore, scalability and applicability of these adsorbents for preparative-scale chromatographic separation of bioactive compounds was evaluated. Separation of soybean isoflavones and tea catechin derivatives had revealed that fine-grade synthetic adsorbents could be well applied with scalability under the same elution conditions used for analytical use. Scalability up to a 22 400-fold loading amount was achieved from a small column packed with analytical-grade adsorbent used for method development to a scale-up preparative column packed with fine-grade adsorbent used for preparative purification. These results showed the usefulness of the fine-grade synthetic adsorbents for more precise purification of bioactive compounds, including pharmaceuticals and functional food additives with higher recovery.

Keywords: Preparative chromatography; Adsorbents; Stationary phases, LC; Food analysis; Soybean; Tea; Particle Size; Phthalates; Curcumin; Isoflavones; Catechins

1. Introduction

Synthetic adsorbents are widely used as polymeric media for recovery and separation of pharmaceuticals and their intermediates [1–3]. For example, they are used for separation of antibiotics such as penicillin, cephalosporin and their derivatives, because of their higher adsorption capacity [4,5]. And the other favorable characteristics of synthetic adsorbents are high mechanical strength for industrial operations and chemical stability at higher or lower pH regions, for recycled use of adsorption–elution–regeneration recovery system [4,5].

On the other hand, analytical-grade polymeric packing materials are used as well as octadecyl silica media. They are used in reversed-phase chromatographic mode for precise and selective separation to specify chemical compounds. However, amounts

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Table 1
Characteristics of polystyrenic adsorbents with various particle sizes

No.	Adsorbent name	Particle size distribution (μm)	Average particle diameter of a referential lot (μm)	Specific surface area of a referential lot (m^2/g)	Specific pore volume of a referential lot (ml/g)	Pore radius of a referential lot (nm)
1	MCI GEL CHP5C	9–11	10	540	1.39	14.0
2	MCI GEL CHP55A	15–20	18	580	1.54	14.0
3	MCI GEL CHP55Y	25–35	30	590	1.55	14.0
4	MCI GEL CHP20P	37–75	55	520	1.17	30.0
5	SEPABEADS SP20SS	63–75	70	560	1.40	29.0
6	DIAION HP20SS	63–150	100	540	1.35	30.0
7	DIAION HP20	200–600	440	580	1.30	30.0
8	DIAION HP21	200–600	440	630	1.39	12.0

recovered by analytical-grade packing materials are small compared to preparative and industrial scale separations.

Recently, more precise purification with higher recovery amounts of pharmaceuticals or functional food additives has become an interest of researchers and chemical engineers in pharmaceutical or food processing fields, and one of the requirements is fine-grade (15–30 μm) synthetic adsorbent.

To fulfill the requirement, synthetic adsorbents made of poly(styrene–divinylbenzene) and polymethacrylate with fine-particle grades were manufactured. Tables 1 and 2 show the characteristics of polystyrenic and polymethacrylic adsorbents with various particle sizes, respectively.

Polystyrenic adsorbents with diameters of 15–20 μm (no. 2) and 25–35 μm (no. 3) were newly manufactured. Both adsorbents have the same chemical structure as analytical-grade adsorbent with diameter of 10 μm (no. 1) or industrial-grade adsorbent with diameter of 200–600 μm (no. 8), and also have almost identical porous structure measured by N_2 adsorption method. On the other hand, semi-industrial and industrial grades of adsorbents with diameters of 35–75 μm (no. 4), 63–75 μm (no. 5), 63–150 μm (no. 6) and 200–600 μm (no. 7) have larger pores and wider pore distributions despite of the same chemical structure as above. The fine-grade polystyrenic adsorbents with almost identical pore structure to these adsorbents are under development.

As for polymethacrylic adsorbents, the adsorbent with diameter of 25–35 μm (no. 11) was newly manufactured. It has the same chemical structure as analytical-grade adsorbent with 10 μm (no. 9) or industrial-grade adsorbent with diameter of 200–600 μm (no. 13), and also has an almost identical porous structure measured by N_2 adsorption method. For this study, polymethacrylic adsorbents with average particle diameters of 17 μm (no. 10) and 115 μm (no. 12) were developmentally prepared.

In this study, chromatographic separation characteristics of newly synthesized fine-grade synthetic adsorbents were evaluated and compared to those of analytical- or industrial-grade adsorbents. Scalability

Table 2
Characteristics of polymethacrylic adsorbents with various particle sizes

No.	Adsorbent name	Particle size distribution (μm)	Average particle diameter of a referential lot (μm)	Specific surface area of a referential lot (m^2/g)	Specific pore volume of a referential lot (ml/g)	Pore radius of a referential lot (nm)
9	MCI GEL CHP2MG	9–11	10	590	1.13	20.0
10	Developmentally prepared	15–20	17	470	1.16	19.0
11	MCI GEL CHP2MGY	25–35	31	510	1.15	23.0
12	Developmentally prepared	63–150	115	520	1.28	29.0
13	DIAION HP2MG	200–600	490	560	1.16	20.0

and applicability of those adsorbents for preparative-scale chromatographic separation in pharmaceutical and food processing fields have been also discussed.

2. Experimental

2.1. Materials

All synthetic adsorbents made of poly(styrene–divinylbenzene) and polymethacrylate were obtained from Mitsubishi Chemical Corporation (Tokyo, Japan).

2.2. Columns

Stainless steel columns of 150×4.6 mm I.D. were used for analytical-grade adsorbents. Stainless steel columns of 250×10 mm I.D. were packed with adsorbents of various particle sizes and used for semi-preparative chromatography. The details of glass columns used for preparative chromatography are shown in the Procedures sections. All of those columns were packed in our laboratory by the slurry packing method.

2.3. Reagents and chemicals

Catechin derivatives, caffeine, curcumin, daidzein and genistein were purchased from Sigma (St. Louis, MO, USA). Polyphenon 60 (extract of green tea leaves) was purchased from Kurita Water Industries (Tokyo, Japan). Daidzin and genistin were purchased from Fujicco (Kobe, Japan). Other reagents and chemicals were of the highest quality available, and were purchased from various suppliers. Demineralized water was prepared by a Milli-Q system (Millipore, Bedford, MA, USA).

2.4. Apparatus

Surface area measurement of the synthetic adsorbents was operated by use of a Micromeritics (Norcross, GA, USA) FlowSorb 2300 with a single-point BET method. Pore size distribution of the synthetic adsorbents was measured using a Micromeritics ASAP 2400 instrument. N_2 was used for both the above measurements.

Hydraulic properties were measured at 25°C using a Shimadzu (Kyoto, Japan) LC-8A pump and a 1000×10 mm I.D. glass column.

For analytical and semi-preparative chromatography, an HPLC system consisting of a Hitachi (Tokyo, Japan) L-7100 pump, a Hitachi L-7200 automatic sample injector, a Shimadzu SPD-6A UV detector and a Shimadzu C-R4A integrator was used.

Preparative chromatography was operated using a Hitachi L-6250 pump, a Shimadzu SIL-8A automatic sample injector with an 80-ml sample loop, a Shimadzu SPD-7A UV detector, a Hitachi L-5200 fraction collector and a Shimadzu C-R4A integrator.

For scale-up preparative chromatography, a medium pressure liquid chromatograph system consisting of an EYELA (Tokyo, Japan) VSP-2200 pump, a Millipore Vantage A2 VA90×500 (500×90 mm I.D.) column, a Soma (Tokyo, Japan) S-310A model-II UV detector and a Shimadzu C-R4A integrator was used. These systems were operated at 25°C unless otherwise mentioned.

2.5. Procedures

2.5.1. Hydraulic properties

Approximately 50 ml of each adsorbent was packed in a 1000×10 mm I.D. glass column. Various flow-rates of water were applied to the column and the pressure drop was measured.

2.5.2. Separation of dialkyl phthalates

A mixture of dimethyl phthalate, dipropyl phthalate and dibutyl phthalate was separated on columns packed with polystyrenic and polymethacrylic adsorbents of various particle sizes, respectively. The eluent for the polystyrenic adsorbents was acetonitrile (ACN)–water (80:20), and that for polymethacrylic adsorbents was ACN–water (60:40). The flow-rates were 0.46 ml/min for 150×4.6 mm I.D. columns and 2.18 ml/min for 250×10 mm I.D. columns to apply the same linear velocity of 166 cm/h. Sample concentration was 5 mg/ml for each dialkyl phthalate. Sample injection volumes were 12.5 μl for 150×4.6 mm I.D. columns and 100 μl for 250×10 mm I.D. columns. The wavelength of the UV detector was 254 nm and the determination of t_0 was carried out by use of sodium nitrate.

2.5.3. Separation of curcumin derivatives

Curcumin (Sigma C-1386, 1 mg/ml) was separated on columns packed with polystyrenic and polymethacrylic adsorbents of various particle sizes. The eluent was ACN–40 m*M* phosphoric acid (50:50). The flow-rates were 0.46 ml/min for 150×4.6 mm I.D. columns and 2.18 ml/min for 250×10 mm I.D. columns to apply the same linear velocity of 166 cm/h. Sample injection volumes were 12.5 μl for 150×4.6 mm I.D. columns and 100 μl for 250×10 mm I.D. columns. The wavelength used for UV detection was 250 nm.

2.5.4. Separation of soybean isoflavones

Isoflavones from soybeans were extracted by use of modified method of Obata et al. [6]. Dried soybeans (30 g) were milled and extracted with 150 ml of Milli-Q water for 2 h at 50°C. The extract solution was centrifuged at 4500 *g* for 10 min, and the supernatant was applied to an Econo-Column (200×15 mm I.D., Bio-Rad Labs, Hercules, CA, USA) packed with 6.0 g of DIAION HP20 (Mitsubishi Chemical Corporation) industrial polystyrenic adsorbent. The column was washed with 100 ml of Milli-Q water and 50 ml of 20% aqueous EtOH, and the bound materials were eluted from the column with 50 ml of 80% aqueous EtOH.

The eluted solution was separated on columns packed with polystyrenic adsorbents of various particle sizes. To identify the elution peaks separated on them, each isoflavone (1 mg/ml each) was chromatographed on an analytical column (150×4.6 mm I.D.) packed with adsorbent of 10 μm diameter. Eluent was MeOH–100 m*M* ammonium acetate (80:20), and the flow-rates were 0.46 ml/min for 150×4.6 mm I.D. column and 2.18 ml/min for 250×10 mm I.D. columns to apply the same linear velocity of 166 cm/h unless otherwise noted. Sample injection volumes were 12.5 μl for 150×4.6 mm I.D. column and 100 μl for 250×10 mm I.D. columns. The wavelength used for UV detection was 254 nm.

2.5.5. Preparative separation of isoflavones

Polystyrenic adsorbent of 18 μm was packed into a Millipore Vantage L VL32×500 (500×32 mm I.D.) column. The packed bed height of the column was 465 mm and the column volume was 374 ml. MeOH–100 m*M* ammonium acetate (80:20) was used as eluent, and the flow-rate was 7.48 ml/min to apply the linear velocity of 56 cm/h. A 37.4-ml sample was applied. The wavelength used for UV detection was 254 nm.

Each fraction collected was analyzed by HPLC and the content of each isoflavone was determined. To reconfirm the each isoflavone content, an ODS-2 (150×4.6 mm I.D., GL Science, Tokyo, Japan) column that has the different retention factors of isoflavones from those of polystyrenic adsorbent was used for HPLC analysis.

2.5.6. Separation of catechin derivatives

Polyphenon 60 (extract of green tea leaves, 10 mg/ml) was separated on columns packed with polystyrenic adsorbents and polymethacrylic adsorbents of various particle sizes, respectively. To identify the elution peaks separated on both adsorbents, catechin derivatives and caffeine (1 mg/ml each) were chromatographed on analytical columns (150×4.6 mm I.D.) packed with both adsorbents of 10 μm in diameter.

Eluent was MeOH–10 m*M* acetic acid (35:65) or MeOH–10 m*M* acetic acid (40:60) for polystyrenic adsorbents, and MeOH–10 m*M* acetic acid (60:40) for polymethacrylic adsorbents. The flow-rates were 0.46 ml/min for 150×4.6 mm I.D. columns and 2.18 ml/min for 250×10 mm I.D. columns to apply the same linear velocity of 166 cm/h. Sample injection volumes were 10 μl for 150×4.6 mm I.D. columns and 47 μl for 250×10 mm I.D. columns, respectively. The wavelength used for UV detecion was 280 nm.

2.5.7. Preparative separation of catechin derivatives

Polyphenon 60 (extract of green tea leaves) was separated on columns packed with polystyrenic and polymethacrylic adsorbents of various particle sizes, respectively.

Polystyrenic adsorbents of 18 μm and 30 μm were packed into Millipore Vantage L VL32×500 (500×32 mm I.D.) columns. The packed bed height of each column was 465 mm and the column volume was 374 ml. The flow-rate was 7.48 ml/min to apply the linear velocity of 56 cm/h. Stepwise gradient elution system consisted of MeOH–10 m*M* acetic acid

(35:65) and MeOH–10 m*M* acetic acid (50:50) was adopted for polystyrenic adsorbents. Various sample concentrations and injection volumes were applied. The wavelength used for UV detection was 280 nm.

Polymethacrylic adsorbent of 31 μm was packed into a 500×30 mm I.D. glass column (Kyoshin kogyo, Tokyo, Japan). The column volume was 353 ml. Isocratic elution with MeOH–10 m*M* acetic acid (60:40) was operated for polymethacrylic adsorbent. The flow-rate was 6.48 ml/min for polymethacrylic adsorbent to apply the linear velocity of 56 cm/h. As for sample injection, 1.63 ml of 100 mg/ml sample solution was applied to polymethacrylic adsorbent.

Furthermore, polystyrenic adsorbent (30 μm) was packed into a Millipore Vantage A2 VA90×500 (500×90 mm I.D.) column. Axial compression packing was carried out under an air pressure of 0.25 MPa. The packed bed height of the column was 435 mm and the column volume was 2770 ml. The flow-rate was 55 ml/min to apply the linear velocity of 52 cm/h. Stepwise gradient elution system consisted of MeOH–10 m*M* acetic acid (35:65) and MeOH–10 m*M* acetic acid (50:50) was adopted. A 140-ml volume of a 20-mg/ml sample solution was applied and chromatographed. The wavelength used for UV detection was 280 nm.

Each fraction was collected and analyzed by HPLC to determine the content of each catechin derivative. To reconfirm each content, an ODS-2 column that has a different selectivity for catechin derivatives from that of both polystyrenic and polymethacrylic adsorbents was used for HPLC analysis.

3. Results and discussion

3.1. Hydraulic properties

Figs. 1 and 2 show the relationship between the linear velocity of water and the pressure drop of polystyrenic and polymethacrylic adsorbents with fine-particle grades, respectively. All of the adsorbents show a linear relationship up to 1 MPa/m. These results prove the mechanical stability of those adsorbents when applied to preparative chromatographic operations.

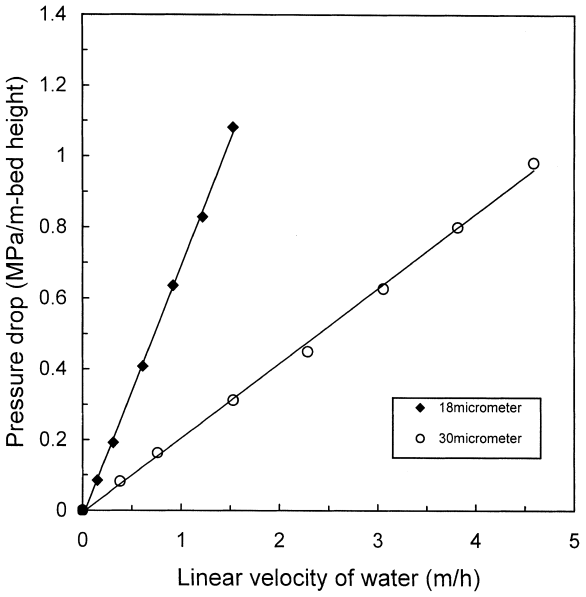

Fig. 1. Relationship between linear velocity of water and pressure drop of fine-grade polystyrenic adsorbents. (Temperature: 25°C).

3.2. Separation of dialkyl phthalates

Separation of dialkyl phthalate mixtures shows the

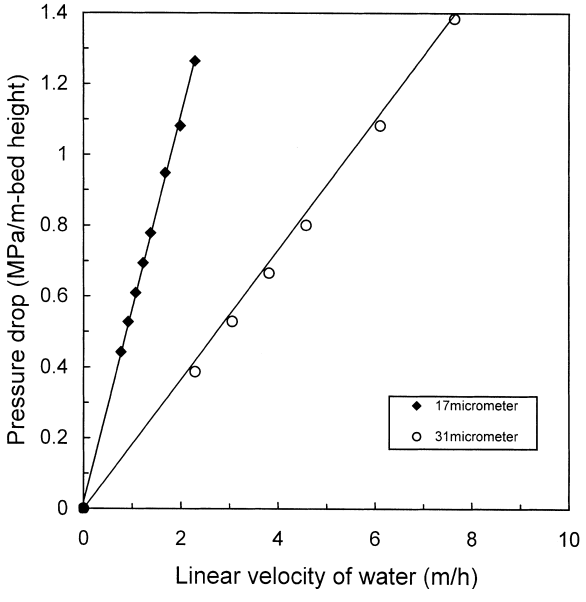

Fig. 2. Relationship between linear velocity of water and pressure drop of fine-grade polymethacrylic adsorbents. (Temperature: 25°C).

basic characteristics of polystyrenic and polymethacrylic adsorbents of various particle sizes, respectively. Fig. 3 shows the separation of dimethyl phthalate, dipropyl phthalate and dibutyl phthalate on polystyrenic adsorbents with various particle sizes. All the chromatograms show the same elution profile, and the resolution becomes higher with smaller particle diameter of the adsorbents. It should be

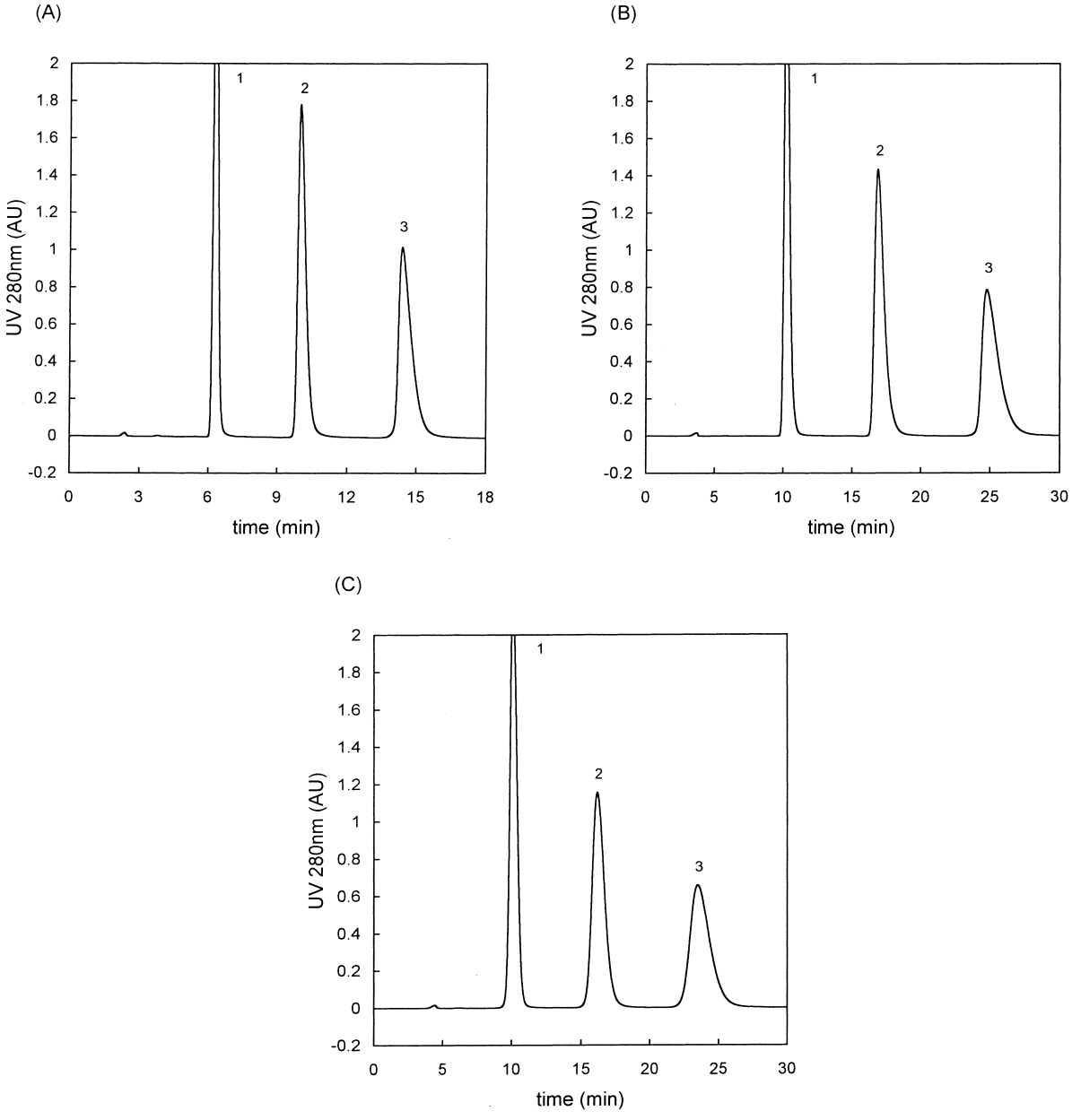

Fig. 3. Separation of dialkyl phthalates on polystyrenic adsorbents with various particle sizes. (A): 10 μm, (B): 18 μm, (C): 30 μm. Conditions: column, 150×4.6 mm I.D. for (A), 250×10 mm I.D. for (B) and (C); eluent, ACN–water (80:20); linear velocity, 166 cm/h. Samples: 1, dimethyl phthalate; 2, dipropyl phthalate; 3, dibutyl phthalate (5 mg/ml each). Injection: 12.5 μl for (A); 100 μl for (B) and (C).

noted that the resolution of analytical adsorbent seems to be lower in appearance, because the column length is shorter (150 mm) than that of adsorbents with fine grades (250 mm). Capacity factors of the dialkyl phthalates for adsorbents nos. 1–6 were plotted in Fig. 4. From Fig. 4, it is revealed that these polystyrenic adsorbents with various particle sizes show almost the same capacity factors in spite of the difference in porosity. It is supposed as below. The molecular mass of the dialkyl phthalates chromatographed in this study is relatively small (less than 300). On the other hand, the pore diameter of the adsorbents is larger than about 30 nm. Therefore, the effective surface area for interacting with these dialkyl phthalates does not differ so much among the adsorbents, and the capacity factors might be almost the same. But to the larger compounds like polypeptides or proteins, the porosity difference among the adsorbents should affect the capacity factors. In this respect, further investigation must be carried out.

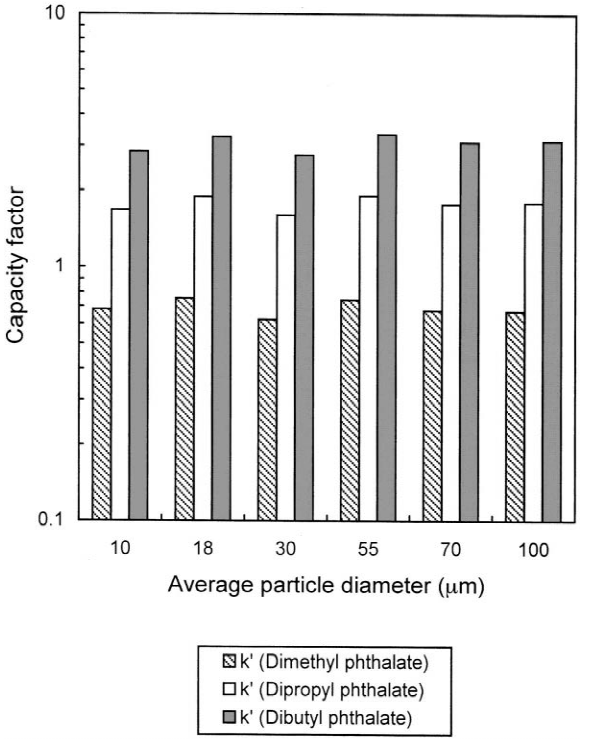

Fig. 4. Capacity factors of dimethyl phthalate, dipropyl phthalate and dibutyl phthalate for polystyrenic adsorbents with various average particle diameters. Conditions: column, 150×4.6 mm I.D. for adsorbent no. 1, 250×10 mm I.D. for adsorbents nos. 2–6; eluent, ACN–water (80:20); linear velocity, 166 cm/h. Samples: dimethyl phthalate, dipropyl phthalate and dibutyl phthalate (5 mg/ml each). Injection: 12.5 μl for adsorbent no.1; 100 μl for adsorbents nos. 2–6.

Fig. 5 also shows the separation of dialkyl phthalates on polymethacrylic adsorbents with various particle sizes. In this case, the same elution profile is also obtained and the resolution becomes higher with smaller particle diameter of the adsorbents. In Fig. 6, the relationship between acetonitrile concentration and capacity factor of dibutyl phthalate on polymethacrylic adsorbents with various particle sizes is described. This result also shows the same retentivity of these adsorbents. It should be noted that the lower acetonitrile concentration was adopted to obtain relatively same retention as in the case of polystyrenic adsorbents. This means that polymethacrylic adsorbents fundamentally have weaker retentivity than polystyrenic ones.

3.3. Separation of curcumin derivatives

Curcumin and its derivatives are main constituents of turmeric (*Curcuma longa*) [7]. Not only for food coloring or flavoring use, turmeric begins to be used for functional food additive or pharmaceutical use because of the clinical effects of curcumin [8,9].

Separations of reagent-grade curcumin (Sigma C-1386) on analytical columns packed with polystyrenic and polymethacrylic adsorbents of 10 μm are indicated in Fig. 7. In both cases, there are three major peaks, but the elution profiles are reversed. On the polystyrenic adsorbent, curcumin eluted as the last peak, and this observation coincided with the result of Taylor and McDowell [10]. The first and the second peaks were thought to be bisdemethoxycurcumin and demethoxycurcumin, respectively. The same elution order was observed on the separation on YMC-Pak ProC_{18} (150×4.6 mm I.D., YMC, Kyoto, Japan, data not shown). On the other hand, polymethacrylic adsorbents eluted curcumin as the first main peak, and the elution order of curcumins was reversed. From these selectivity differences, it should be mentioned that both adsorbents could be used complimentarily. This means that if one of these adsorbents shows poor resolution against certain compounds, another adsorbent might

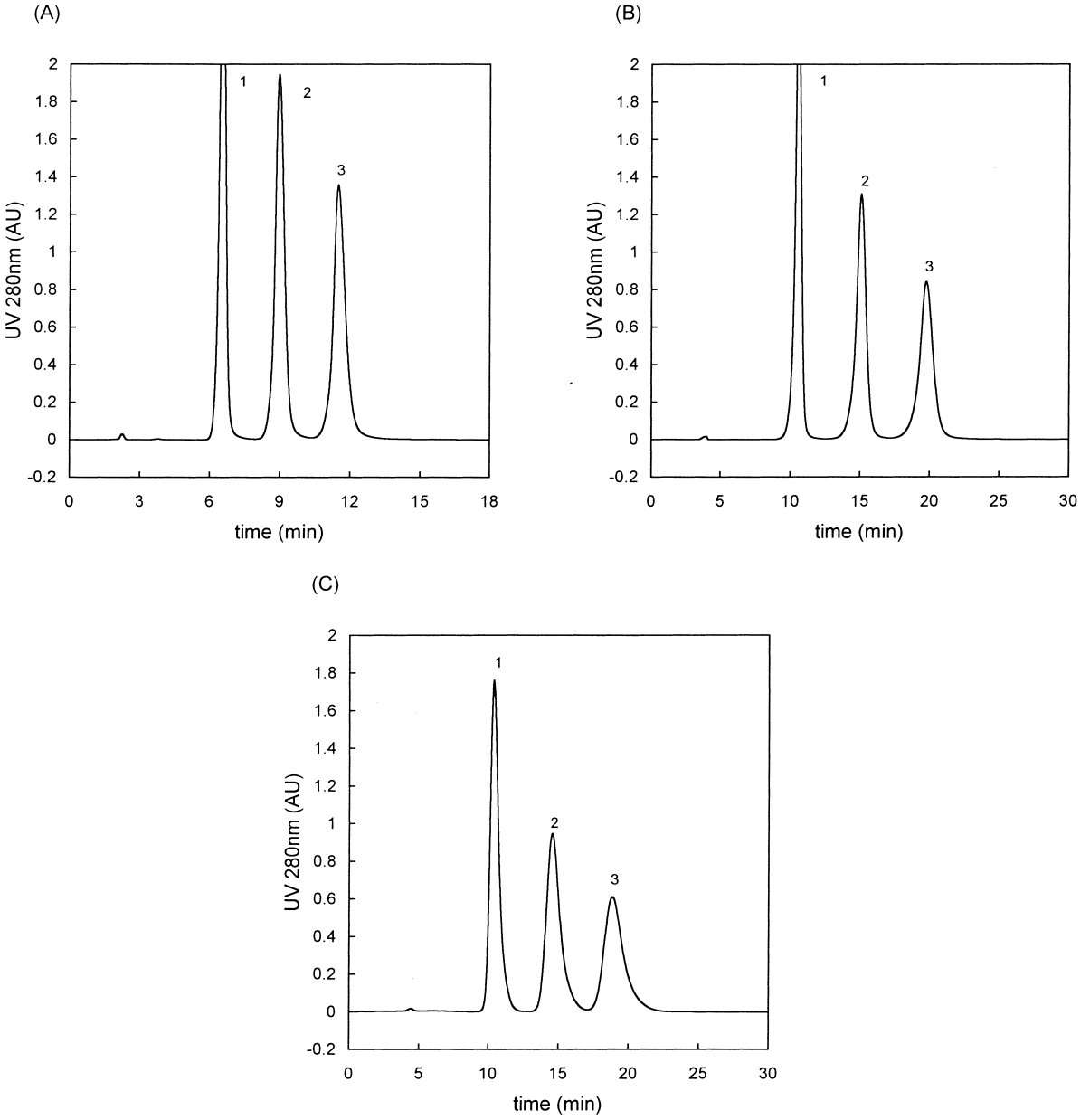

Fig. 5. Separation of dialkyl phthalates on polymethacrylic adsorbents with various particle sizes. (A): 10 μm, (B): 17 μm, (C): 31 μm. Conditions: column, 150×4.6 mm I.D. for (A), 250×10 mm I.D. for (B) and (C); eluent, ACN–H_2O (60:40); linear velocity, 166 cm/h. Samples: 1, dimethyl phthalate; 2, dipropyl phthalate; 3, dibutyl phthalate (5 mg/ml each). Injection: 12.5 μl for (A); 100 μl for (B) and (C).

be able to give desired resolution because of the reversed selectivity.

Chromatograms of curcumin derivatives on polymethacrylic adsorbents with various particle sizes are depicted in Fig. 8. It could be said that, if the elution conditions were established by use of analytical-grade adsorbent, the same retention behavior could be easily achieved at preparative scales by use of the

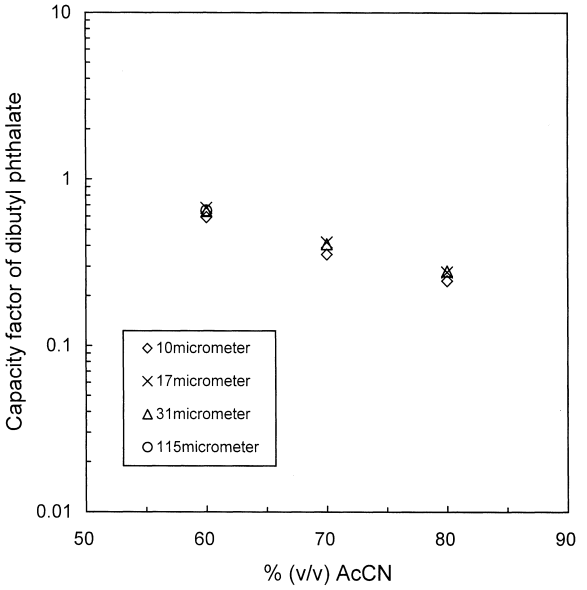

Fig. 6. Relationship between acetonitrile concentration and capacity factor of dibutyl phthalate on polymethacrylic adsorbents with various average particle diameters. Conditions: column, 150×4.6 mm I.D. for adsorbent no. 9, 250×10 mm I.D. for adsorbents nos. 10–12; eluent, ACN–water; linear velocity, 166 cm/h. Sample: dibutyl phthalate (5 mg/ml). Injection: 12.5 μl for adsorbent no. 9; 100 μl for adsorbents nos. 10–12.

fine-grade adsorbents with the same elution conditions.

3.4. Separation of soybean isoflavones

Recently, isoflavones have become more attractive compounds because they have many biological effects such as cancer inhibition [11] or antioxidant [12]. The main constituents of soybean isoflavones are daidzin and genistin, that is to say, glycosides of daidzein and genistein, respectively [13]. The content of isoflavones is relatively low, so the concentration step by industrial-grade synthetic adsorbent was adopted prior to the chromatographic separation. Dried soybeans were milled and extracted with Milli-Q water for 2 h at 50°C. The extracted solution was centrifuged at 4500 *g* for 10 min, but the supernatant was not transparent after this step. Then the supernatant was applied to a DIAION HP20 (industrial-grade polystyrenic adsorbent) column, and the column was washed with Milli-Q water until the eluate

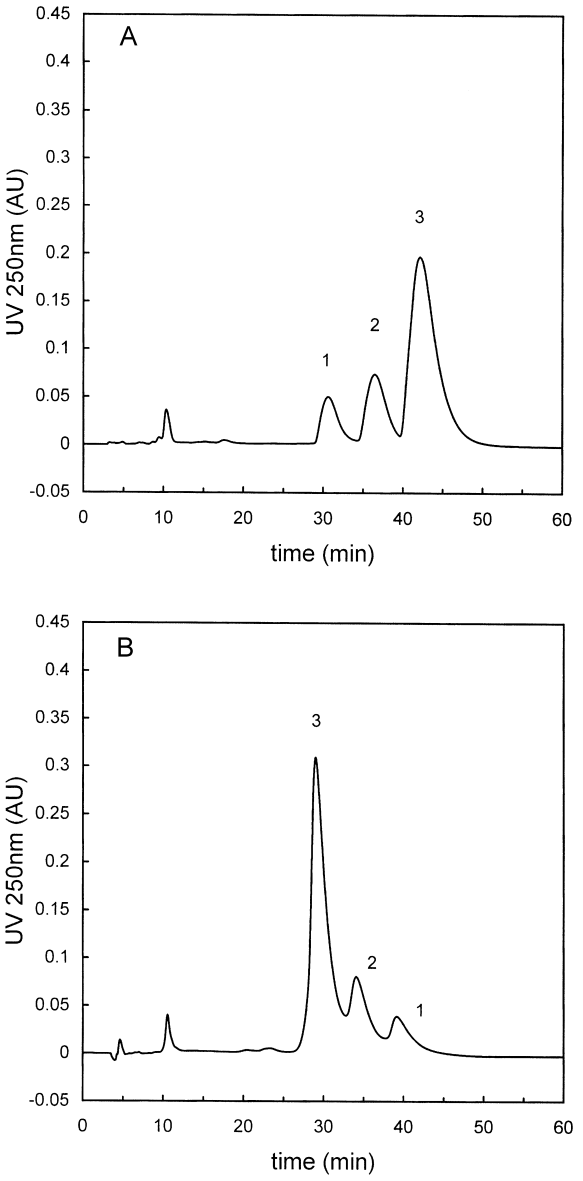

Fig. 7. Analytical separation of curcumin derivatives on polystyrenic and polymethacrylic adsorbents. (A): polystyrenic adsorbent of 10 μm, (B): polymethacrylic adsorbent of 10 μm. Conditions: column, 150×4.6 mm I.D.; eluent, ACN–0.04*M* phosphoric acid (50:50); flow-rate, 0.46 ml/min. Sample: curcumin (Sigma C-1386, 1 mg/ml). Injection: 12.5 μl. Peak identification: 1, bisdemethoxycurcumin; 2, demethoxycurcumin; 3, curcumin.

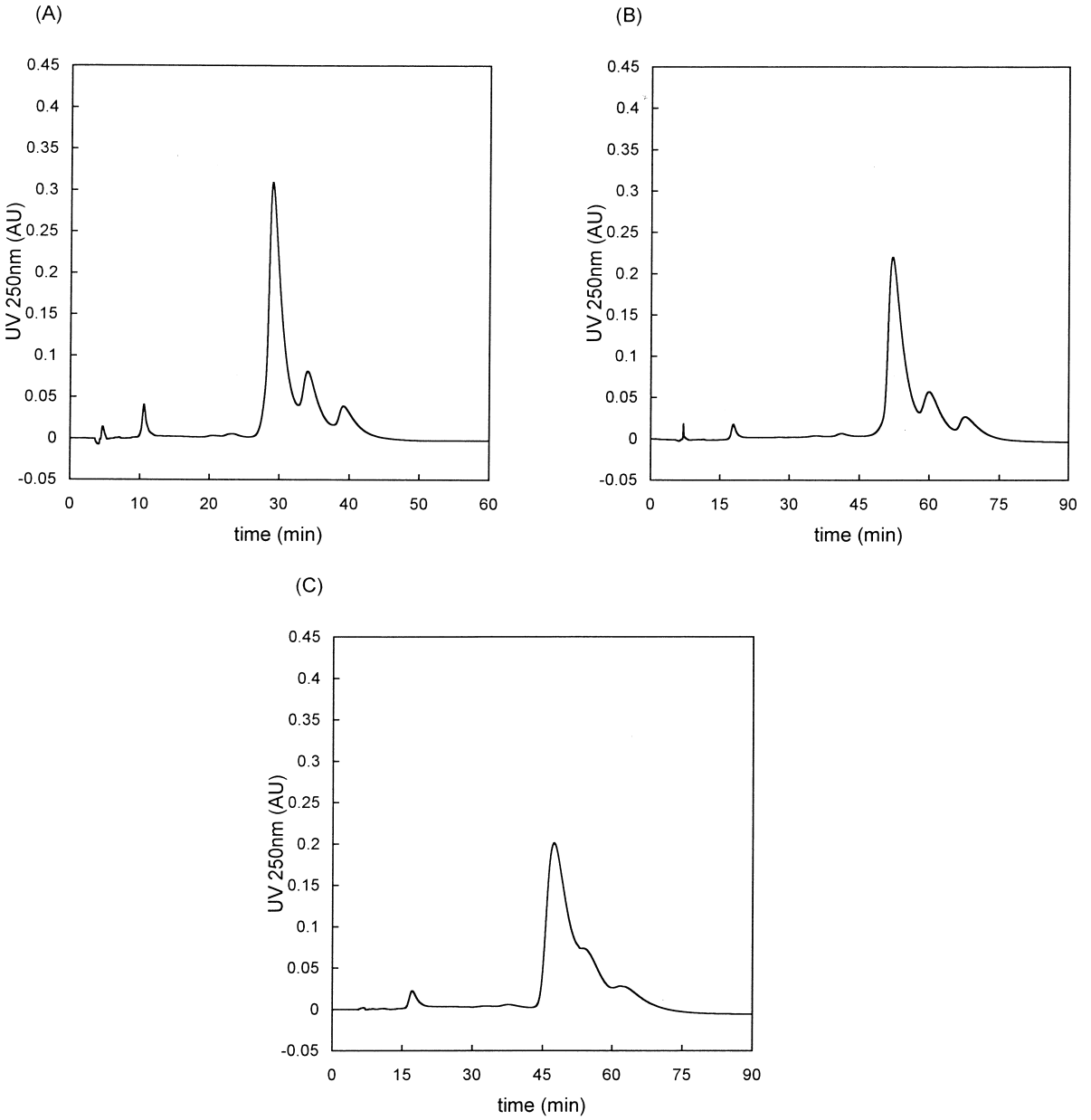

Fig. 8. Chromatographic separation of curcumin derivatives on polymethacrylic adsorbents with various particle sizes. (A): 10 μm, (B): 17 μm, (C): 31 μm. Conditions: column, 150×4.6 mm I.D. for (A), 250×10 mm I.D. for (B) and (C); eluent, ACN–0.04*M* phosphoric acid (50:50); linear velocity, 166 cm/h. Sample: curcumin (Sigma C-1386, 1 mg/ml). Injection: 12.5 μl for (A); 100 μl for (B) and (C).

became transparent. The HP20 column was colored yellow after the adsorption step. Desorption of bound materials was done by two-step elution. The effluent of 20% EtOH showed yellowish color, but the effluent of 80% EtOH had slightly pink color.

Separation of the effluent of 80% EtOH on analytical polystyrenic adsorbent of 10 μm is shown in Fig. 9 (in this case, the flow-rate was 1.00 ml/min). Compared with the chromatograms of daidzein and genistein, it could be said that the effluent of

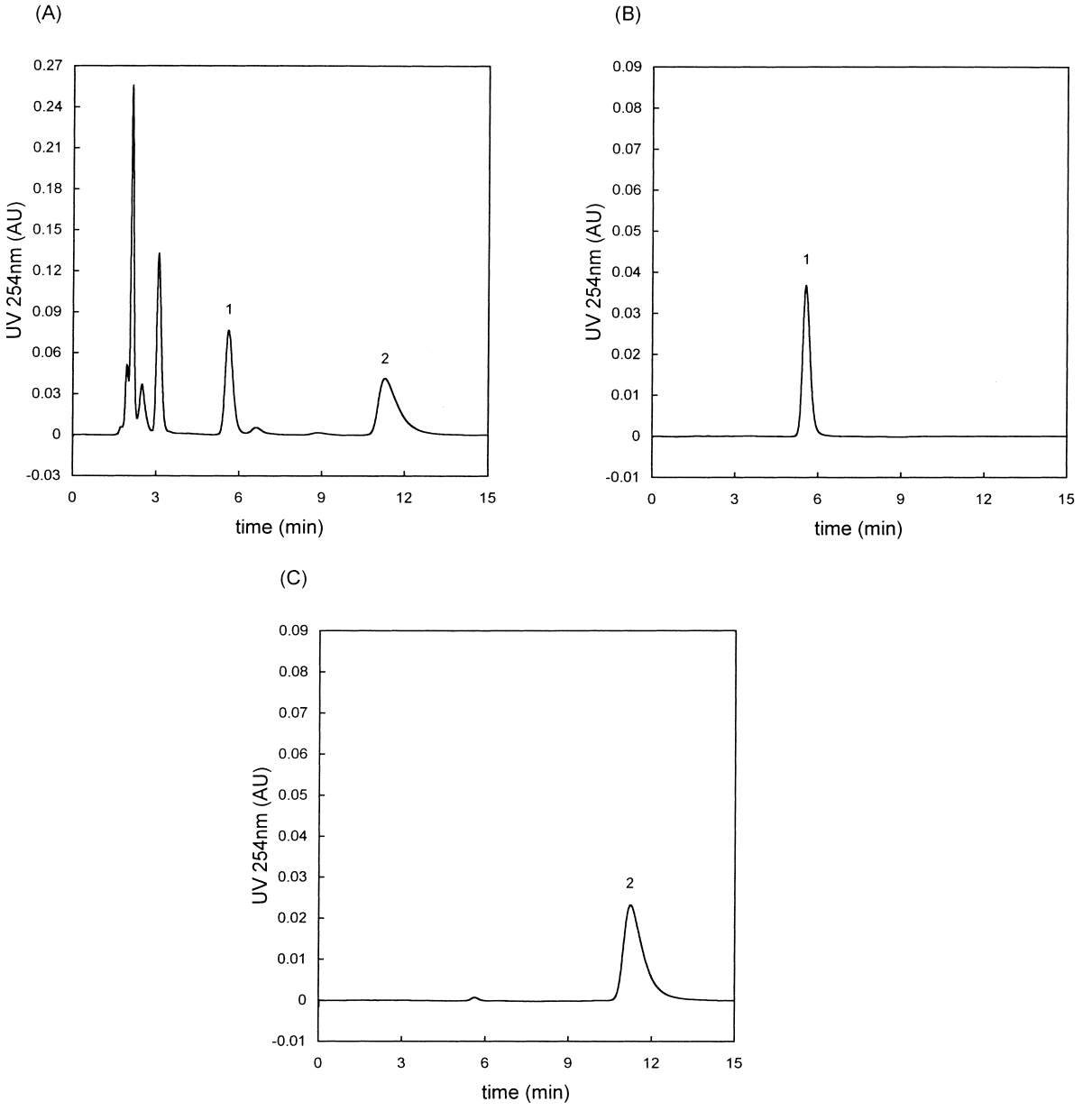

Fig. 9. Chromatograms of soybean crude extract and isoflavones on analytical-grade polystyrenic adsorbent. Conditions: column, 150×4.6 mm I.D.; eluent, MeOH–0.1*M* ammonium acetate (80:20); flow-rate, 1.00 ml/min. Samples: (A), effluent of 80% EtOH from HP20 column; (B), daidzein (100 μg/ml); (C), genistein (100 μg/ml). Injection: 10 μl for (A); 1.0 μl for (B) and (C). Peak identification: 1, daidzein; 2, genistein.

80% EtOH contained daidzein and genistein, and these isoflavones were able to be separated on polystyrenic adsorbent. From Fig. 9, it was recognized that the content of daidzein and genistein was relatively high, though the content of them is low in dried soybeans [13]. The reason of this observation was thought by Obata et al. that daidzin and genistin, the major constituents of isoflavones, were converted

to daidzein and genistein by β-glucosidase in dried soybeans during the extraction [6]. Fig. 10 shows the separation of the effluent of 80% EtOH on polystyrenic adsorbents with various particle sizes. The same elution profile of these adsorbents proved the usefulness of them for the purification of natural

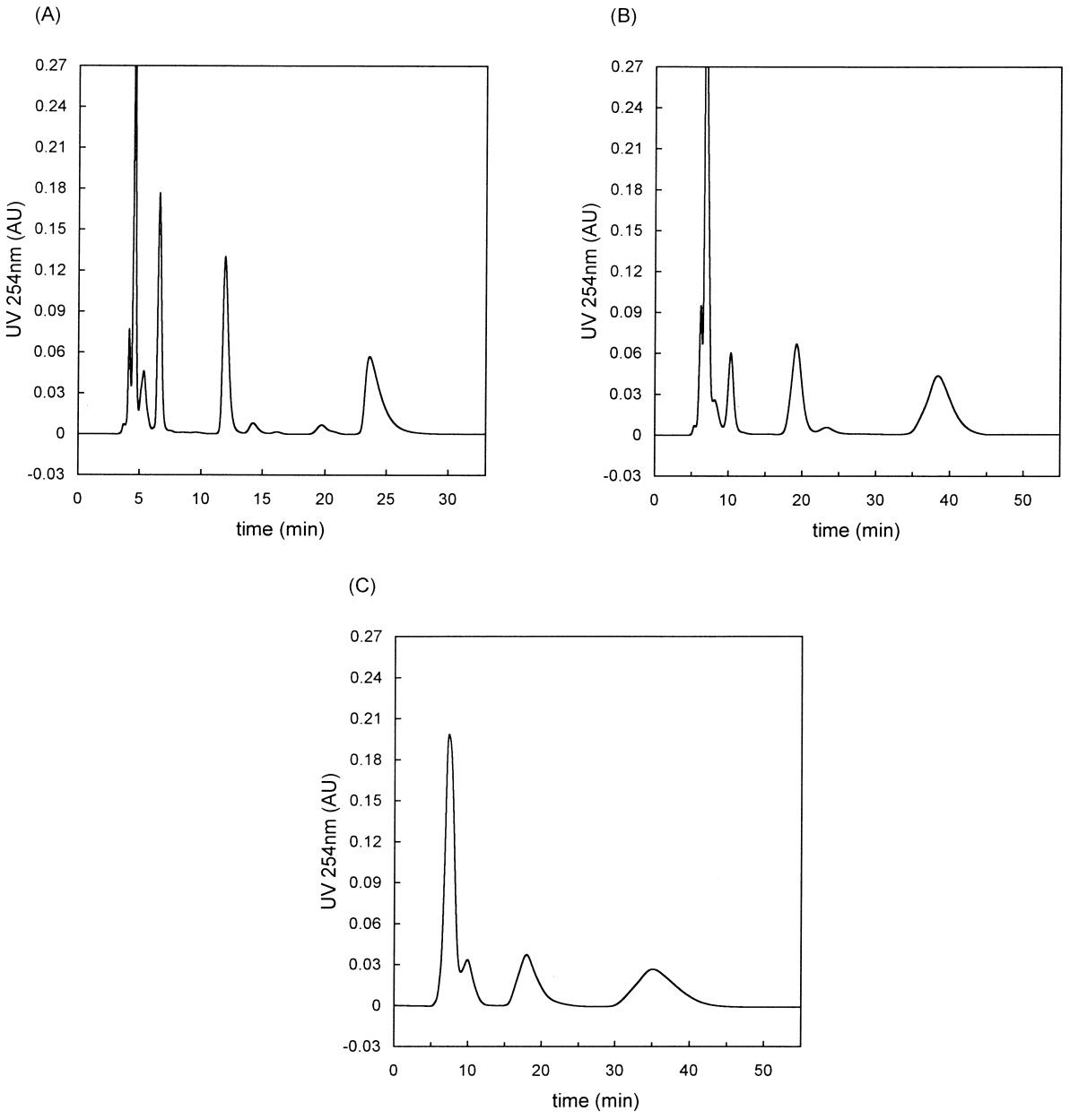

Fig. 10. Chromatographic separation of soybean crude extract on polystyrenic adsorbents with various particle sizes. (A): 10 μm, (B): 18 μm, (C): 30 μm. Conditions: column, 150×4.6 mm I.D. for (A), 250×10 mm I.D. for (B) and (C); eluent, MeOH–0.1 *M* ammonium acetate (80:20); linear velocity, 166 cm/h. Sample: effluent of 80% EtOH from HP20 column. Injection: 12.5 μl for (A); 100 μl for (B) and (C).

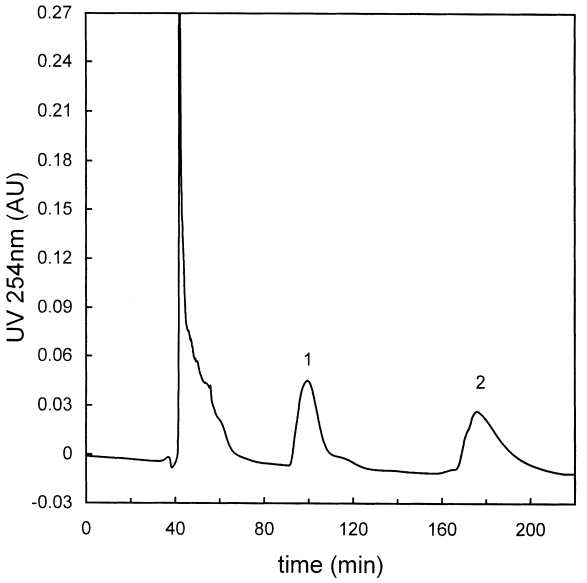

Fig. 11. Preparative chromatographic separation of soybean crude extract on polystyrenic adsorbent with fine-grade particle size of 18 μm. Conditions: column, 465×32 mm I.D.; eluent, MeOH–0.1 *M* ammonium acetate (80:20); flow-rate, 7.48 ml/min. Sample: effluent of 80% EtOH from HP20 column. Injection: 37.4 ml. Peak identification: 1, daidzein; 2, genistein.

products from analytical to semi-preparative scales with consistency.

3.5. Preparative separation of isoflavones

Preparative separation of crude extract of soybeans operated on columns packed with polystyrenic adsorbents of 18 μm was shown in Fig. 11. The same eluent of MeOH–100 m*M* ammonium acetate (80:20) as used for analytical and semi-preparative separation was used. The flow-rate was reduced to give one third of linear velocity compared to analytical and semi-preparative conditions because of the low maximum pressure of the glass column. Compared to analytical and semi-preparative conditions, a double amount of sample per column bed volume was applied, but the separation profile was maintained. From this result, it is revealed that the fine-particle polystyrenic adsorbents can be applied for the precise separation of bioactive compounds with the same elution conditions developed by the use of a column packed with analytical-grade adsorbent made of the same chemistry.

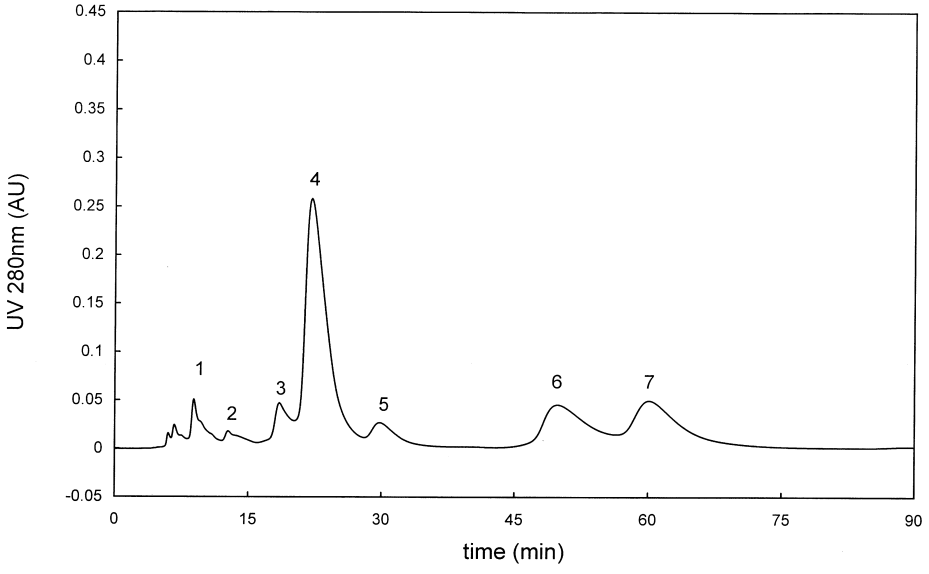

Fig. 12. Separation of tea extract on a column packed with analytical polystyrenic adsorbent of 10 μm. Conditions: column, 150 mm×4.6 mm I.D.; eluent, MeOH–0.01*M* acetic acid (35:65); flow-rate, 0.46 ml/min. Sample: Polyphenon 60 (10 mg/ml). Injection: 10 μl. Peak identification: 1, (−)-epigallocatechin; 2, (+)-catechin; 3, (−)-epicatechin; 4, (−)-epigallocatechin gallate; 5, (−)-gallocatechin gallate; 6, caffeine; 7, (−)-epicatechin gallate.

3.6. Separation of catechin derivatives

Catechin and its derivatives are one of the groups of natural polyphenol compounds and they are much contained in the leaves of green tea [14]. An increasing attention has been given to the effects of catechin derivatives on human health [15,16]. Therefore, there seems to be more demand for the purification of individual catechin derivatives.

Fig. 12 shows the separation of Polyphenon 60 on

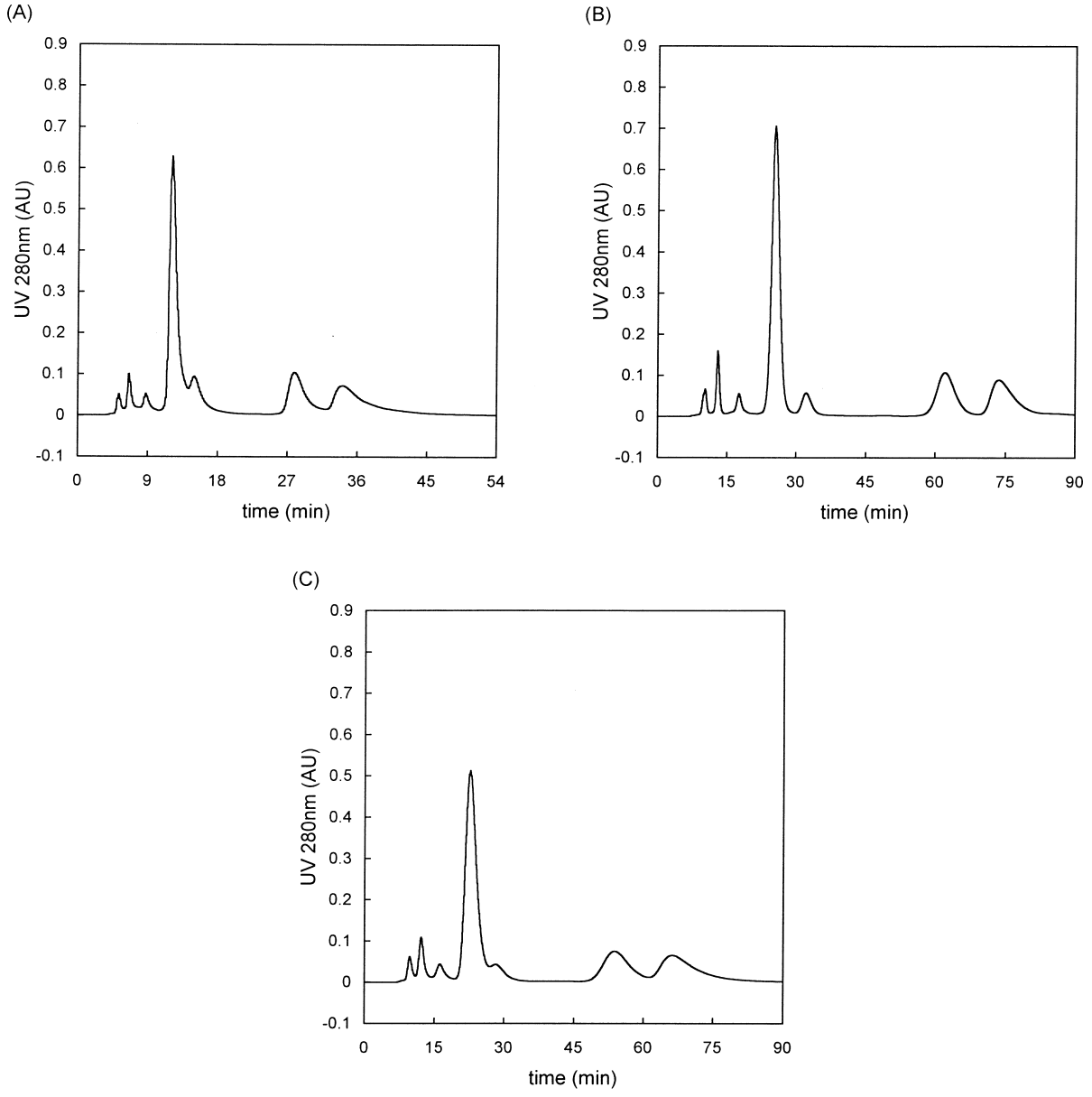

Fig. 13. Chromatographic separation of tea extract on polystyrenic adsorbents with various particle sizes. (A): 10 μm, (B): 18 μm, (C): 30 μm. Conditions: column, 150×4.6 mm I.D. for (A), 250×10 mm I.D. for (B) and (C); eluent, MeOH–0.01*M* acetic acid (40:60); linear velocity, 166 cm/h. Sample: Polyphenon 60 (10 mg/ml). Injection: 10 μl for (A); 47 μl for (B) and (C).

analytical polystyrenic adsorbent. It was found that (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate, (−)-gallocatechin gallate, caffeine and (−)-epicatechin gallate were well separated on polystyrenic adsorbent using the eluent of MeOH–10 m*M* acetic acid (35:65). Not only this eluent consists of common and cost-effective chemicals, but also both MeOH and acetic acid are easy to remove. The effect of particle sizes of polystyrenic adsorbents on separation is shown in Fig. 13. In these cases, eluent of MeOH–10 m*M* acetic acid (40:60) was used. Identical separation profile proved the consistency and scalability of these adsorbents.

The separation of Polyphenon 60 on analytical polymethacrylic adsorbent is shown in Fig. 14. In this case, retention of catechin derivatives was longer than in the case of polystyrenic adsorbents under the same elution system (data not shown), so the content of organic modifier was increased to MeOH–10 m*M* acetic acid (60:40). Usually, retention of the compounds on polystyrenic adsorbents is longer than that on polymethacrylic ones, so this result seems to be a specific case for the combination of polymethacrylic adsorbents and polyphenolic compounds. Moreover, the selectivity of caffeine was considerably different. The reason of those results might be attributed to the effect of hydrogen bonding, but further investigation must be required to clarify the retention difference between polystyrenic and polymethacrylic adsorbents. The scalability of polymethacrylic adsorbents with various particle sizes was also recognized from Fig. 15.

With regard to resolution under this elution condition, analytical polymethacrylic adsorbent could not separate (+)-catechin and (−)-epicatechin. Furthermore, (−)-gallocatechin gallate and (−)-epicatechin gallate could not be separated. But it cannot be concluded that polymethacrylic adsorbent has a resolution performance inferior to polystyrenic adsorbent, because the optimum separation conditions differs for each adsorbent or each compound to be separated. Scale-up separation of (−)-epigallocatechin gallate on both adsorbents will be discussed later.

3.7. Preparative separation of catechin derivatives

To estimate the capability of fine-grade synthetic adsorbents for preparative purification of bioactive compounds such as pharmaceuticals or functional

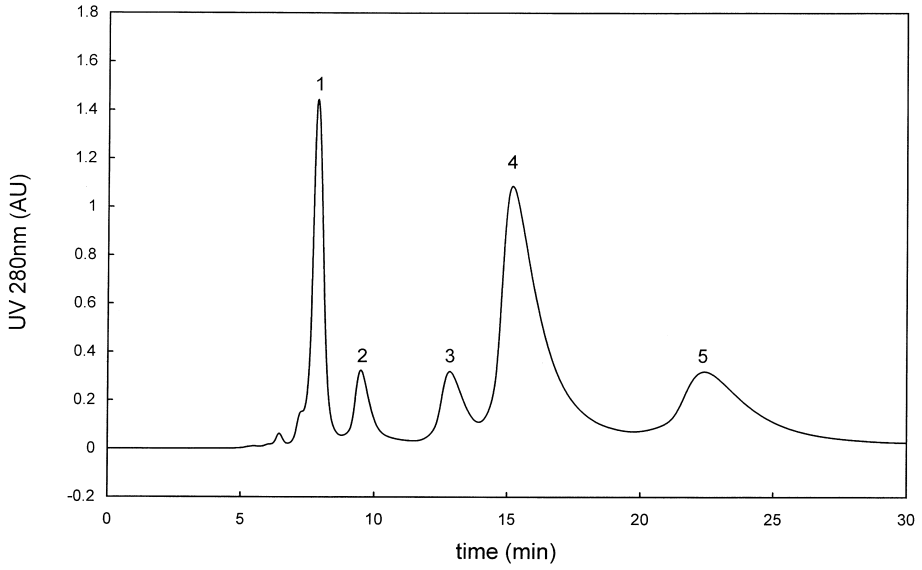

Fig. 14. Separation of tea extract on a column packed with analytical polymethacrylic adsorbent of 10 μm. Conditions: column, 150 mm×4.6 mm I.D.; eluent, MeOH–0.01*M* acetic acid (60:40); flow-rate, 0.46 ml/min. Sample: Polyphenon 60 (10 mg/ml). Injection: 12.5 μl. Peak identification: 1, caffeine; 2, (−)-epigallocatechin; 3, (+)-catechin and (−)-epicatechin; 4, (−)-epigallocatechin gallate; 5, (−)-gallocatechin gallate and (−)-epicatechin gallate.

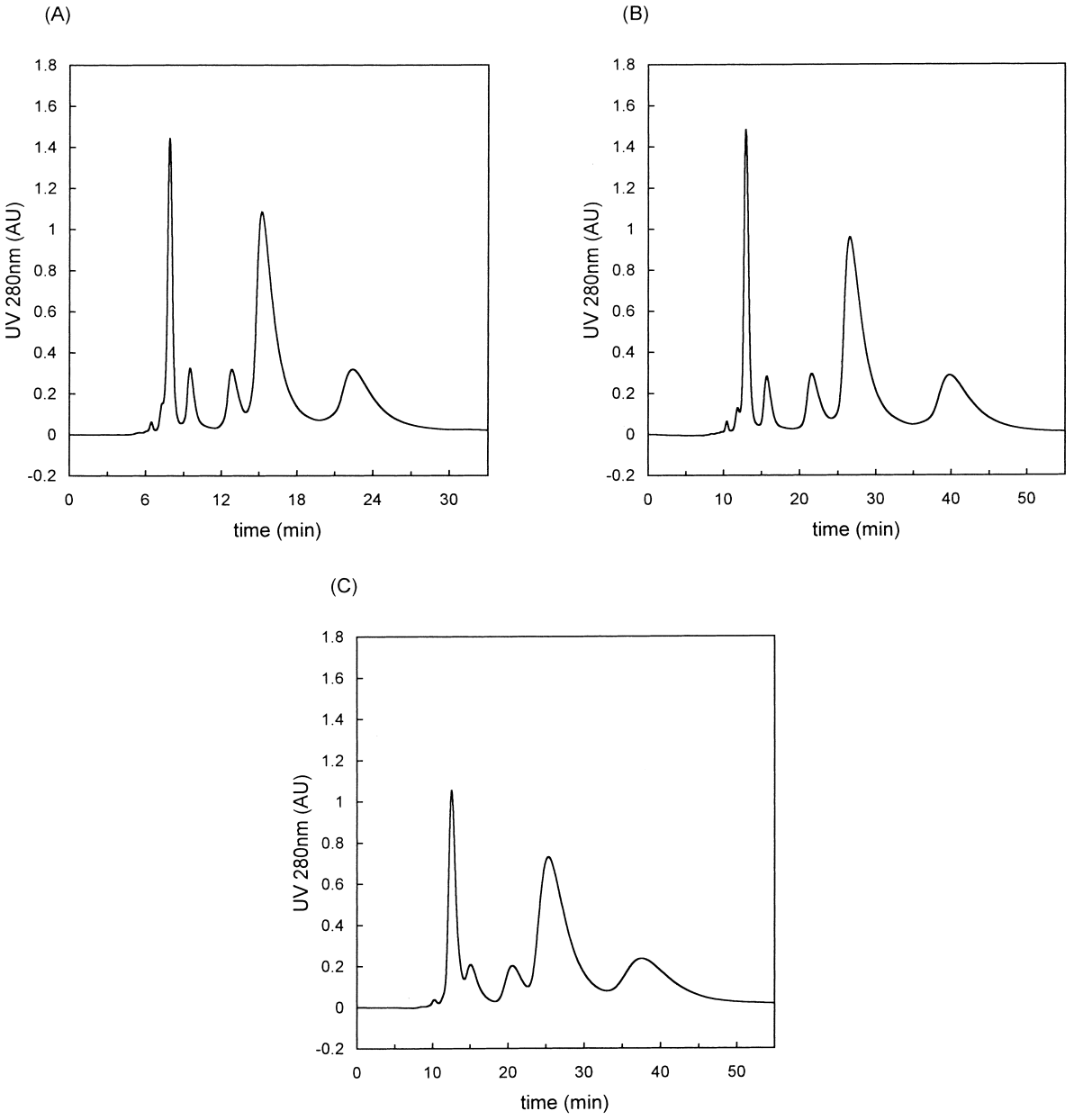

Fig. 15. Chromatographic separation of tea extract on polymethacrylic adsorbents with various particle sizes. (A): 10 μm, (B): 17 μm, (C): 31 μm. Conditions: column, 150×4.6 mm I.D. for (A), 250×10 mm I.D. for (B) and (C); eluent, MeOH–0.01*M* acetic acid (60:40); linear velocity, 166 cm/h. Sample: Polyphenon 60 (10 mg/ml). Injection: 12.5 μl for (A); 100 μl for (B) and (C).

food additives, (−)-epigallocatechin gallate was purified from Polyphenon 60.

Various elution conditions, including stepwise elution and loading amounts for fine-grade polystyrenic adsorbents, were investigated. Fig. 16 shows the optimized chromatogram on preparative column packed with polystyrenic adsorbent of 18 μm. In this case, stepwise elution from MeOH–10 m*M* acetic

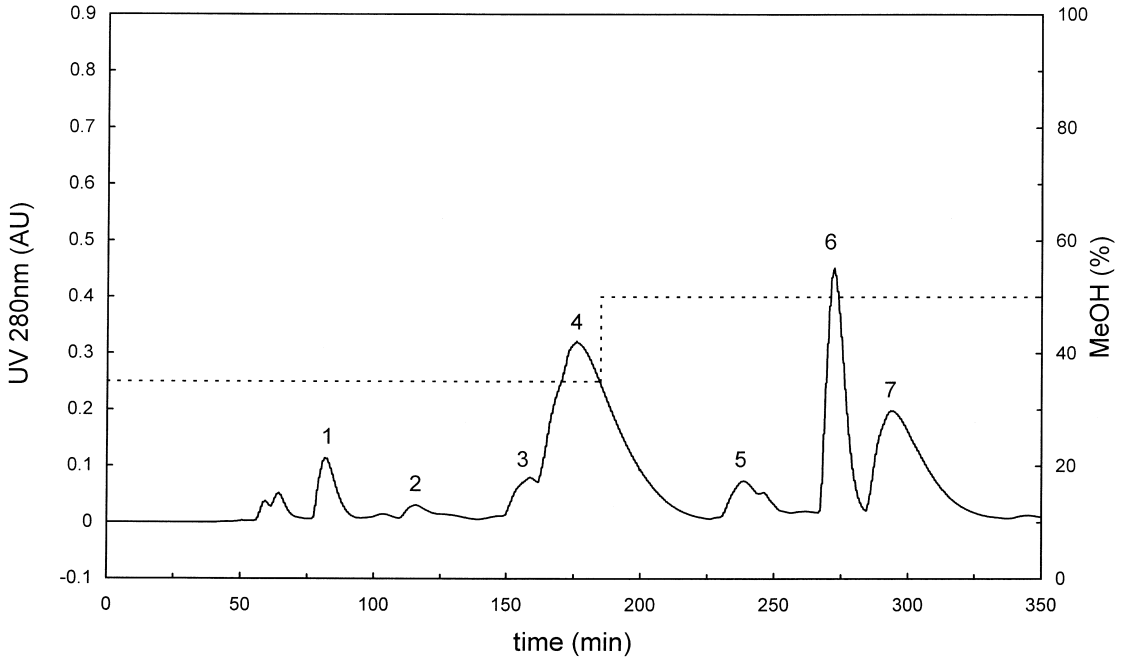

Fig. 16. Preparative separation of tea extract on a column packed with polystyrenic adsorbents of 18 μm. Conditions: column, 465 mm×32 mm I.D.; eluent, 0–185 min, MeOH–0.01*M* acetic acid (35:65); 185–350 min, MeOH–0.01 *M* acetic acid (50:50); flow-rate, 7.48 ml/min. Sample: Polyphenon 60 (10 mg/ml). Injection: 18.7 ml. Peak identification: 1, (−)-epigallocatechin; 2, (+)-catechin; 3, (−)-epicatechin; 4, (−)-epigallocatechin gallate; 5, (−)-gallocatechin gallate; 6, (−)-epicatechin gallate; 7, caffeine.

acid (35:65) to MeOH–10 m*M* acetic acid (50:50) was operated at 185 min to fasten the elution of (−)-epicatechin gallate and caffeine and to diminish the total time of purification. Note that the elution order of (−)-epicatechin gallate and caffeine was reversed compared to Fig. 12 because of the effect of stepwise elution. From the fraction analysis, it was found that (−)-epigallocatechin gallate was isolated with a purity of 99% and a recovery of 82%.

Preparative separation of (−)-epigallocatechin gallate on polymethacrylic adsorbent with 31 μm particle was operated under the same isocratic elution as operated on analytical or semi-preparative columns. The chromatogram is shown in Fig. 17. An almost identical elution profile was maintained in spite of the 9.2-fold amount of loading per column volume compared to analytical or semi-preparative separations. The (−)-epigallocatechin gallate purity of main fractions is also plotted in Fig. 17. Fractions with high purity were obtained. From Fig. 17, it can be said that fine-grade polymethacrylic adsorbents possess almost the same feasibility for the separation of bioactive compounds as polystyrenic ones.

Further scale-up preparative purification of (−)-epigallocatechin gallate was operated on polystyrenic adsorbent of 30 μm packed into a Millipore Vantage A2 VA90×500 (500×90 mm I.D.) column. The chromatogram shown in Fig. 18 demonstrates the identical elution profile to that in Fig. 16. As for loading amount, 0.05 column volume of 20 mg/ml solution was applied and that was twice that of the system described in Fig. 16. Fraction analysis showed that (−)-epigallocatechin gallate was isolated with a purity of 99% and a recovery of 61%. The recovery was lower than in the case of Fig. 16 because of the effect of the large particle diameter and the higher loading amount. But almost 50% improved throughput was obtained on account of the double loading amount.

Finally, scalability up to a 22 400-fold loading amount was achieved from a small column packed with analytical-grade adsorbent used for method

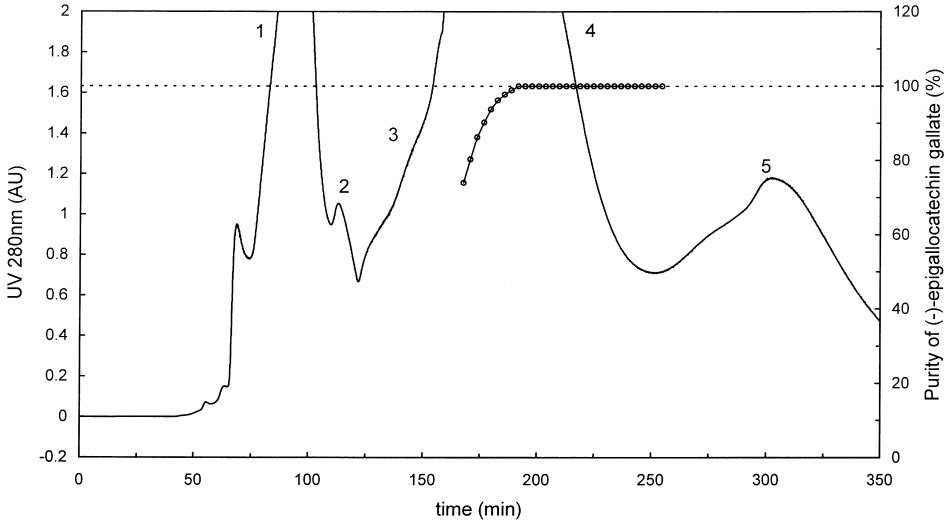

Fig. 17. Preparative separation of tea extract on a column packed with polymethacrylic adsorbents of 31 μm. Conditions: column, 500 mm×30 mm I.D.; eluent, MeOH–0.01*M* acetic acid (60:40); flow-rate, 6.54 ml/min. Sample: Polyphenon 60 (100 mg/ml). Injection: 1.63 ml. Peak identification: 1, caffeine; 2, (−)-epigallocatechin; 3, (+)-catechin and (−)-epicatechin; 4, (−)-epigallocatechin gallate; 5, (−)-gallocatechin gallate and (−)-epicatechin gallate.

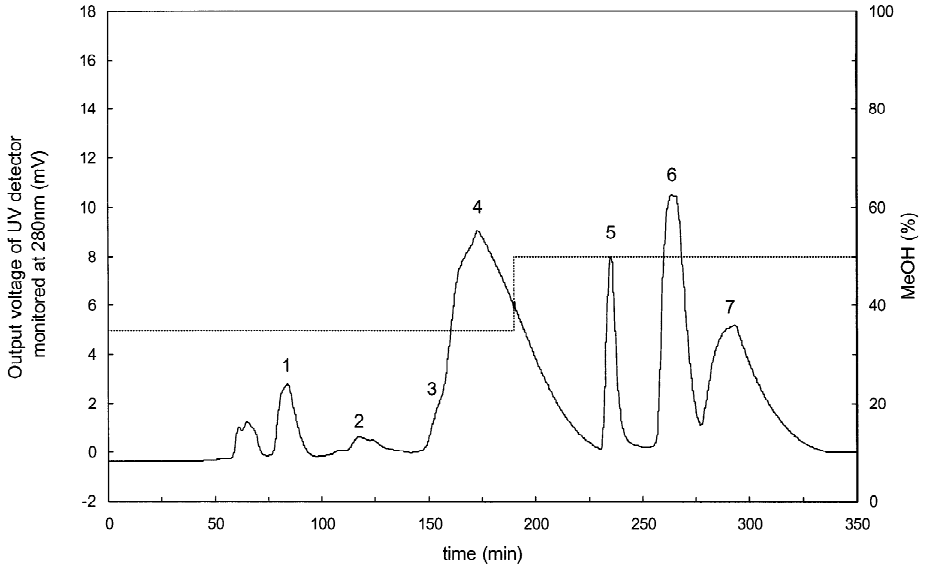

Fig. 18. Scale-up preparative separation of tea extract on a column packed with polystyrenic adsorbent of 30 μm. Conditions: column, 435 mm×90 mm I.D.; eluent, 0–195 min, MeOH–0.01*M* acetic acid (35:65); 195–350 min, MeOH–0.01*M* acetic acid (50:50); flow-rate, 55 ml/min. Sample: Polyphenon 60 (20 mg/ml). Injection: 140 ml. Peak identification: 1, (−)-epigallocatechin; 2, (+)-catechin; 3, (−)-epicatechin; 4, (−)-epigallocatechin gallate; 5, (−)-gallocatechin gallate; 6, (−)-epicatechin gallate; 7, caffeine.

development to a scale-up preparative column packed with fine-grade adsorbent used for preparative purification.

4. Conclusions

Newly manufactured synthetic adsorbents with fine-particle grades were characterized. The chemical structure of fine-grade polystyrenic or polymethacrylic adsorbents was the same as analytical- or industrial-grade adsorbents, respectively. Their porosity was almost identical to that of analytical- or industrial-grade adsorbents, too. Their hydraulic properties indicated that they had sufficient mechanical strength for use in preparative chromatographic operations. Chromatographic evaluations, including retention characteristics and scalability, revealed the consistency of those adsorbents with various particle sizes and the scalability from analytical-grade adsorbents to fine-grade adsorbents. Up to a 22 400-fold loading amount was achieved from a small column packed with analytical-grade adsorbent used for method development to a scale-up preparative column packed with fine-grade adsorbent used for preparative purification. In conclusion, it was found that the fine-grade synthetic adsorbents were exceedingly useful for more precise purification of bioactive compounds, including pharmaceuticals and functional food additives with higher recovery.

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