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Use of synthetic adsorbents in preparative normal-phase liquid chromatography

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Abstract

Normal-phase liquid chromatographic separation using polymeric synthetic adsorbents was investigated. The retention behavior of dialkyl phthalates under a hexane–isopropanol eluent system revealed that both polystyrenic and polymethacrylic adsorbents can be used for normal-phase liquid chromatography, and that the polymethacrylic adsorbent has stronger retentivity than the polystyrenic adsorbent. Applicability of these synthetic adsorbents, especially the polymethacrylic adsorbents, was verified by the separation of polyunsaturated fatty acids, schizandrin in *Schisandra chinensis* fruit, tocopherols and tocotrienols in vegetable oils using a 10-μm adsorbent packed into an HPLC column. Furthermore, the separation was expanded to preparative scale by using polymethacrylic adsorbents with 17- and 31-μm fine grade particle sizes. The effect of loading volume on the retention or separation was also examined, and separation was maintained up to the loading of several grams per liter adsorbent. These results demonstrate that commercially available synthetic adsorbents possess considerable performance as normal-phase chromatographic media for more precise purification of bioactive compounds, including pharmaceuticals and nutraceuticals with scale-up possibilities.
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Keywords: Preparative chromatography; Adsorbents; *Schisandra chinensis*; Vegetable oils; Dialkyl phthalates; Phthalates; Fatty acids; Schizandrin; Tocopherols; Tocotrienols

1. Introduction

For a few decades, it has been known that polymer-based synthetic adsorbents could be used as normal-phase liquid chromatographic support materials [1]. However, most researchers usually use silica gels in the early stages of normal-phase liquid chromatographic separation of natural products from fermentation broth, herbal drugs, etc. But when they have to consider scale-up separation, they will have difficulties using silica gels because of problems including batch-to-batch reproducibility of silica-based media with a chemically heterogeneous surface and the high sensitivity of the media to moisture or polar impurities in the mobile phase [2].

A better batch-to-batch reproducibility can be expected using synthetic adsorbents because they are synthesized by a simple polymerization process. Moreover, more reproducible operation and longer life can be expected due to the superior chemical stability of synthetic adsorbents compared to silica-

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based materials [3,4]. But only a few applications have been reported using polymer-based separation media [1,5]. The objective of this study is to demonstrate that commercially available synthetic adsorbents can be applied to normal-phase liquid chromatographic separation of natural products with significant resolution and scalability.

2. Experimental

2.1. Materials

All synthetic adsorbents made of poly(styrene–divinylbenzene) and polymethacrylate listed in Table 1 were from Mitsubishi Chemical Corporation (Tokyo, Japan). The fundamental characteristics of these adsorbents have been described elsewhere [6].

2.2. Columns

Stainless steel columns of 150×4.6 mm I.D. were used for analytical grade adsorbents, while stainless steel columns of 250×10 mm I.D. and 500×20 mm I.D. were packed with adsorbents of various particle sizes and used for semi-preparative and preparative chromatography, respectively. All these columns were packed in our laboratory by the slurry packing method. An ultrasphere Si (250×4.6 mm I.D.) column packed with 5-μm bare silica gel was obtained from Beckman Coulter (Fullerton, CA, USA).

2.3. Reagents and chemicals

Stearic acid (octadecanoic acid), linoleic acid (*cis*,*cis*-9,12-octadecadienoic acid) and γ-linolenic acid (*cis*,*cis*,*cis*-6,9,12-octadecatrienoic acid) were purchased from Sigma (St. Louis, MO, USA). Fruit of *Schisandra chinensis* Baill. (Japanese Pharmacopoeia) and schizandrin were supplied by Tochimoto (Osaka, Japan) and Koshiro (Osaka, Japan), respectively. A tocopherol set (catalogue No. 613424) containing DL-α-tocopherol, DL-β-tocopherol, DL-γ-tocopherol and DL-δ-tocopherol and a tocotrienol set (catalogue No. 613432) containing DL-α-tocotrienol, DL-β-tocotrienol, DL-γ-tocotrienol and DL-δ-tocotrienol were purchased from Calbiochem-Navabiochem (San Diego, CA, USA). A commercially available tocotrienol mixture from rice bran oil, ''Oryza Tocotrienol-30G'' was kindly donated by Oryza Oil and Fat Chemical (Aichi, Japan). A commercially available tocopherol mixture from rapeseed oil ''E mix-70L'' was kindly donated by Eisai (Tokyo, Japan). Other reagents and chemicals were of the highest quality available, and were purchased from various suppliers.

2.4. Apparatus

For analytical and semi-preparative chromatography, an HPLC system consisted of a Hitachi (Tokyo, Japan) L-7100 pump, a Hitachi L-7200 automatic sample injector with a 100-μl sample loop, a Hitachi L-7300 column oven, and a Hitachi L-7400 UV detector. Preparative chromatography was carried out using a Shimadzu (Kyoto, Japan) LC-10AS pump, a Shimadzu SIL-6B automatic sample injector with a 2.0-ml sample loop, and a Shimadzu SPD-6A UV detector with a preparative cell. These chromatographic systems were operated at 25 °C. Data were collected into a personal computer through a Shimadzu CBM-10A communica-

Table 1
Characteristics of polystyrenic and polymethacrylic synthetic adsorbents

No.	Adsorbent name	Type	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	Polystyrenic	9–11	10	540	1.39	14.0
2	MCI GEL CHP2MG	Polymethacrylic	9–11	10	590	1.13	20.0
3	Developmentally prepared	Polymethacrylic	15–20	17	470	1.16	19.0
4	MCI GEL CHP2MGY	Polymethacrylic	25–35	31	510	1.15	23.0

tions bus module and analyzed by Shimadzu Class-LC10 software.

2.5. Procedures

2.5.1. Separation of dialkyl phthalates

A mixture of diethyl phthalate, dipropyl phthalate and dibutyl phthalate was separated on columns packed with polystyrenic and polymethacrylic adsorbents of 10-μm particle size. The mixture was also separated on a 5-μm silica-based column. The mobile phase was hexane–isopropanol of varying ratios. The flow-rate was 1.00 ml/min. The sample concentration was 5 μl/ml for each dialkyl phthalate. The injection volumes were 5.0 μl for 150×4.6 mm I.D. columns and 2.0 μl for a 250×4.6 mm I.D. column, respectively. Wavelength of UV detector was 254 nm for the separation of dialkyl phthalates and t_0 was determined by use of CCl_4 at wavelength of 230 nm.

2.5.2. Analytical chromatography of polyunsaturated fatty acids

Stearic acid, linoleic acid and γ-linolenic acid were dissolved in hexane to give a concentration of 2 mg/ml. The mobile phase was hexane–EtOH (99:1). The flow-rate was 0.50 ml/min for the 150×4.6 mm I.D. analytical column to apply a linear velocity of 180 cm/h. The injection volume was 10 μl. The wavelengths of UV detection were 220 nm for stearic acid and 210 nm for both linoleic acid and γ-linolenic acid.

2.5.3. Separation of schizandrin from S. chinensis

A 20-g sample of *S. chinensis* Baill. fruit was milled and extracted with 200 ml of hexane for 2 h at 50 °C. The extracted solution was filtered by Millicup-LH (Millipore, Bedford, MA, USA), and evaporated to give 1.95 g of residue. The residue was dissolved in hexane to give concentration of 20 mg/ml. The concentration of reagent grade schizandrin was 0.5 mg/ml. The mobile phase was hexane–EtOH (90:10). Flow-rates were 0.50 ml/min for the 150×4.6 mm I.D. analytical column and 2.36 ml/min for 250×10 mm I.D. semi-preparative columns to apply the same linear velocity of 180 cm/h. The injection volumes were 10 μl for the 150×4.6 mm I.D. column and 80 μl for the 250×10 mm I.D. columns.

For preparative separation of schizandrin, a polymethacrylic adsorbent with 31-μm particle diameter was packed into a 500×20 mm I.D. column and separation was carried out at a flow-rate of 4.7 ml/min to give a linear velocity of 90 cm/h. Various sample concentrations and injection volumes were applied to observe the effect of loading volume on retention and separation. The wavelength of UV detection was 254 nm.

2.5.4. Separation of tocopherols and tocotrienols

Analytical chromatography of each tocopherol or tocotrienol was carried out on the 150×4.6 mm I.D. column packed with the polymethacrylic adsorbent of 10-μm particle size and on the 250×4.6 mm I.D. column packed with the silica-based medium of 5-μm particle size, respectively. Mobile phases were hexane–EtOH (98:2) for the polymethacrylic adsorbent and hexane–EtOH (99:1) for the silica-based medium. The flow-rate was 1.00 ml/min to apply a linear velocity of 360 cm/h. The sample concentration of each tocopherol or tocotrienol was 1 mg/ml, and the injection volume of each sample was 10 μl.

Analytical and semi-preparative chromatography of the tocotrienol mixture was carried out on the columns packed with polymethacrylic adsorbents of various particle sizes. The mobile phase was hexane–EtOH (98:2). The sample concentration of the tocotrienol mixture was 50 mg/ml. The column sizes, flow-rates and injection volumes were the same as described in Section 2.5.3.

For preparative chromatography of the tocotrienol mixture and tocopherol mixtures, a mobile phase of hexane–EtOH (98:2) was adopted for the separation of the tocotrienol mixture and hexane–EtOH (99:1) was used for the separation of the tocopherol mixture. The column size and flow-rate were as described in Section 2.5.3.

Various sample concentrations and injection volumes were applied to observe the effect of loading volume on retention and separation. The wavelength of UV detection was 295 nm.

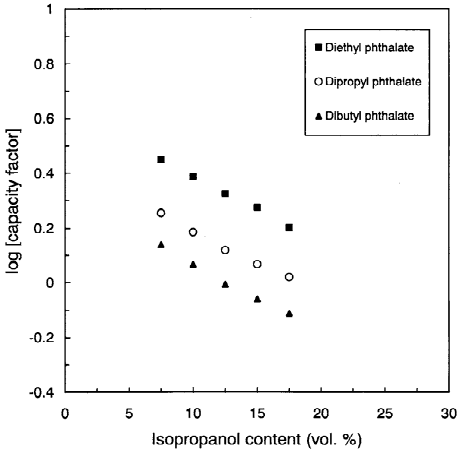

Fig. 1. Relationship between isopropanol content in the hexane–isopropanol eluent and capacity factors of dialkyl phthalates on a polymethacrylic adsorbent of 10-μm diameter. Conditions: column, 150×4.6 mm I.D.; eluent, hexane–isopropanol; flow-rate, 1.00 ml/min. Samples: diethyl phthalate, dipropyl phthalate and dibutyl phthalate (5 μl/ml each). Injection: 5.0 μl.

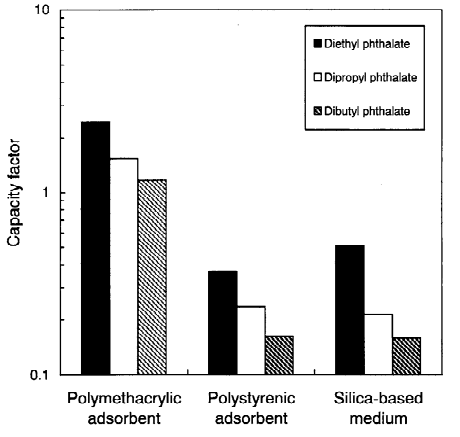

Fig. 2. Capacity factors of dialkyl phthalates chromatographed on each adsorbent under the elution condition of hexane–isopropanol (90:10). Conditions: column, 150×4.6 mm I.D. for polystyrenic adsorbent of 10 μm and polymethacrylic adsorbent of 10-μm diameter, 250×10 mm I.D. for silica-based medium of 5 μm; eluent, hexane–isopropanol (90:10); flow-rate, 1.00 ml/min. Samples: diethyl phthalate, dipropyl phthalate and dibutyl phthalate (5 μl/ml each). Injection: 5.0 μl for polystyrenic adsorbent of 10 μm and polymethacrylic adsorbent of 10-μm diameter; and 2.0 μl for silica-based medium of 5-μm diameter.

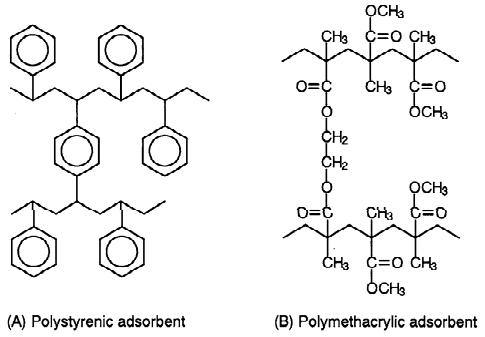

Fig. 3. Chemical structure of (A) polystyrenic and (B) polymethacrylic adsorbent.

3. Results and discussion

3.1. Separation of dialkyl phthalates

Separation of dialkyl phthalates mixture shows the basic characteristics of polystyrenic and polymethacrylic adsorbents as normal-phase chromatographic support materials. Fig. 1 shows the relationship between isopropanol content in the eluent and capacity factors of diethyl phthalate, dipropyl phthalate and dibutyl phthalate on the polymethacrylic adsorbent with 10-μm diameter. The capacity factors

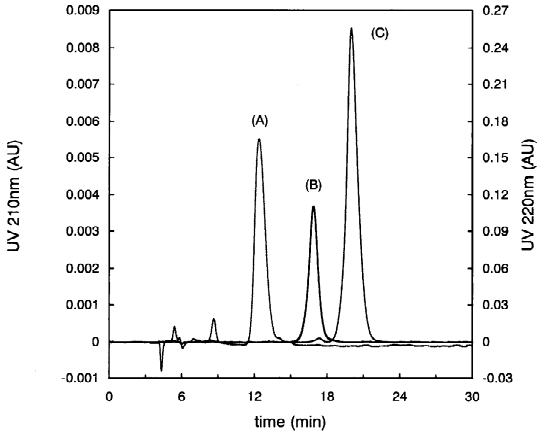

Fig. 4. Chromatograms of (A) stearic acid, (B) linoleic acid and (C) γ-linolenic acid on an analytical polymethacrylic adsorbent of 10-μm diameter. Conditions: column, 150×4.6 mm I.D.; eluent, hexane–EtOH (99:1); flow-rate, 0.50 ml/min. Samples: (A) stearic acid, (B) linoleic acid, and (C) γ-linolenic acid (2 mg/ml each). Injection: 10 μl. Detection: UV 210 nm for (A), and UV 220 nm for (B) and (C).

of each dialkyl phthalate were increased with decreasing isopropanol content in the eluent, and the elution of dialkyl phthalate with longer alkyl chain was shorter than that of dialkyl phthalate with shorter alkyl chain. These results proved that the separation mode is normal-phase. The capacity factors of dialkyl phthalates chromatographed on each adsorbent under the same elution condition of hexane–isopropanol (90:10) are shown in Fig. 2. From Fig. 2, it is found that the polymethacrylic adsorbent has stronger retentivity than the polystyrenic adsorbent. This retentivity difference is due to the difference in polarity of those adsorbents because of their chemical structures. The polystyrenic adsorbents have benzene rings, which are not very polar, whereas polymethacrylic adsorbents have more polar ester groups (Fig. 3). Furthermore, the retentivity of the polymethacrylic adsorbent is stronger than that of a

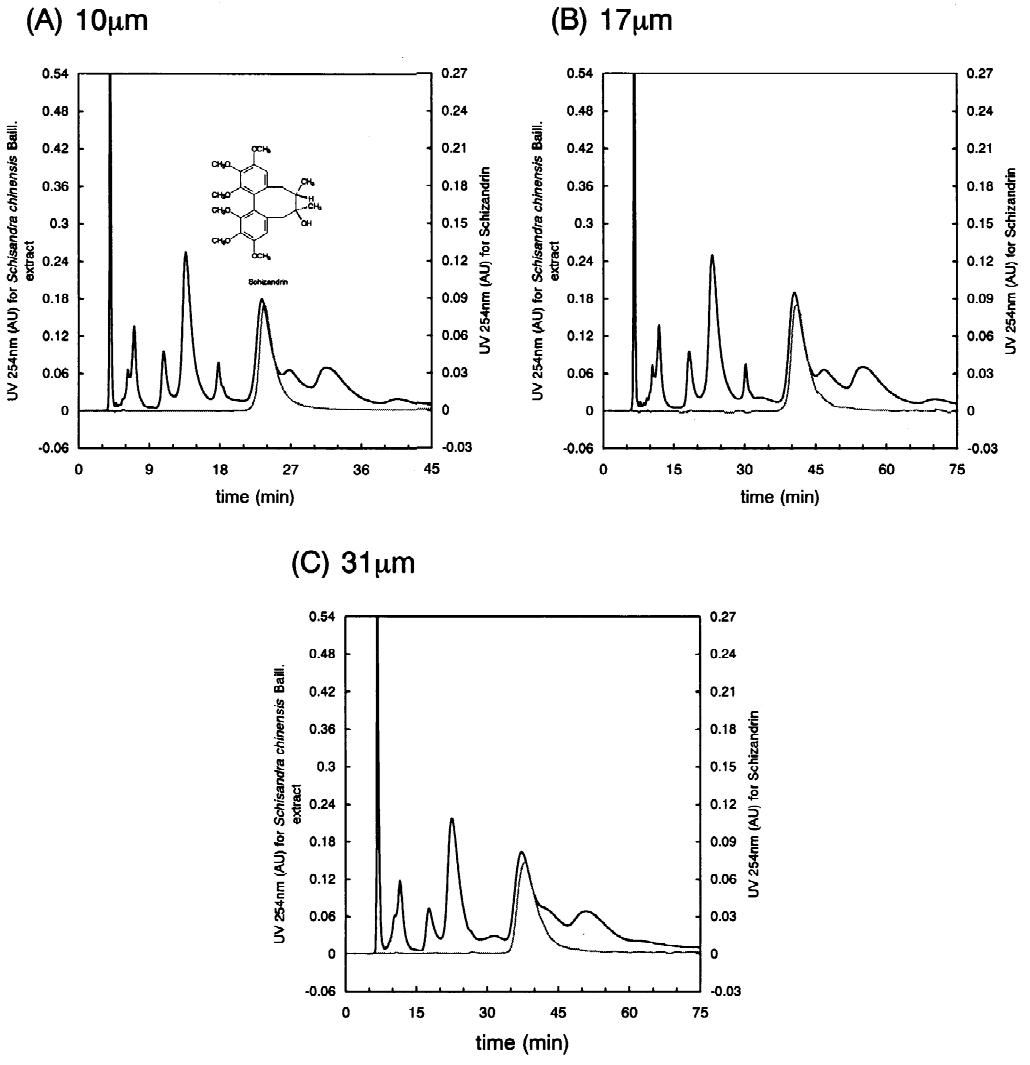

Fig. 5. Chromatograms of the extract of *S. chinensis* Baill. and schizandrin on polymethacrylic adsorbents with various particle sizes: (A) 10 μm, (B) 17 μm, (C) 31 μm. Conditions: column, 150×4.6 mm I.D. for (A), and 250×10 mm I.D. for (B) and (C); eluent, hexane–EtOH (90:10); linear velocity, 180 cm/h. Samples: black line represents extract of *S. chinensis* Baill. (20 mg/ml); gray line represents schizandrin (0.5 mg/ml). Injection: 10 μl for (A), and 80 μl for (B) and (C).

typical silica-based medium. The high retentivity of the polymethacrylic adsorbent enables the usage of a wide range of eluent systems including the selection of less polar solvents as main component, the selection of polar solvents used as modifiers, and the range of compositions of modifiers.

3.2. Analytical chromatography of polyunsaturated fatty acids

Polyunsaturated fatty acids are contained in various natural oils. The dietary and pharmaceutical effects of individual polyunsaturated fatty acids have been widely investigated [7,8], and therefore, preparative separation of these fatty acids is required both for investigations and industrial applications.

Polyunsaturated fatty acids have been chromatographically separated using bare silica gels [9], argentated silica gels [10] and octadecyl silica gels [11], but there have been few reports using polymeric stationary phases.

Chromatograms of stearic acid, linoleic acid and

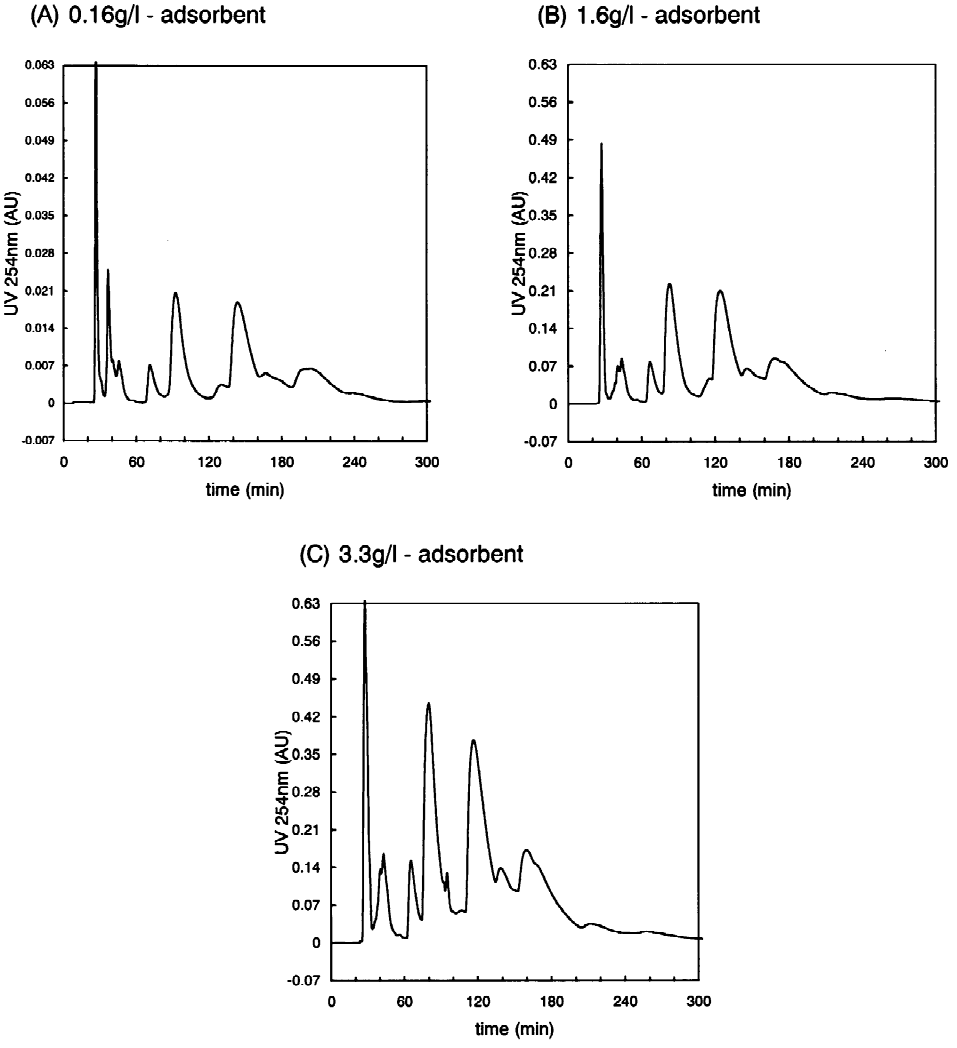

Fig. 6. Preparative separation of the extract of *S. chinensis* Baill. on a column packed with a polymethacrylic adsorbent of 31-μm diameter. Conditions: column, 500×20 mm I.D.; eluent, hexane–EtOH (90:10); flow-rate, 4.7 ml/min. Sample: extract of *S. chinensis* Baill. Sample concentration: (A) 20 mg/ml, (B) 200 mg/ml and (C) 400 mg/ml. Injection: 1280 μl.

γ-linolenic acid on the analytical polymethacrylic adsorbent are shown in Fig. 4. From Fig. 4, it is found that the polymethacrylic adsorbent can separate fatty acids of the same chain length utilizing their polarity differences associated with the numbers of double bonds.

3.3. Separation of schizandrin from S. chinensis

S. chinensis Baill. has been used for centuries as a traditional Chinese medicine effective as a tonic, sedative, astringent and antitussive. Recently, in addition to these effects, many biological activities such as platelet activating factor antagonist activity [12] and protective effect against myocardial ischemia–reperfusion injury [13] have been found in *S. chinensis* Baill. extract. *S. chinensis* Baill. contains many dibenzocyclooctadiene lignans such as schizandrin, gomisin A, gomisin N, wuweizisu C, etc. [14]. Consequently, the precise separation of schizandrin and its analogues is of great importance.

Separation of the extract of *S. chinensis* Baill. and schizandrin on polymethacrylic adsorbents with various particle sizes is shown in Fig. 5. It can be seen that almost identical elution profiles are maintained with increasing particle sizes of these polymethacrylic adsorbents. This means that once the separation condition is determined using the analytical adsorbent, the same condition can be directly applied to preparative separation on the larger sized adsorbents of identical chemistry.

In Fig. 6, preparative chromatograms of the extract of *S. chinensis* Baill. on the polymethacrylic adsorbent of 31-μm diameter at various loading volumes are described. Even at the loading volume of 3.3 g/l adsorbent, the separation of schizandrin was still sufficient for the high purity fractionation.

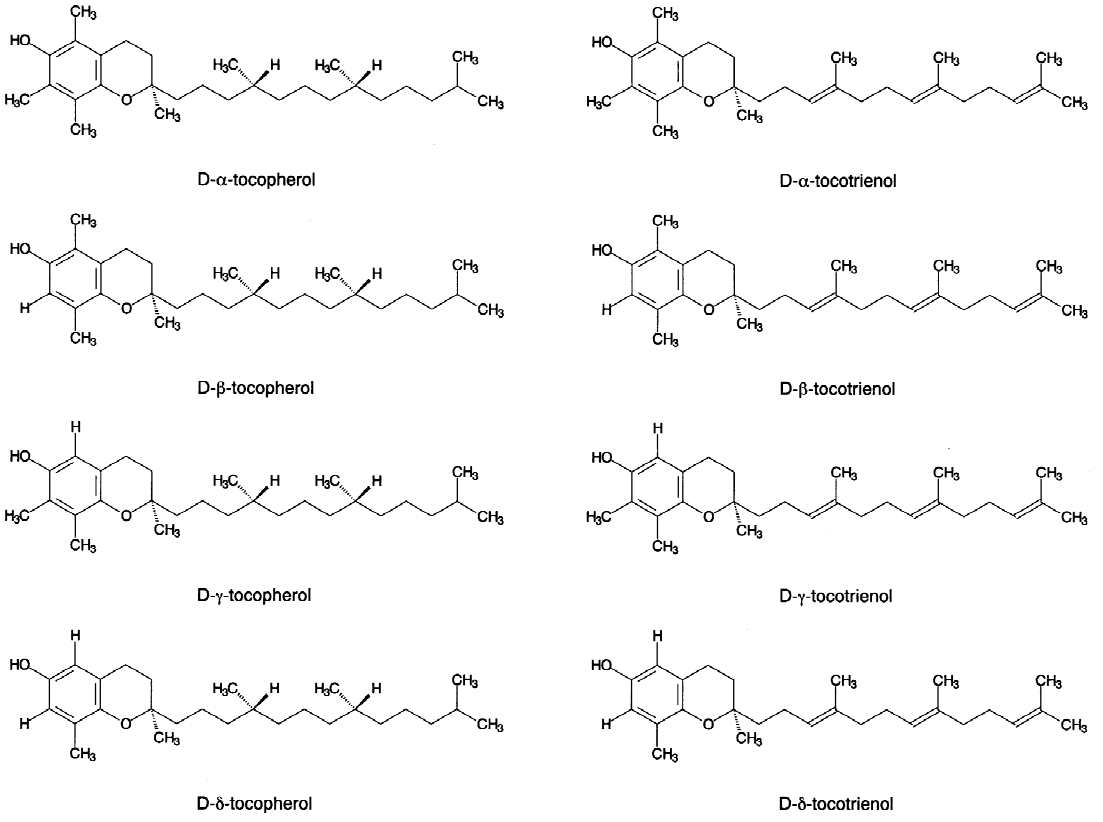

Fig. 7. Chemical structure of natural vitamin E.

3.4. Separation of tocopherols and tocotrienols

Vitamin E is a natural antioxidant and plays an important role by scavenging free radicals in the living body. Natural vitamin E consists of four tocopherols and four tocotrienols (Fig. 7), and each of them has different antioxidant activity or other biological activities. For example, D-α-tocotrienol, D-γ-tocotrienol and D-δ-tocotrienol showed strong inhibitory effect against proliferation of estrogen receptor-negative MDA-MB-435 human breast cancer, whereas D-α-tocopherol had no effect [15].

Many methods including TLC, GC, normal-phase LC and reversed-phase LC for the separation of vitamin E have been studied [16]. But few applications of polymeric media have been reported, and silica-based materials are prevalent especially in the field of normal-phase separation of vitamin E.

But when considering scale-up separation, silica-based media present difficulties because of their

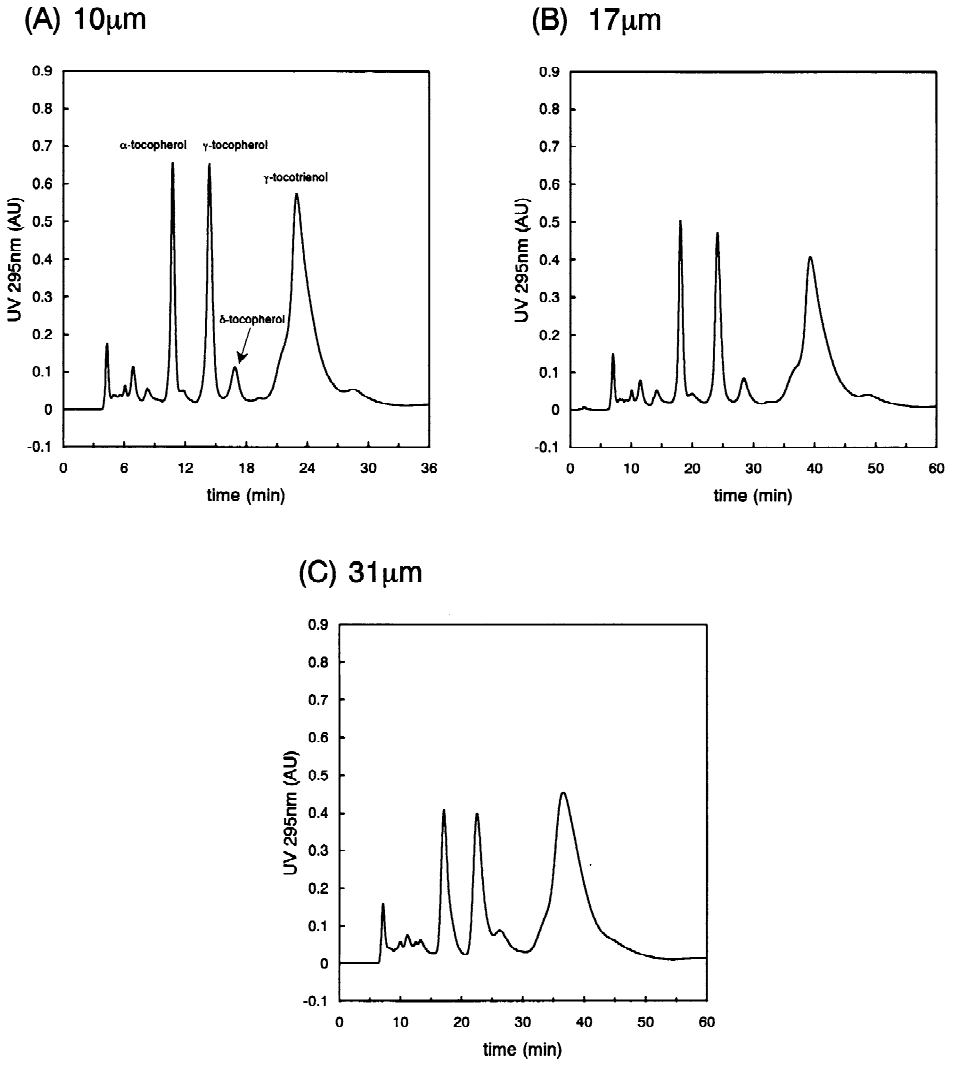

Fig. 8. Chromatographic separation of rice bran oil on polymethacrylic adsorbents with various particle sizes: (A) 10 μm, (B) 17 μm, (C) 31 μm. Conditions: column, 150×4.6 mm I.D. for (A), and 250×10 mm I.D. for (B) and (C); eluent, hexane–EtOH (98:2); linear velocity, 180 cm/h. Sample: rice bran oil (Oryza Tocotrienol-30G, 50 mg/ml). Injection: 10 μl for (A), and 80 μl for (B) and (C).

Fig. 9. Capacity factor of each tocopherol and tocotrienol chromatographed on a polymethacrylic adsorbent of 10-μm diameter and a silica-based medium of 5 μm. Conditions: column, 150× 4.6 mm I.D. for polymethacrylic adsorbent of 10-μm diameter, 250×10 mm I.D. for silica-based medium of 5-μm diameter; eluent, hexane–isopropanol (98:2) for polymethacrylic adsorbent of 10-μm diameter, and hexane–isopropanol (99:1) for silica-based medium of 5-μm diameter; flow-rate, 1.00 ml/min. Sample: DL-tocopherols and DL-tocotrienols (1 mg/ml each). Injection: 10 μl for polymethacrylic adsorbent of 10-μm diameter and 5 μl for silica-based medium of 5-μm diameter.

short lifetime and high sensitivity to moisture or polar impurities in the eluent systems [2]. Therefore, anion-exchange method is adopted in the field of industrial production of vitamin E despite its lower separation efficiency [17].

If synthetic adsorbents can be applied to normal-phase separation of vitamin E, better solutions would be expected. Fig. 8 shows the separation of rice bran oil containing tocopherols and tocotrienols on the polymethacrylic adsorbents with various particle sizes. From Fig. 8, it is found that all the polymethacrylic adsorbents can separate D-α-tocopherol, D-γ-tocopherol, D-δ-tocopherol and D-tocotrienols, and the elution profiles are almost identical. These chromatograms show the scalability of the polymethacrylic adsorbents.

Precise elution of each tocopherol and tocotrienol on the analytical polymethacrylic adsorbent and the common silica medium was investigated. The capacity factor of each tocopherol and tocotrienol is shown in Fig. 9. It should be noted that the elution order of tocopherols and tocotrienols on the polymethacrylic adsorbent differs from that on the silica medium. The polymethacrylic adsorbent eluted tocopherols first and then tocotrienols. This elution order may be attributed to the strong affinity of the polymethacrylic adsorbent for the double bonds of tocotrienols. The stronger retentivity of the polymethacrylic adsorbent for tocotrienols than for tocopherols seems to favor fractionation of tocopherols and tocotrienols as regards industrial applications. In contrast, the retention difference between each tocopherol and its related tocotrienol is smaller than the retention difference among four tocopherols in

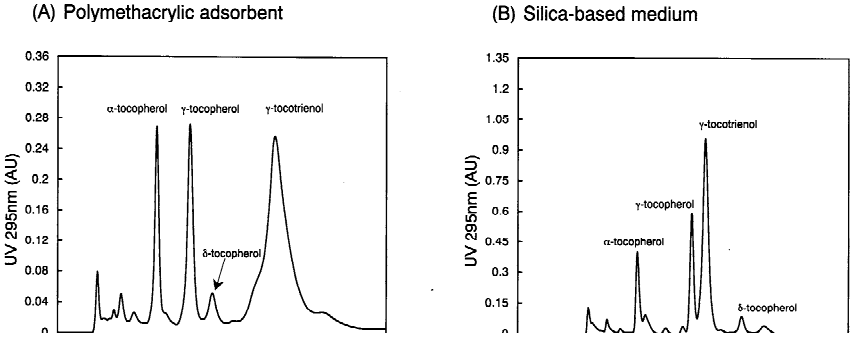

Fig. 10. Elution profiles of rice bran oil chromatographed on a polymethacrylic adsorbent of 10 μm diameter and a silica-based medium of 5-μm diameter. Conditions: column, 150×4.6 mm I.D. for polymethacrylic adsorbent of 10-μm diameter, 250×10 mm I.D. for silica-based medium of 5-μm diameter; eluent, hexane–isopropanol (98:2) for polymethacrylic adsorbent of 10-μm diameter, hexane–isopropanol (99:1) for silica-based medium of 5-μm diameter; flow-rate, 1.00 ml/min. Sample: rice bran oil (Oryza Tocotrienol-30G, 50 mg/ml). Injection: 5 μl.

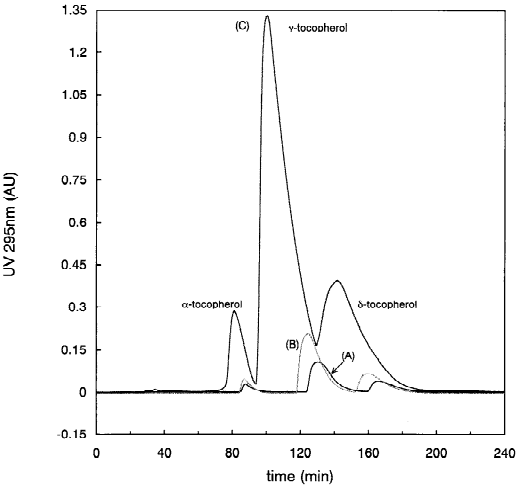

Fig. 12. Preparative separation of rapeseed oil on a column packed with a polymethacrylic adsorbent of 31-μm diameter. Conditions: column, 500×20 mm I.D.; eluent, hexane–EtOH (99:1); flow-rate, 4.7 ml/min. Sample: rapeseed oil (E mix-70L). Concentration and injection: 50 mg/ml, 640 μl for (A), 50 mg/ml, 1280 μl for (B), and 500 mg/ml, 1280 μl for (C).

and resolution of rapeseed oil separated on the preparative column. In this case, the larger the loading volume applied, the shorter the retention observed. This observation results both from non-linear adsorption behavior and from kinetic effect of the polymethacrylic adsorbent. Similar phenomena have been reported for the normal-phase separation of cyclopentanone and cycloheptanone using silica-based media [18]. But it should be emphasized that the separation of tocopherols was still maintained at the loading amount of 4.1 g/l adsorbent. This value of loading amount is sufficient for industrial separation of high value added organic compounds.

4. Conclusions

Polymeric synthetic adsorbents as normal-phase chromatographic support materials were characterized. Among them, the polymethacrylic adsorbents showed higher retentivity than conventional silica media or polystyrenic adsorbents.

Separation of natural products such as polyunsaturated fatty acids, schizandrin in *S. chinensis* fruit, tocopherols and tocotrienols in vegetable oil on the 10-μm polymethacrylic adsorbent packed into the HPLC column revealed the applicability of the polymethacrylic adsorbents. Furthermore, preparative separation using the polymethacrylic adsorbents with 17- and 31-μm fine grade particle sizes confirmed the consistency of the polymethacrylic adsorbents of different particle sizes. Loadability of several grams per liter polymethacrylic adsorbent was sufficient for cost effective separation of natural products.

We conclude that the polymeric synthetic adsorbents are industrially comparable to conventional silica media. Besides, application of normal-phase chromatographic separation could be expanded due to the chemical and mechanical stability of the synthetic adsorbents. The results obtained in this study may help researchers pursuing more precise purification of bioactive compounds, including pharmaceuticals and nutraceuticals with scale-up considerations.

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