C18 Attached Silica Monolith Microcolumns Made in Stainless Steel Tubing and Their Application in Analysis of Flavonoids in Green Tea Extracts

Joung Ho Ko, Gyoung Won Kang, You Jin Seo, and Won Jo Cheong*

Department of Chemistry and Institute of Basic Research, Inha University, Incheon 402-751, Korea
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Recently, the monolith columns have raised a lot of interest[1-5] in liquid chromatography. The whole stationary phase of the monolith column is one body with numerous multiple porous channels, thus it does not need any frit since the whole stationary phase functions as a frit. A monolith column is prepared by in situ polymerization or condensation of organic or inorganic monomers inside the column tubing and, if necessary, the surface is functionalized.[3]

We reported a disposable microcolumn that enabled removing the need of sample pretreatment in a previous study.[6] We disposed of the stationary phase after using the column for a day or so and repacked the column with fresh stationary phase. We were unable to suggest a feasible frit technique but a rather tedious one at that time.

The technique of making fixed frits[7] in the column main body tubing is generally used in production of microcolumns, especially microcolumns made of silica capillary. The recent trend of silica capillary microcolumns is shifting swiftly toward monolithic columns. Details of monolith columns have been introduced in some review articles,[3-5] and patents of various monolith columns have appeared. A method of preparing fixed frits at both ends of the column by partial heat treatment of packed silica capillary was introduced in the literature,[8,9] and a research group packed a silica capillary with porous silica and sintered the stationary phase by heating, leaving the whole stationary phase combined but the porous spherical structures of the powders unchanged.[10] A peculiar monolith column was prepared by putting the dispersed solution of stationary phase powders, water, a solvent such as alcohols, and a metal alkoxide in silica capillary, and by applying heat and vacuum.[11]

Study on formation of monoliths in stainless steel tubing is scarce in the literature. There was once a report on formation of organic monolith in LC/MS spray tube of 0.1 mm ID,[12] however there has been no report on formation of silica monoliths in a metal tube. The commercially available silica monolith rod is produced in a mold and sold only in the form encased in shrinkable poly(ethyl ether ketone) and equipped with column end fittings.[4,5] The Merck Chromolith columns released to the market in 2000 have been extensively applied in various fields, including analysis of drugs and metabolites, analysis of environmentally relevant substances, food additives, chiral separations, as well as bioanalytical separations.[5] The ID of this column is 4.6 mm. The advantage of fast analysis of this monolith column is due to high eluent flow rate enabled by much lower column back pressure compared to conventional columns. Increased consumption of mobile phase, however, is a problem of this column. Decrease of mobile phase consumption can only be enabled by decrease of column diameter. Production of silica monolith rod of small ID clad in poly(ethyl ether ketone) is known to suffer from technical problems.

This study has been carried out in expectation of developing stainless steel silica monolith microcolumns of 0.5 mm ID

Experimental Section

Chemicals. Methanol and water were of HPLC grade and purchased from Fisher (Pittsburg, PA, USA) and used without purification. The standard catechin compounds were purchased from Sigma (St. Louise, MO, USA). All the other chmical reagents were purchased from Alldrich (Milwakee, IL, USA).

HPLC. Two Shimadzu (Tokyo, Japan) 10AD pumps, a Shimadzu DGU-14A membrane degasser, a Tee union with a 1/16 inch ID stainless steel frit(as a micromixer), a Rheodyne (Cotati, CA, USA) 7520 injector with a 0.5 μL injection loop, an Isco (Lincoln, NE, USA) CV4 capillary window detector, and the home-made 0.5 mm ID microcolumn were combined to compose the HPLC system. The chromatographic data were obtained by a PC system, and a software Multichro 2000 from Youlin-Gisul (Sungnam, Korea) was used to acquire and process the data.

Preparation of C18 attached silica monolith microcolumns in stainless steel tubing. Plain stainless steel tubing of 0.5 mm ID (1.6 mm OD) was used as a cheap column material in this study. The stainless steel tubing was first washed with 1 N NaOH, 1 N HCl, water, and methanol in sequence, dried and filled with the monolith reaction mixture to form the silica monolith. The silica monolith in stainless steel tubing was then treated with a solution of a C18 ligand to give a C18 attached silica monolith microcolumn.

The reaction mixture was prepared by stirring acetic acid, polyethyleneglycol (PEG: MW 10,000), and urea in a predetermined ratio (see Table 1) in a flask followed by adding trimethoxysilane (TMOS) drop by drop with vigorous stirring at 0 °C for 30 min. The reaction mixture was immediately transferred in the stainless steel tubing and

Table 1. The summary of compositions of monolith mixtures, reaction (polymerization) conditions, and conditions of C18 attachment reaction for different C18 attached silica monolith columns

	Reagent or process	Column S1	Column S2	Column S3
Silica monolith	TMOS (mL)	0.4	0.4	1.0
	PEG (g)	0.108	0.088	0.220
	0.01 N Acetic acid (mL)	1.0	1.0	2.5
	Urea (g)	X	0.090	0.225
Washing Solvent	Xylene (washing)	O	X	X
	Toluene (washing)	X	O	O
C18 ligand End-capping	Pyridine/Toluene (mL/mL)	X	0.05	0.05
	Octadecylchlorodimethyl-silane/Toluene	0.25 g/mL	0.25 g/mL	0.25 g/mL
	TMCS/Toluene (g/mL)	X	0.25	0.25
Polymerizati-on hours at each sequential steps	40 °C (h)	24	24	24
	110 °C (h)	24	X	X
	120 °C (h)	X	3	3
	200 °C (h)	6	X	X
	330 °C (h)	X	25	25

X: not used

each tubing end was sealed by union fitting with a plug. The column was placed in an oven at 40 °C for 24 hours. Next the oven temperature was raised to 110 °C for 24 hours to form hardened silica monolith. The column was then calcined in an electric furnace at 330 °C for more than 6 hours to remove residual solvent and unreacted material. The column was connected to a HPLC pump and washed with ethanol. The column was dried, washed with xylene or toluene, and filled with dimethylchlorooctadecylsilane dissolved in toluene. The column was sealed by union fitting with a plug and placed in an oven at 110 °C for more than 8 hours. Addition of pyridine in the reagent solution was helpful to complete the reaction. For end-capping, the column was filled with trimethylchlorosilane (TMCS) dissolved in toluene and heated at 110 °C for more than 8 hours. The column was finally washed with ethanol thoroughly.

Analysis of catechin compounds in green tea extracts. An exact amount of 250 mL distilled water was poured into a 500 mL three neck round bottom flask, a reflux condenser, a thermometer, and a glass stopper were placed at the three necks respectively, and an oil bath over a magnetic hot plate was placed below the flask. The content was stirred with a magnetic bar. The bottle was heated and allowed to be in temperature equilibrium at 50 or 100 °C. Once the temperature equilibrium was achieved, an exact amount of 5 g green tea was transferred in the flask, and extraction took place while stirring. An exact amount of 1.0 mL supernatant aqueous solution was taken when the elapsed time was 10, 20, 30 min, 1, 2, 4, 6, 8, 12, 24, 36, and 48 hours. The sampled extract was filtered through a 0.45 μm membrane filter to remove dispersed minute green tea powders. The sample was washed with 1.0 mL chloroform in a separatory funnel to remove caffeine, pigments, and other nonpolar impurities. This step was repeated three times and the chloroform phase was found with negligible catechin compounds owing to their low solubility in chloroform. Next, the catechin compounds in the water phase were extracted to 1.0 mL ethyl acetate, and this step was also repeated three times. The water phase now was found with negligible catechin compounds. The ethyl acetate phase was evaporated to dryness and dissolved in 0.30 mL methanol and injected for chromatographic analysis.

Results and Discussion

The compositions of monolith mixtures, reaction (polymerization) conditions, and conditions of C18 attachment reaction for different monolith microcolumns are summarized in Table 1.

The generally known method of making silica monolith in silica capillary was first used to make silica monolith in stainless steel tubing. The separation efficiency of this monolith column (Column S1) was found very low, and there was only one peak observed for a mixture of benzene, toluene, and ethylbenzene.

Variation of composition of reaction mixture and polymerization process was tried to improve column efficiency (Column S2 and S3). Major changes were addition of urea in the reaction mixture, incorporation of heating process at 330 °C for 24 hours as the final step of polymerization, and addition of pyridine to the reaction mixture of ligand attachment. Urea was added to help formation of porous monolith structure by generating ammonia at 120 °C.[13] Heating at 330 °C was for securing hard silica monolith frame. Addition of pyridine was to neutralize HCl formed during C18 ligand attachment reaction. The column efficiency (Column S2) was somewhat improved, but was still disagreeable.

The major reason for such low column efficiency was found incomplete mixing of the reaction mixture. Thus we increased the batch size and secured complete mixing of the reaction mixture by vigorous stirring. C18 attachment reaction and end-capping reaction were also secured by extending reaction time from 6 hours to overnight. We were able to obtain better microcolumns (Column S3) owing to such improvements. The chromatograms of benzene, toluene, ethylbenzene obtained by the Column S2 and S3 are comparatively shown in Figure 1. A few more bathes of microcolumns were made in the improved procedure with even more extreme care, and column efficiency was further improved.

Improved C18 attached silica monolith microcolumns were used to determine catechin (flavonoid) components in green tea extracts. Total 5 flavonoids were identified in the extract at 50 °C, and 8 flavonoids in the extract at 100 °C. The chromatograms of the standard mixture of 5 flavonoids and the green tea extract at 50 °C are shown in Figure 2. The chromatograms of the standard mixture of 8 flavonoids and the green tea extract at 100 °C are shown in Figures 3. The molecular structures of the flavonoids are given in Figure 4.

Variation of each flavonoid content in green tea extracts at 50 °C with respect to extraction time was also observed.

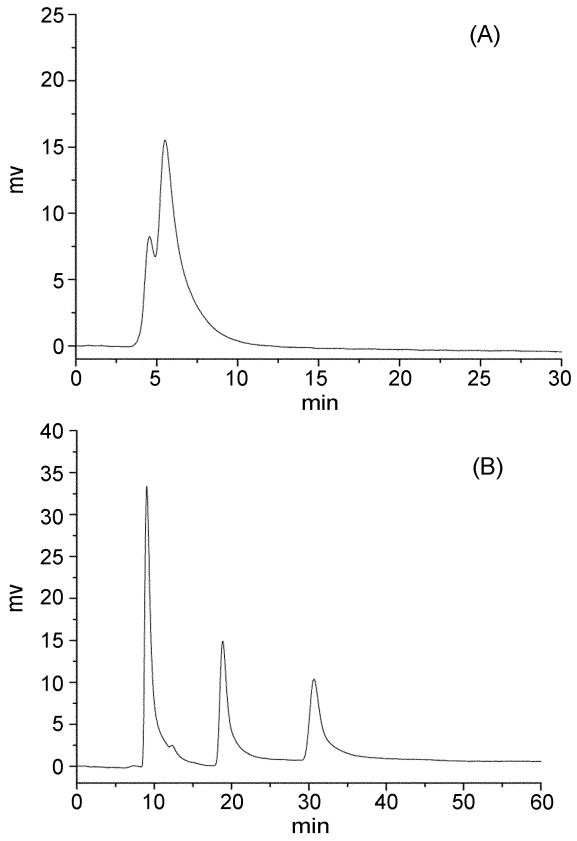

Figure 1. (A) The chromatogram obtained with the S2 column (Silica monolith column, 16 cm × 0.5 mm) at a flow rate of 0.01 mL/min (11 bar) in 60% MeOH with 0.1% TFA. (B) The chromatogram obtained with the S3 column (Silica monolith column, 23 cm × 0.5 mm, first batch) at a flow rate of 0.01 mL/min in 50% MeOH with 0.1% TFA. The solutes were benzene, toluene, ethylbenzene.

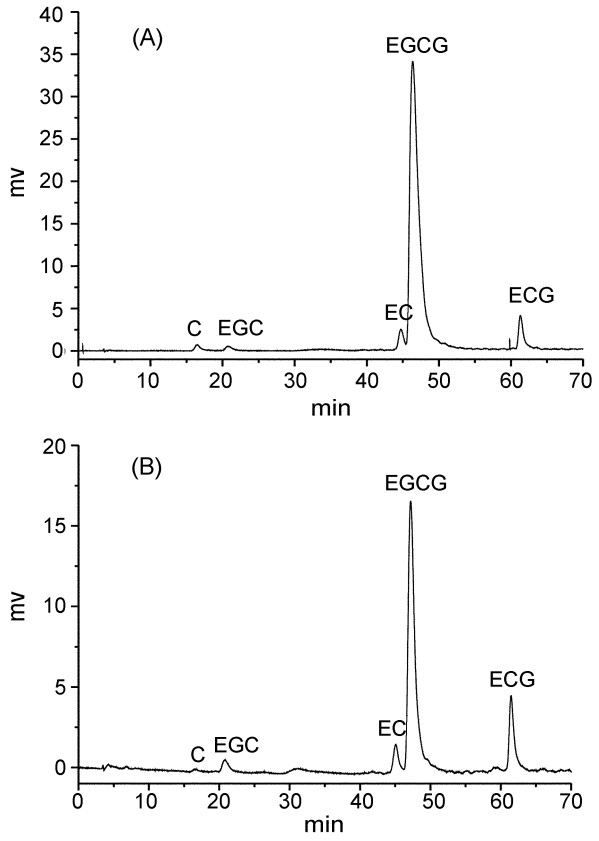

Figure 2. The chromatogram of flavonoid standards (A) and the chromatogram of the extract of 5 g green tea powder in 250 mL water at 50 °C for 20 min (B) obtained with the S3 column (Silica monolith column, 31 cm × 0.5 mm, second batch) by gradient elution at a flow rate of 0.02 mL/min. Gradient elution: 2% (30 min)-19% (45 min)-19% (50 min)-25% (60 min)-2% (70 min) MeCN with 0.1% TFA.

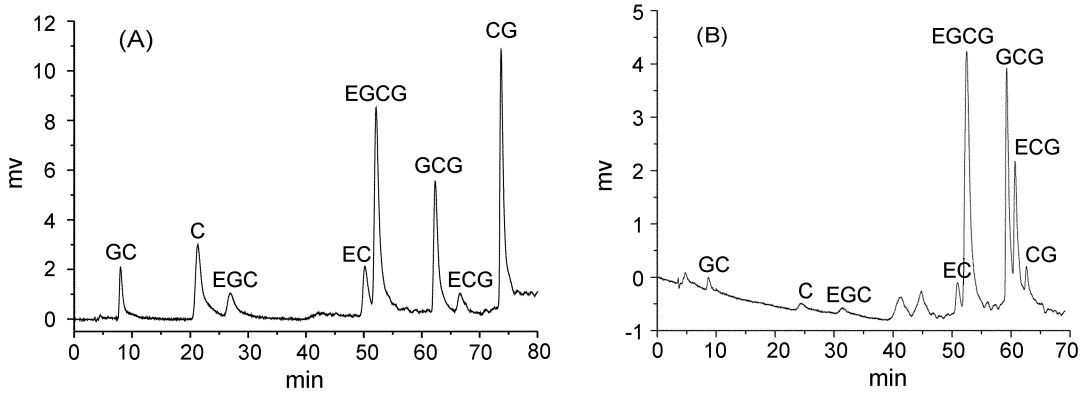

Figure 3. The chromatogram of flavonoid standards (A) and the chromatogram of the extract of 5 g green tea power in 250 mL water at 100 °C for 10 min (B) obtained with the S3 column (Silica monolith column, 30 cm × 0.5 mm, third batch) by gradient elution at a flow rate of 0.02 mL/min. The retention of each flavonoid was reproducible for the standard sample while retention was not reproducible for the green tea sample extracted at 100 °C, probably owing to accumulation of some impurities in the column. The flavonoid in suspicion was confirmed by spiking the component of interest to the sample followed by obtaining the chromatogram. Gradient elution: 1% (30 min)-5% (45 min)-5% (45 min)-8% (55 min)-8% (65 min)-25% (75 min)-1% (85 min) MeCN with 0.1% TFA.

Variations of 5 flavonoids against extraction time are shown in Figure 5. The content of each flavonoid increased abruptly at first, leveled off at about 12 hours, and decreased for prolonged extraction. It implies that flavonoids decomposed slowly.

It should be noted that the monolith columns prepared in this study are different from the commercial(Choromolith) columns. First of all, our monoliths are prepared in stainless

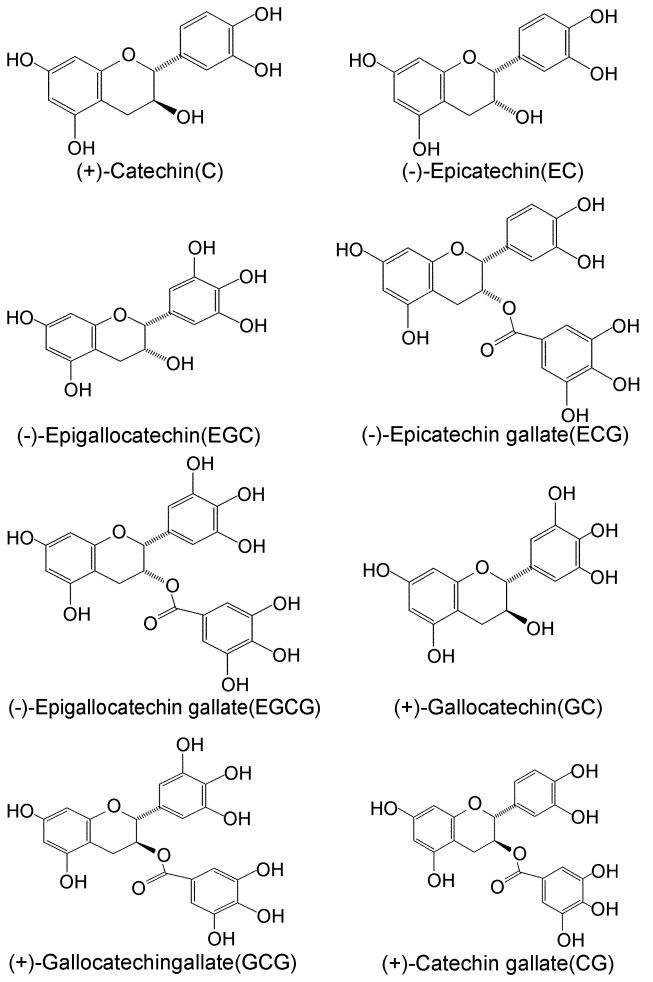

Figure 4. Molecular structures of catechin compounds.

steel tubing while the commercial ones are made in PEEK (poly ethyl ether ketone). Ours are much smaller in diameter, 0.5 mm against 4.6 mm (Chromolith). Nonpolar solute such as alkylbenzenes show bad peak shapes with severe tailing in our monolith column while they do not in a Chromolith column.[4,5] On the other hand, satisfactory separation with good peak shapes was achieved with our monolith column for a real biological sample of some polarity such as flavonoids in green tea extracts. The above phenomena imply that there exist differences in physicochemical properties between the monolith columns prepared in this study and the commercial columns. The details are not clear at present and are under investigation.

Thus C18 attached silica monolith microcolumn in stainless steel tubing has been successfully prepared and showed good performance in analysis of flavonoids in green tea extracts. We regret, however, that this column may not serve as a disposable microcolumn of a commercial value. Despite the use of cheap stainless steel tubing, the production cost would be rather high owing to C18 attachment and end-capping. It should be noted that the column developed in this study can be used like conventional columns. In such a case, the production cost would be lower than that of conventional microcolumns since the column structure of this study is much simpler.

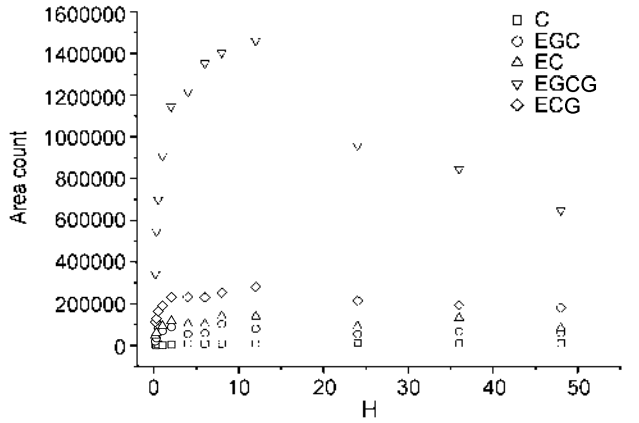

Figure 5. Variation trends of contents (area counts) of individual flavonoid components in the green extracts (50 °C) as a function of extraction time.

The idea of cheap disposable microcolumns may be realized if underivatized silica monolith columns are made in stainless steel tubing by one-step process. However, such a column should be used in normal phase mode and performance and/or versatility of its use is in question. Relevant study is underway.

Conclusion

New C18 attached silica monolith microcolumn has been prepared in stainless steel tubing by optimizing reaction mixture, polymerization processes, and C18 attachment reaction. Such a column showed satisfactory column efficiency for solutes of some polarity and was successfully employed in determination of flavonoids in green tea extracts.

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