Short Communications

Preparation and characterization of surfactin-modified silica stationary phase for reversed-phase and hydrophilic interaction liquid chromatography

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Keywords: hydrophilic interaction; mixed-mode; surfactin; reversed-phase

Abstract

Surfactants are good candidates as selectors in mixed-mode reversed-phase liquid chromatography (RPLC)/hydrophilic interaction liquid chromatography (HILIC) because they contain both a hydrophobic and a hydrophilic moiety. Surfactin, a cyclic heptapeptide, is an efficient biosurfactant produced by *Bacillus subtilis* that comprises seven amino acids and a β-hydroxyl fatty acid. A surfactin-modified silica (SMS) stationary phase was prepared by amide bond formation between amino groups on aminopropyl silica and the carboxylic acid groups of L-Glu and L-Asp residues in surfactin. The resulting SMS stationary phase was characterized in both RPLC and HILIC mode using different mobile phases. The SMS column was found to separate analytes in both modes. The retention of polar solutes exhibited "U-shaped" curves, depending on the acetonitrile content. "U-shaped" curves are an indicator of RPLC/HILIC mixed-mode retention behavior. The presence of hydrophobic and hydrophilic moieties in surfactin provides unique properties that allow the SMS column to be used for both RPLC and HILIC separations, simply by changing the mobile phase composition.

1 Introduction

Reversed-phase liquid chromatography (RPLC) is widely used to retain and separate hydrophobic and moderately hydrophobic compounds; however, it often cannot be used to separate polar compounds due to lack of retention on the column. Normal phase liquid chromatography is another choice, but non-polar mobile phases are poor solvents for polar compounds. Recently, hydrophilic interaction chromatography (HILIC), where a bare silica or polar group (amine, amide, cyano, diol)-bonded silica stationary phase and a hydro-organic mobile phase are used, has become a valuable alternative for the separation of polar compounds. Separation in HILIC mode is believed to result from the partitioning of analytes between a water-rich layer on the surface of the hydrophilic stationary phase and the hydro-organic mobile phase, and from the interaction based on hydrogen bonds between the analytes and the functional group on the stationary phase [1].

Mixed-mode chromatographic separation based on more than one retention mechanism would likely provide better separation than single mode separation. This new concept is gaining attention, and several mixed-mode separation materials have been reported. Most mixed-mode separation methods combine RP and anion- or cation-exchange. However, the column-packing materials available for RPLC and HILIC mixed-mode separations are limited, although the combination of these two chromatographic approaches may expand their applicability [2-6]. Several researchers have used materials possessing a long alkyl chain (hydrophobic moiety) and an ionizable group (hydrophilic moiety) for RPLC/HILIC mixed-mode separation [3]. However, ionized groups can electrostatically interact with ionized analytes, resulting in a severe peak tailing. In contrast, Wu *et al.* synthesized nonionic polar stationary phases with hydroxyl and sulfoxide groups, and reported that some of these stationary phases were effective for RPLC and HILIC mode separations [4].

Surfactants are good candidates as selectors for RPLC/HILIC mixed-mode separation because they consist of both a hydrophobic and hydrophilic moiety. Lin *et al.* prepared a hydrophobic/strong cation-exchange monolithic column by copolymerization of 3-sulfopropyl methacrylate and pentaerythritol triacrylate [7]. Gu *et al.* prepared a methacrylate-derived surfactant-bound monolithic column by copolymerization of 11-acrylaminoundecanoic acid and ethylene dimethacrylate. They evaluated its potential in the RP separation of three model proteins [8]. However, to our knowledge, an RPLC/HILIC mixed-mode stationary phase immobilized with surfactant has yet to be reported.

Surfactin is an efficient biosurfactant produced by *Bacillus subtilis* [9]. It is a cyclic heptapeptide consisting of seven amino acids and a β-hydroxyl fatty acid. Compared with chemical surfactants, surfactin has some unique advantages such as lower toxicity, biodegradability, and effectiveness at extreme temperature or pH values [10]. Surfactin is also known for its antiviral, antitumor and hemolytic activities [11-13].

In the present study, a surfactin-modified silica (SMS) stationary phase (Fig. 1) was prepared by amide bond formation between the amino groups on aminopropyl silica (APS) and the carboxylic acid groups of L-Glu and L-Asp residues in surfactin. An SMS-packed column was characterized in both RPLC and HILIC mode using different mobile phase compositions.

2 Materials and methods

2.1 *Chemicals*

APS (particle size, 5 μm; pore size, 120 Å) was a kind gift from Daiso Chemical (Osaka, Japan). Surfactin, HPLC grade of acetonitrile (ACN), *tris*(hydroxymethyl)aminomethane (Tris),

hydrochloric acid, ammonium acetate, acetic acid, benzene, naphthalene, toluene, phenol, aniline, 4,5-dimethyl-1,2-phenylenediamine, uracil, thymidine, cytosine, uridine, adenosine, pyridoxine, thymine, 1-methylxanthine, 1,7-dimethylxanthine, 1,3,7-trimethylxanthine (caffeine) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Wako Pure Chemicals (Osaka, Japan). *N*,*N*-Dimethylformamide (DMF) was from Nacalai Tesque (Kyoto, Japan). *N*,*N*-dimethyl-4-aminopyridine (DMAP) was purchased from Merck KGaA (Darmstadt, Germany). Propylbenzene and butylbenzene were from Tokyo Chemical Industry (Tokyo, Japan). Dichloromethane (DCM), ethanol, sodium perchlorate, perchloric acid, ethylbenzene and adenine were from Kishida Chemicals (Osaka, Japan). Riboflavin was obtained from Sigma (St. Louis, MO, USA).

2.2 *Preparation of SMS stationary phase*

SMS was obtained by a single-step reaction as follows: surfactin (1.54 g), EDC (1.12 g) and DMAP (0.036 g) were added to a suspension of APS (0.77 mmol/g, 0.22 g) in DMF (145 mL) and the mixture was shaken at 30 °C for 15 h. After the reaction, SMS was filtered and washed with DMF. Elemental analysis: C 5.00%; H 0.67%; N 1.08% for APS; C 13.24%, H 1.76%, N 2.94% for SMS. The modification ratio of surfactin on APS was estimated from the value of nitrogen by elemental analysis as follows: 29.4 (mg/g) / 14x8 = 0.26 (mmol/g). Surfactin involves 7 nitrogen atoms and aminopropyl group on APS involves 1 nitrogen atom.

2.3 *Chromatography*

A slurry of SMS in the mixture of glycerine and methanol (1/5, v/v) was prepared with

ultrasonication (1 min) and was pumped into an HPLC column (150 mm x 1.5 mm I.D.) at 35 MPa using an HPLC pump, methanol (flushing solvent) and stainless-steel reservoir (75 x 6 mm I.D.). During packing, the pressure decreased because glycerine was flushed out from the packed column. Then, the packed HPLC column was flushed with methanol at 1.0 mL/min for 30 min; at 0.5 mL/min for 30 min; finally, at 0.2 mL/min overnight.

The HPLC system included a Shimadzu LC-20AD pump, SPD-20A UV detector and CR-8A recorder (Kyoto, Japan). Flow rate was set at 0.2 mL/min with UV detection at 260 nm. All aqueous solutions were made with the water that was deionized and distilled using WG 203 (Yamato Scientific, Tokyo, Japan) and then passed through a water purification system (Puric-Z, Organo, Tokyo, Japan).

The column efficiency N was calculated from the number of theoretical plates per meter:

$$N = 5.55 \times (t_r / w_{0.5})^2$$

where t_r is retention time of analytes; $w_{0.5}$ and peak width at half height. A measure of the symmetry of a peak, given by the following equation:

$$S = W_{0.05}/2f$$

where S is symmetry factor, $W_{0.05}$ is the peak width at 5% height and f is the distance from peak front to apex point at 5% height.

3. Results and discussion

3.1 Retention properties in RPLC mode

The influence of ACN content in the mobile phase on the retention of hydrophobic compounds

was studied by varying the percentage of ACN from 30% to 70%. Fig. 2 depicts the plots of log *k* and the percentage of ACN. The retention of test compounds decreased as the content of ACN increased. A linear relationship between log *k* and ACN content was obtained, typical of the RPLC mode in which the retention of hydrophobic compounds is dominated by hydrophobic interactions. Fig. 2 also shows that the retention of test compounds increased with their increasing hydrophobicity. The retention factors of test compounds on SMS stationary phase were one-third of those on RP C8 stationary phase (data not shown), but were stronger than the hydrophobic retention on bare silica, conventional diol and long alkyl hydroxyl group-bonded stationary phases [3, 4].

3.2 Retention properties in HILIC mode

HILIC separation commonly employs a hydro-organic mobile phase with an organic content above 60%. The effect of ACN content in the mobile phase on the retention of polar compounds (nucleic acids, nucleosides, vitamins and xanthines) was investigated (Fig. 3). The retention factors increased, either drastically or slightly, when the ACN content increased from 90% to 98%, which is typical of HILIC retention behavior. Hence, SMS stationary phase acts as an HILIC phase at high ACN content. A representative chromatogram is shown in Fig. 4. For the 13 tested polar compounds, the column efficiency ranged from 2300 to 11000 (*N*/m) and the symmetry factors ranged from 1.0 to 2.1. Furthermore, retention times and elution orders of tested compounds on SMS column were compared with those on silica gel column. Retention factors for 10 analytes on SMS column was larger than those on silica gel column, especially retention factors for uridine, thiamine and rivoflavin were more than 4-fold larger (data not shown). The elution

orders were fully different from each column.

3.3 RPLC/HILIC mixed-mode

RPLC/HILIC mixed-mode retention behavior was investigated with a set of moderately polar and polar compounds. As shown in Fig. 5, SMS stationary phase provided a “U-curve” retention profile, an indicator of RPLC/HILIC mixed-mode retention behavior [2, 5]. The retention time of the test compounds decreased with an increase in ACN content at low and intermediate contents, according to the RPLC mode. However, the retention times increased as the ACN content increased from 80% to 95%, indicating that retention was governed by hydrophilic interactions between the stationary phase and the compounds. An ACN content of about 50% affords the weakest retention and is the boundary between the two retention modes. This feature may provide greater flexibility in real sample analyses compared to conventional RPLC and HILIC columns.

Caffeine, 1,7-dimethylxanthine and 1-methylxanthine are purine derivatives with different numbers of methyl groups. Despite their methylated sites, they retain a degree of polarity. This makes them suitable for separation by HILIC, although their separation is commonly performed by RPLC [4, 14]. Their separation in RPLC mode (5% ACN) caused these analytes to be eluted in the order of their hydrophobicity; thus, caffeine showed the strongest retention (data not shown). These compounds could not be well resolved in RPLC mode. On the other hand, by changing the stationary phase, the elution order could be reversed, and good separation was obtained in HILIC mode (95% ACN). This suggests that the SMS column can be used for both RPLC and HILIC separation modes simply by changing the mobile phase composition.

3.4 Comparison between SMS and APS

In order to illustrate the impact of surfactin modification to APS, the retention factors and the elution orders of test compounds on APS-packed column were studied in both RPLC and HILIC modes. In RPLC mode, hydrophobic compounds (benzene, naphthalene and alkylbenzenes) were hardly retained on APS column even though mobile phase with high water content (50%) was used. In HILIC mode, among 12 test polar compounds (nucleic acids, nucleosides, vitamins and xanthines), the elution orders of 4 early eluting (caffeine, 4,5-dimethyl-1,2-phenylenediamine, thymine and uracil) on APS were same as SMS and their retention factors on APS were almost same as SMS. However, the elution orders of the other compounds were clearly different between SMS and APS. Furthermore, the retention factors of thymine, uridine and pyridoxine on SMS were 27%, 56%, 26% smaller than those on APS, while the retention factors of adenosine, thiamine, adenine and riboflavin on SMS were 1.1-, 6.3-, 1.5- and 2.1-fold larger than those on APS. Based on these observations, the modification of surfactin to APS was found to contribute to the characteristic mixed-mode retention behavior.

Conclusion

This is the first report to use a biosurfactant for modifying a silica stationary phase. SMS stationary phase was synthesized and characterized in RP and HILIC mode, and shown to function in both modes. The retention of polar solutes depended on the ACN content and exhibited “U-shaped” curves, an indicator of RPLC/HILIC mixed-mode retention behavior. The SMS column may be useful for both RP and HILIC mode separations, providing flexibility for real sample analyses.

6 References

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Figure captions

Fig. 1 Surfactin-modified silica stationary phase.

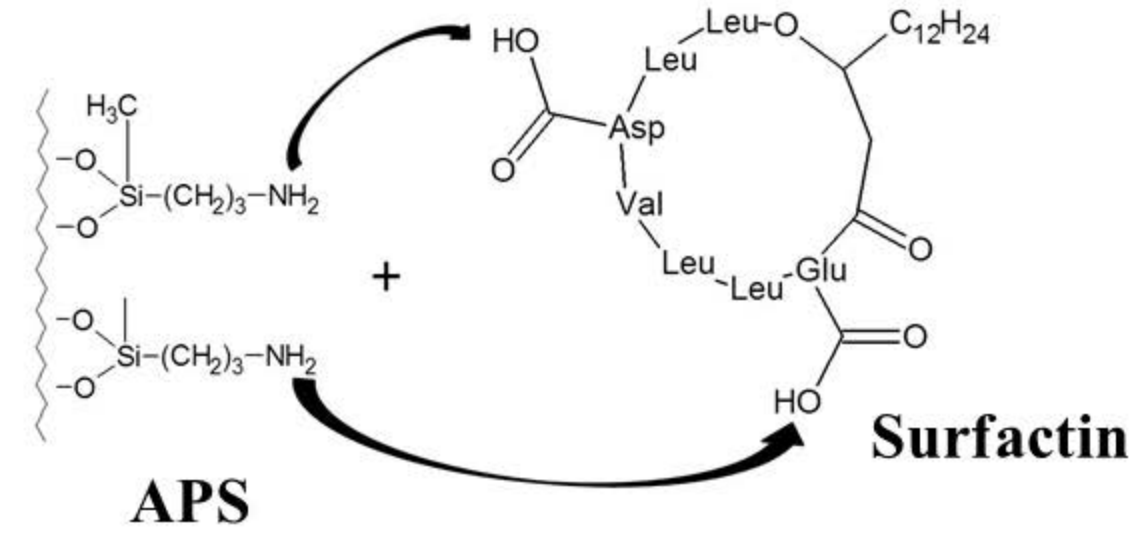
Fig. 2 Effect of ACN content in the mobile phase on log *k*. Conditions: mobile phase, Tris-HCl buffer (pH 7.0)/ACN; flow rate, 0.2 ml/min; detection wavelength, 260 nm; injection volume, 5 μl.

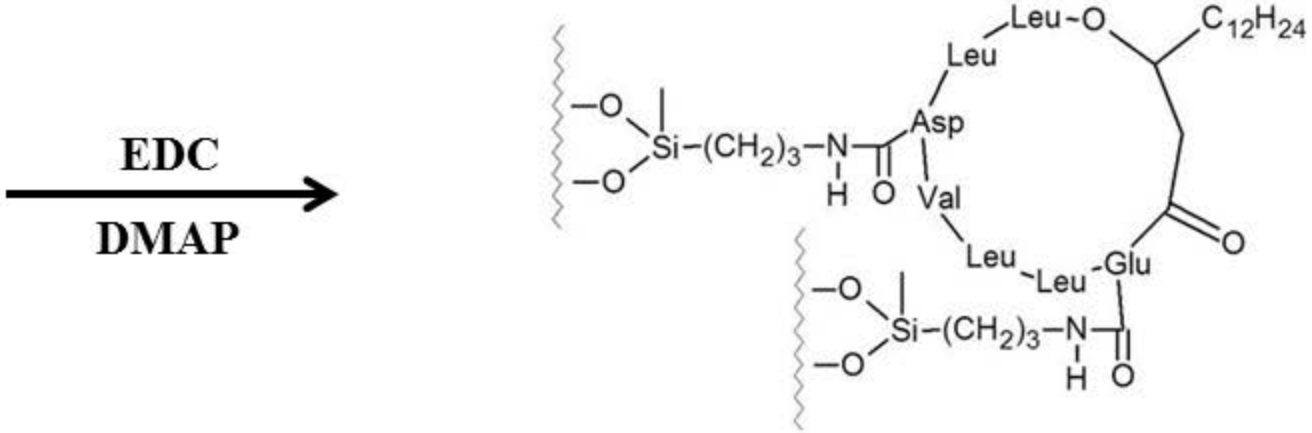
Fig. 3 Effect of ACN content in the mobile phase on the retention factors. Conditions are the same as those given in Fig. 2.

Fig. 4 Separation of test compounds. Conditions: mobile phase, Tris-HCl buffer/ACN=5/95 (v/v %). Other conditions are the same as those given in Fig. 3. Peaks (retention time, min): 1, aniline (1.43 min); 2, caffeine (1.52 min); 3, thymidine (2.23 min); 4, uridine (3.09 min); 5, thiamine (3.84 min); 6, adenine (6.30 min); 7, riboflavin (6.39 min); 8, cytosine (8.64 min).

Fig. 5 Effect of ACN content in the mobile phase on retention times. Conditions: mobile phase, 5 mM ammonium acetate (pH 5.0)-ACN. Other conditions are the same as Fig. 4.

Fig. 1





Surfactin-modified silica

Fig. 2

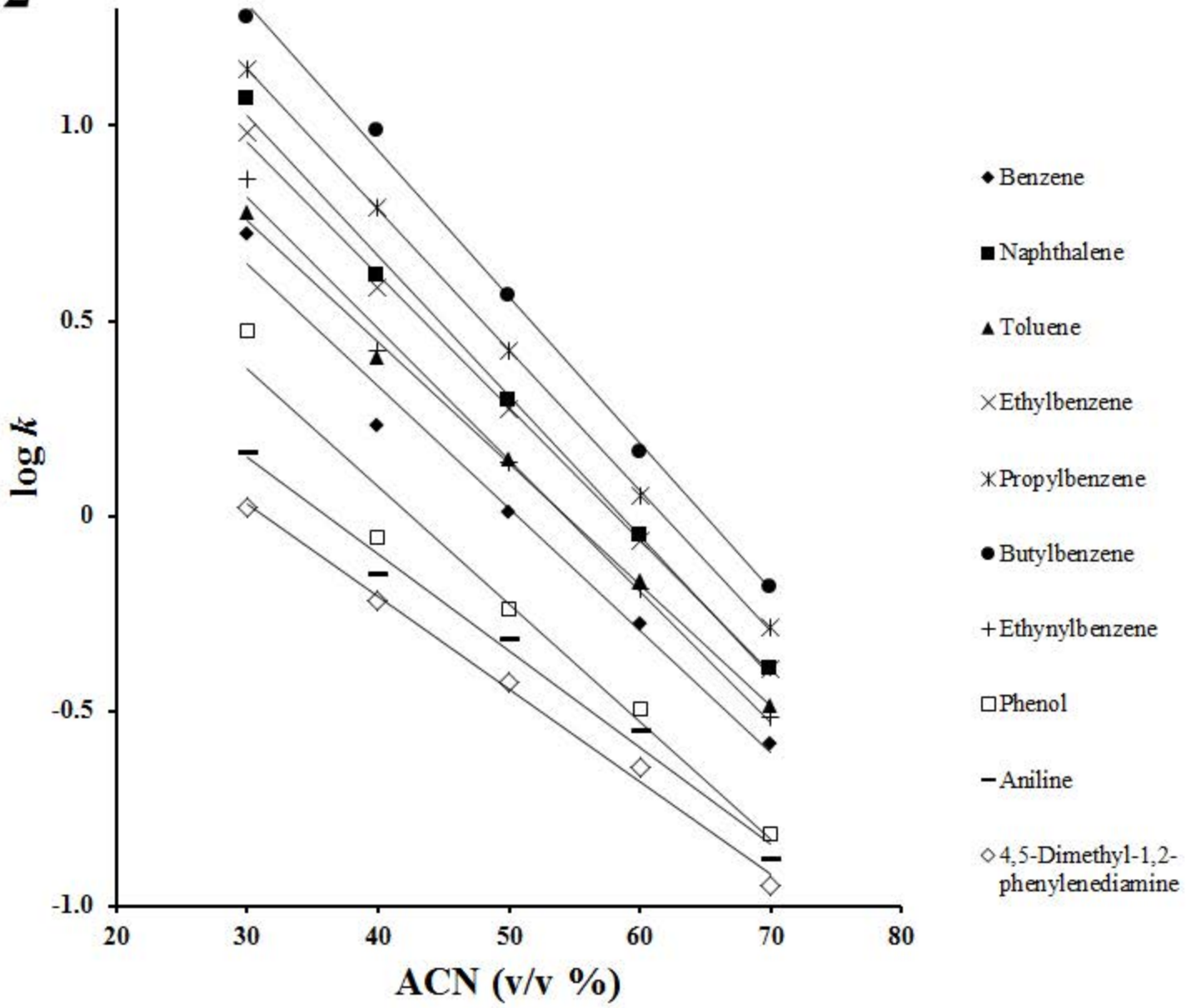

1.0
0.5
0
-0.5
-1.0
log k
20
30
40
50
60
70
80
ACN (v/v %)
Benzene
Naphthalene
Toluene
Ethylbenzene
Propylbenzene
Butylbenzene
Ethynylbenzene
Phenol
Aniline
4,5-Dimethyl-1,2-phenylenediamine

Fig. 3

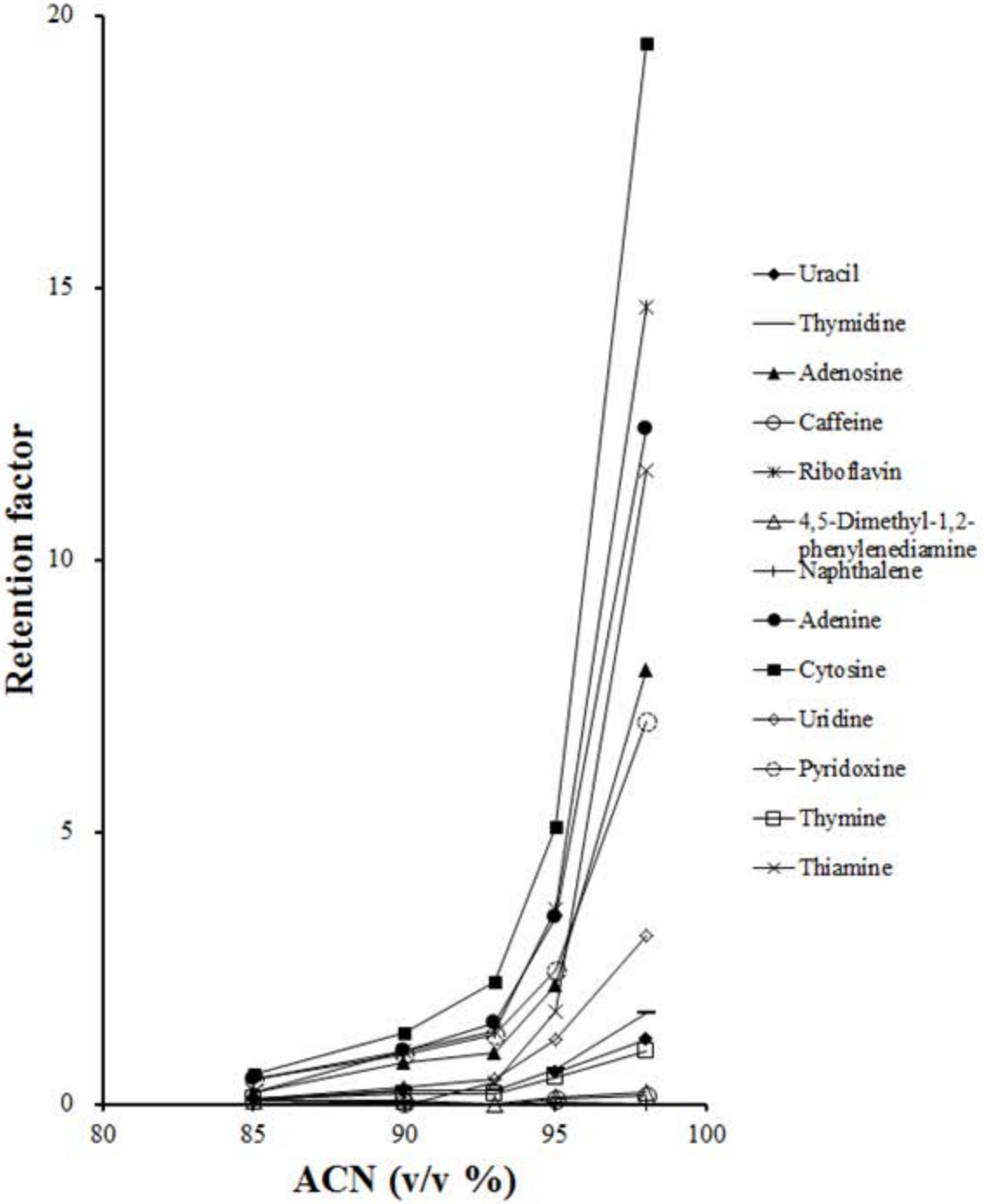

20
15
10
5
0
Retention factor
80
85
90
95
100
ACN (v/v %)
Uracil
Thymidine
Adenosine
Caffeine
Riboflavin
4,5-Dimethyl-1,2-phenylenediamine
Naphthalene
Adenine
Cytosine
Uridine
Pyridoxine
Thymine
Thiamine

Fig. 4

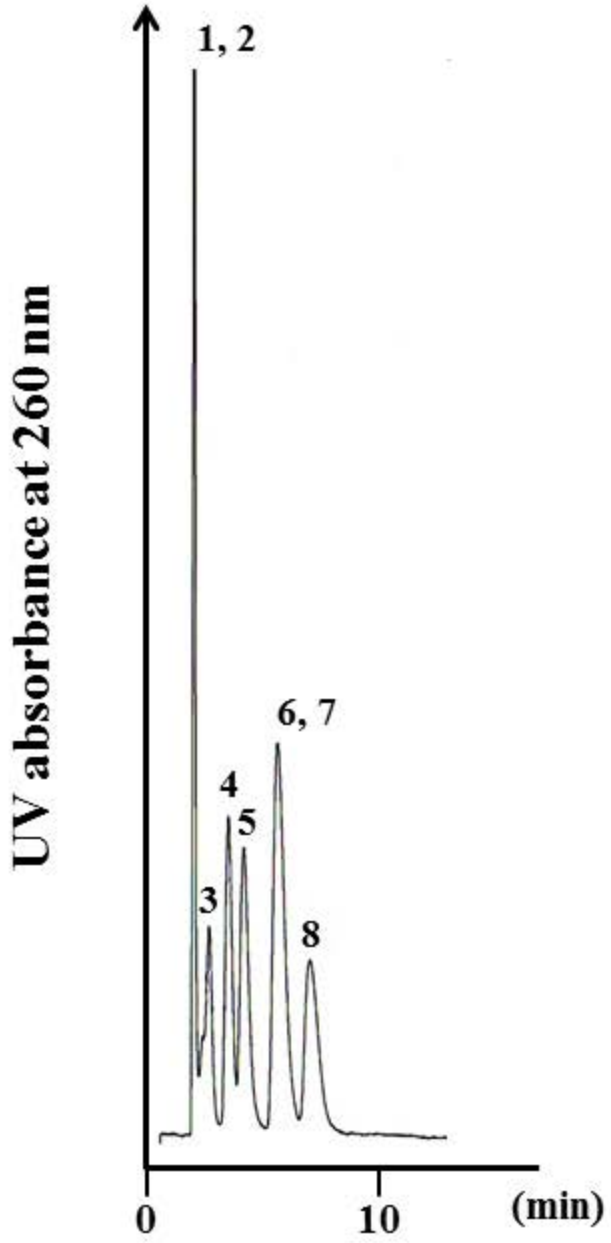
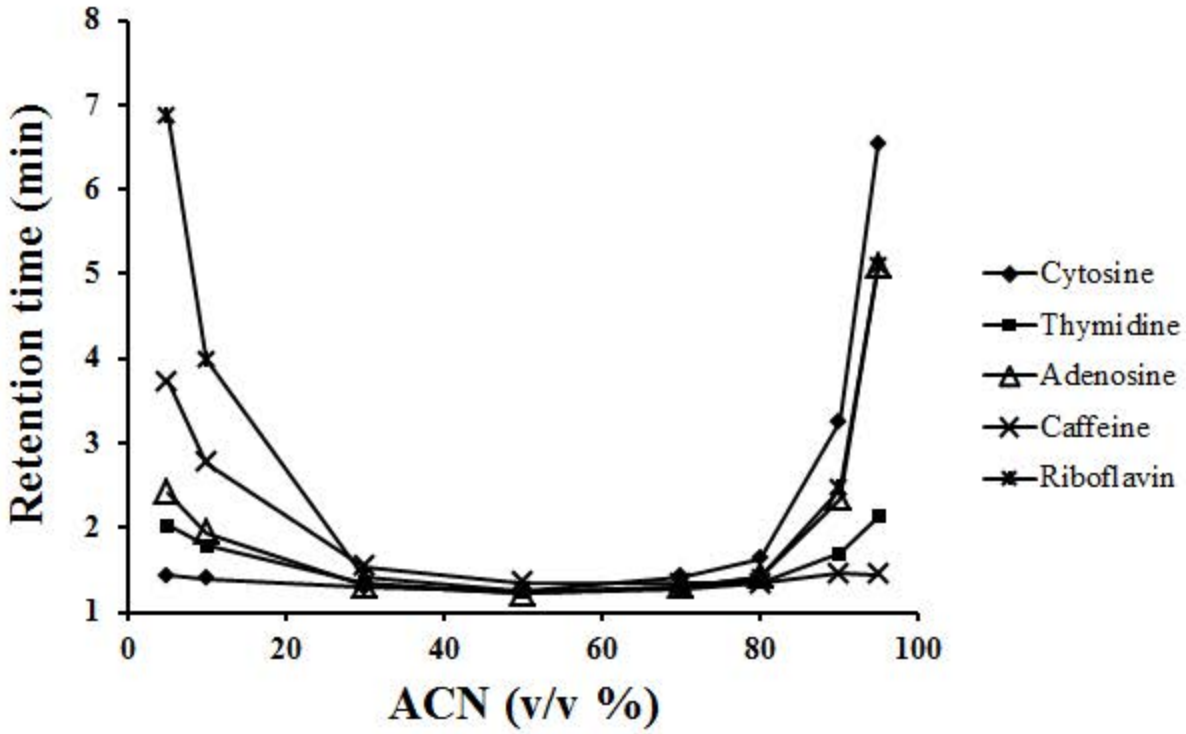


Fig. 5


Retention time (min)
8
7
6
5
4
3
2
1
0
20
40
60
80
100
ACN (v/v %)
Cytosine
Thymidine
Adenosine
Caffeine
Riboflavin