

UNIVERSITY OF PÉCS

Doctoral School of Chemistry

**Investigation of retention mechanism of cavitands in
reversed phase chromatography and the study of
the adsorption of water in hydrophilic interaction
liquid chromatography**

PhD thesis

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1. Introduction

The chromatographic behavior of large molecules such as resorcinarene-based cavitands is an unexplored field of liquid chromatography. The retention behavior depends on the structure of the analyte and the quality of the mobile and stationary phases inclusive of the functional groups of the solute and stationary phase. The enthalpy and entropy changes are estimated by analyzing the temperature dependence of k . The linear van't Hoff plot suggests that the retention mechanism for the analyte is constant over the temperature range studied. The retention mechanism is very complex so linear chromatography does not give detailed information on it. With non-linear chromatography, the adsorption isotherm of the analyte can be determined by the overloading experiments. For the determination of the adsorption isotherms, the most commonly used methods are the frontal analysis. However, the inverse method is popular too, due to the lesser solute consumption and reduced experimental time. It is a numerical method for isotherm determination and estimates adsorption isotherm parameters by fitting simulated elution profiles to experimental ones.

In reversed-phase liquid chromatography the influence of pressure on retention assuming constant flow rate arises from many sources, which include the expansion of the column tube, the compressibility of the mobile and the stationary phases, the change of the partial molar volume of the analyte and structural alteration of the alkyl-bonded layer of the silica. The change of molar volume of the analyte is associated with its passage from the mobile to the stationary phase. If the molar volume change is negative, i.e. the solute has a smaller volume in the stationary phase than in the mobile phase the retention of the analyte should increase with pressure according to the Le Chatelier's principle.

It is generally agreed that in HILIC the retention mechanism involves mostly partitioning between the largely organic mobile phase and a water-rich layer mobile phase on the surface of stationary phase. The water uptake from acetonitrile-water (ACN:H₂O) mobile phases can be studied by frontal analysis using coulometric Karl-Fischer titration. There is no fixed boundary between the adsorbed water-rich layer and the bulk mobile phase. Thus, the amount of water in the stationary phase can be expressed as the volume fraction of excess adsorbed.

2. Aims and overview

The understanding of the retention behavior of medium or large molecules is an area of interest in liquid chromatography. The investigation of the chromatographic behavior of resorcinarenes and cavitands is meager up to now. To understand the chromatographic behavior of cyclic oligomers (such as resorcinarenes and cavitands) the temperature and pressure dependence of retention factor of these analytes were investigated. Furthermore, the thermodynamics of the retention in acetonitrile-water and methanol-water mobile phase for cyclic oligomers were compared. Moreover, their retention mechanism was investigated by generating of adsorption isotherm, applying nonlinear chromatographic methods

Besides the investigation of retention mechanism of resorcinarenes and cavitands on reverse stationary phases, the adsorption of water in hydrophilic interaction chromatographic system has studied, on Cogent Silica C and Cogent Phenyl hydride stationary phases at different temperatures

The main objectives of this PhD research were:

- determination the thermodynamics (changes of enthalpy, entropy, and the Gibbs-free energy) of the retention of resorcinarenes (HRM, MRM) and cavitands (HCM, MCM) in different reverse phased chromatographic system,
- determination the equilibrium adsorption isotherms parameters of resorcinarene (HRM) and cavitand (MCM) by inverse method and compare the Henry constants obtained from adsorption isotherm determination and thermodynamics determination,
- exploration the influence of the change of molar volume on the retention of resorcinarene (HRM) and cavitand (MCM) on alkylsilyl and polar-embedded C8 and C18 reversed phases,
- investigation the adsorption of water in aqueous normal-phase liquid chromatography at different temperature.

3. Experimental

3.1 Chemicals and analytes

Acetonitrile, methanol, tetrahydrofuran (gradient LC grade) and water (gradient LC grade). In water adsorption experiments water was purified using a Milli-Q water purification system – Millipore, Bedford, MA, USA) as well as thiourea, toluene, polystyrene standard ($M_w = 1\,800\,000\text{ g/mol}$) and Coulomat AG (a reagent for coulometric Karl Fischer titration) were purchased from Sigma-Aldrich (Steinheim, Germany).

The macrocyclic test compounds were C-tetra-methylcalix[4]resorcinarene (HRM); 2-methyl-C-tetra-methylcalix[4]resorcinarene (MRM); Cavitand (HCM) and methyl-cavitand (MCM) – shown in Figure 1 – were from the Inorganic Chemistry Department of University of Pécs.

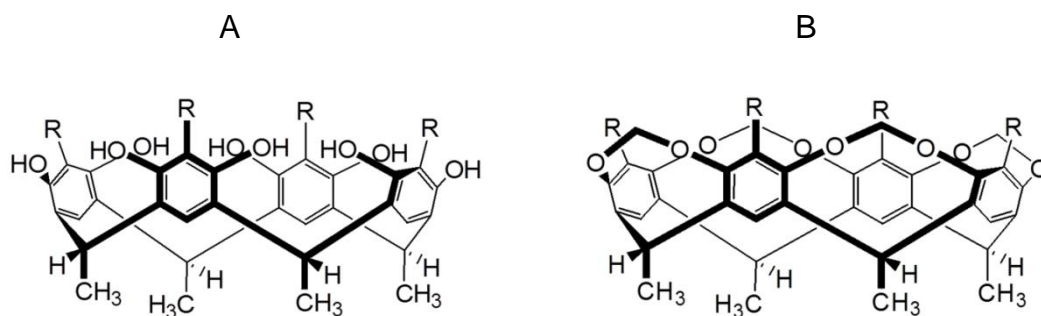


Figure 1. Structures of resorcinarenes and cavitands. The ligands are (A) R = H in HRM R = CH₃ in MRM and (B) R = H in HCM and R = CH₃ in MCM.

The employed columns:

- Hypersil BDS C₈ and C₁₈ (100 × 4.6 mm; particle size 3 μm)
- XTerra RP₈ and RP₁₈ (100 × 4.6 mm; particle size 3.5 μm)
- Cogent Silica C (150 × 4.6 mm; particle size 4 μm)
- Cogent Phenyl hydride (150 × 4.6 mm; particle size 4 μm)

The hold-up times in case of cavitand experiments were determined with thiourea. In water adsorption investigation the hold-up times were determined from injections of toluene in 100% tetrahydrofuran.

3.2 Thermodynamic analysis conditions

The measurements were performed on an Agilent 1290 Infinity HPLC (Agilent Technologies, Waldbronn, Germany) system with a binary pump, a column oven and an

autosampler. The instrument was equipped with a diode array UV-VIS detection system.

The isocratic elution was carried out with ACN:H₂O 55:45 (v/v%), MeOH:H₂O 72:28 and 88:12 (v/v%), the solvents were degassed by ultra-sonication before usage. The mobile phase was delivered at a flow-rate of 1 mL/min. The experiments were carried out at 15, 25, 35, and 45°C and the precision of the temperature adjustment was $\pm 0.1^\circ\text{C}$. The injection volume was 2 μL and triplicate injections were applied. The detection was accomplished at 230 nm. The samples were dissolved in the mobile phase. The concentrations of all samples were 0.5 mg/mL.

3.3 The pressure induced retention changes analysis conditions

These measurements were also performed on the before mentioned Agilent 1290 Infinity HPLC system. The isocratic elution was carried out with MeOH:H₂O 72:28(v/v%) for HRM and 88:12 (v/v%) for MCM taking into account the solubility of the analytes. The solvents were degassed by ultra-sonication before use. Mobile phase was delivered at a flow-rate 0.1 mL/min. The column temperature was adjusted at 25 °C. The injection volume was 2 μL and triplicate injections were applied. The detection was accomplished at 230 nm. The samples were dissolved in the mobile phase. The concentration of each sample was 0.1 mg/mL. The experiments were carried out in the range of 1-320 bar.

3.4 Overloading experiments conditions

The overloaded band profiles were acquired using a Shimadzu HPLC system with two LC-10AD pump, and an SPD-M10A VP diode array UV-VIS detector. Data acquisition, data handling and instrument control were performed with the LabSolutions (Shimadzu) software.

The mobile phases were MeOH:H₂O 72:28 and 88:12 (v/v%) and were degassed by ultra-sonication before use. The measurements were carried out at 25 °C. Manual injector was used. The injection volume was 200 μL . The concentration of MCM was 0.6 g/L in MeOH:H₂O 88:12 (v/v%) and that of HRM was 3 g/L in MeOH:H₂O 72:28 (v/v%).

3.5 Water adsorption experiments condition

All the water adsorption experiments were executed using an HPLC setup including a high-pressure pump (ECOM, Prague, Czech Republic) connected with a variable

wavelength UV detector from the same manufacturer and an automated fraction collector (CF-1 Fraction Collector, Spectrum Chromatography, Houston, TX, USA). The detection wavelength was set to 210 nm. The chromatographic column was placed in a column oven compartment.

The temperature in the experiments was gradually increased up to the limits given by the column manufacturer: 40 °C, 60 °C, 80 °C, 100 °C in case of Cogent Silica C, and 40 °C, 60 °C, 80 °C in case of the Cogent Phenyl hydride columns, respectively. The concentration of water in the collected fractions was determined using an 831 KF Coulometer, a coulometric Karl Fischer titrator equipped with a 728 Ti Stand-magnetic stirrer (Metrohm, Herisau, Switzerland). An LC0101 (ECOM, Prague, Czech Republic) still air column thermostat was used at each experiment.

4. Results

A huge difference was found between the retention of resorcinarenes and cavitands. In some cases, the retention factor of cavitands was even a hundred times larger than that of resorcinarenes. The retention of methylated derivatives (MRM, MCM) was two-to-four times larger than that of demethylated compounds (HRM, HCM) on every column. Based on the results, it is clear that the transfer of the resorcinarenes and cavitands from the mobile phase to the stationary phase is enthalpy-controlled process because the enthalpic contribution for all analytes was found more significant than the entropic contribution of those.

In MeOH:H₂O mobile phase, the hydrogen bonds between the solvated analytes and methanol molecules adsorbed on the surface dominated, therefore every stationary phase was more retentive for resorcinarenes containing hydroxyl groups than the acetonitrile containing mobile phase. In ACN:H₂O eluent the increase of the entropic contribution had a big role to the retention because of the thick adsorbed acetonitrile layer, which was significant in the case of apolar cavitands. The effect of polar embedded groups of the XTerra column could manifest in the ACN:H₂O eluent. There was no difference between the enthalpy changes of resorcinarenes on BDS and XTerra columns in methanol-containing mobile phase.

The retention behavior of resorcinarenes and cavitands was investigated by nonlinear chromatography too. The inverse method was chosen for the determination of adsorption isotherms as the solubility of the examined resorcinarene (HRM), especially the cavitand (MCM) was very poor. The Langmuir isotherm represented well the experimental data, which corresponds to a homogeneous adsorbent. The calculated adsorption–desorption equilibrium constant of HRM and MCM was three times and twice larger on the C₁₈ than on the C₈ column, respectively. The *b* isotherm parameter relates to the adsorption energy of the analytes. According to expectations, the results refer to a larger adsorption energy between the analytes and the octadecyl chains than the octyl chains of the stationary phase. The saturation capacity of HRM was twice larger but this parameter of MCM was smaller on the C₈ than on the C₁₈ column. The Henry constant (*K*) of MCM was approximately 28 times larger than the *K* of HRM on the C₁₈ column. On the C₈ column, the value of *K* of MCM was 15 times larger than that of HRM. The *K* values of HRM and MCM calculated by linear and nonlinear chromatography showed a good agreement.

In reversed-phase chromatography pressure generally increases retention. The retention factors of the more polar resorcinarene (HRM) and the apolar cavitand (MCM) increased with the increase of the average column pressure (1-400 bar) on each stationary phase. The molar volume changes of HRM calculated from the slope of the plot of $\ln k$ versus p (column pressure) were between $-\Delta V_m = 9-19$ mL/mol and these values for MCM were between $-\Delta V_m = 5-12$ mL/mol. The larger molar volume changes of resorcinarene are mostly due to the larger loss of its solvation layer and furthermore its conformational changes when it interacts with the apolar ligands of the stationary phase. Both types of the analytes had twice larger molar volume change on the Hypersil BDS than on the XTerra C₈ or C₁₈ columns.

In water adsorption experiments, the temperature dependence of hydrophobicity was studied on two different types of hydrosilated silica stationary. At full column saturation, the adsorbed water fills 4.02–7.57% pore volume over the studied temperature range, which approximately corresponds to the equivalent of 0.24–0.68 water layer coverage of the adsorbent surface. On the Silica C column, the adsorbed monomolecular water layer increase with increasing temperatures whereas on the Phenyl Hydride column the saturation capacity and the adsorbed monomolecular water layer are not significantly affected by the temperature.

5. Thesis points

1. Based on the determined thermodynamics parameters, the transfer of the resorcinarenes and cavitands from the mobile phase to the stationary phase is enthalpy-controlled process because the enthalpic contribution for all analytes was found more significant than the entropic contribution of those.
2. In ACN:H₂O system the huge difference between the retention of resorcinarenes and cavitands is due to the increase of the entropic contribution of cavitands, which causes the more negative values of Gibbs free energy changes on different stationary phases although the enthalpy changes are comparable. The increase of entropic contribution is the reason of the large retention of methylated derivatives, particularly in the case of MCM, for which the entropy changes (ΔS) are one magnitude smaller than that of the demethylated derivatives on every column.
3. Applying methanol modifier in the mobile phase, the enthalpy changes of the analytes became more favorable but the entropic contributions became more unfavorable compared to that in ACN:H₂O.
4. The determined adsorption–desorption equilibrium constant of HRM and MCM was three times and twice larger on the C₁₈ than on the C₈ column, respectively.
5. It is found that the molar volume changes for both the apolar and more polar analytes were twice larger on the Hypersil BDS than on the XTerra columns and they were independent of the length of the alkyl chains of the stationary phases.
6. In case of the temperature dependence of hydrophobicity experiment, the adsorbed monomolecular water layer was calculated. It enables a comparison of the uptake of excess water.

6. Publications

6.1 Publications related to the thesis

1. I. Prauda, **E. Bartó**, and A. Felinger, "Influence of pressure on the retention of resorcinarene-based cavitands," *Journal of Chromatography A*, vol. 1535, pp. 123-128, 2018. Impact Factor: 3.981
2. **E. Bartó**, A. Felinger, and P. Jandera, "Investigation of the temperature dependence of water adsorption on silica-based stationary phases in hydrophilic interaction liquid chromatography," *Journal of Chromatography A*, vol. 1489, pp. 143-148, 2017. Impact Factor: 3.981
3. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger, "Investigation of retention mechanism of resorcinarene based cavitands by linear and nonlinear chromatography," *Journal of Chromatography A*, vol. 1456, pp. 152-161, 2016. Impact Factor: 3.926
4. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger, "Retention behavior of resorcinarene-based cavitands on C₈ and C₁₈ stationary phases," *Journal of Separation Science*, vol. 38, no. 17, pp. 2975-2982, 2015. Impact Factor: 2.741
5. **E. Bartó**, A. Felinger, F. Kilár, I. Prauda, and I. Kiss, "The Temperature Dependence of the Retention Factor of Cavitands on C₁₈ and C₈ Reversed Stationary Phases in HPLC," *Műszaki Szemle*, vol. 63, pp. 17-22, 2013.

6.2 Posters and presentations related to this thesis

1. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger; Kavítandok nemlineáris izotermájának meghatározása különböző fordított fázisú állófázisokon az inverz módszer segítségével; 20th International conference on chemistry, November 6-9, Kolozsvár (Cluj-Napoca, Romania), 2014
2. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger; Kavítandok retenciósfaktorjának hőmérséklet függése különböző fordított fázisú állófázisokon; 19th International conference on chemistry, November 21-24, Nagybánya(Baia-Mare, Romania), 2013
3. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, V. Sándor and A. Felinger; Study of resorcin[4]arene derivatives in Reversed-Phase High-Performance Liquid Chromatog-

- raphy coupled Mass Spectrometry; 10th János Szentágothai Transdisciplinary Conference and Student Competition, November 4-5, Pécs (Hungary) 2013
4. **E. Bartó**, A. Felinger, and P. Jandera; Investigation of Temperature Dependence of Adsorption of Water from Aqueous Acetonitrile on Silica Based Stationary Phases; 10th Balaton Symposium on High-Performance Separation Methods, September 6-8, Siófok (Hungary) 2015
 5. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger; Kavítandok retenciósjelenségének túlterheléses vizsgálata különböző állófázisokon; Elválasztástudományi Vándorgyűlés, October 12-14, Egerszalók (Hungary) 2014
 6. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger; Investigation of the retention phenomena of cavitands by column overload; 30th International Symposium on Chromatography, September 14-18, Salzburg (Austria) 2014
 7. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger; Temperature dependence of the retention factors of resorcinarene based cavitands on C₈ and C₁₈ reversed stationary phases; 30th international symposium on microscale bioseparations, April 27 – May 1, Pécs (Hungary) 2014
 8. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, V. Sándor and A. Felinger; Analysis of resorcin[4]arene-based cavitand on different stationary phases; 9th Balaton Symposium on High-Performance Separation Methods, September 4-6, Siófok (Hungary) 2013
 9. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, V. Sándor and A. Felinger; Analysis of cavitand derivatives with High Performance Liquid Chromatography – Mass Spectrometry; 39th International Symposium on High Performance Liquid Phase Separations and Related Techniques, June 16-20, Amsterdam (Netherlands) 2013
 10. **E. Bartó**, I. Prauda, F. Kilár, I. Kiss, and A. Felinger; Application of Cavitand Derivatives on High Performance Liquid Chromatography; 10th International Interdisciplinary Meeting on Bioanalysis, April 25-27, Pécs (Hungary) 2013