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Fundamentals of retention and mass flow in supercritical fluid chromatography

PhD thesis

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1. Introduction

Supercritical fluid chromatography (SFC) has gathered increased attention in a wide range of fields, including several academic and industrial areas. The technique has been employed extensively in analytical and preparative chromatography, forensics, chiral separations, pharmaceutical applications and the food industry. Fundamental research is a smaller part of the SFC community focusing on topics such as the effects of the mobile phase density or sample solvent and organic modifier adsorption. The reputation of SFC can be associated with the rapid technological advancements of the last decade that made it a highly viable and comparable, but in the end a complementary technique besides liquid chromatography (LC).

SFC is usually characterized by having three major benefits over LC that can be derived from the physico-chemical properties of carbon dioxide: (1) it is more cost-effective and environmentally friendly compared to LC due to lower organic solvent consumption; (2) the low viscosity of the mobile phase enables higher flow rates and thus shorter separations while also allowing rapid diffusion processes, reduced band broadening effects hence increased efficiency and (3) eluent strength is considered to be “well-tunable” by adjusting temperature, pressure and the concentration of the organic modifier in the mobile phase.

In SFC, the mobile phase is primarily composed of carbon dioxide besides the organic modifier and other additives. The solvents most often employed in LC are generally considered incompressible from a practical point of view. However, this is not the case for SFC due to the compressibility of carbon dioxide which results in the variation of a series of thermodynamic and chromatographic properties along the system, e.g. mobile phase density, viscosity, temperature, velocity, solvation strength, retention factors, column efficiencies. This introduces several difficulties to the work of chromatographers working with SFC, which requires a deeper understanding and careful approach to resolve those effects, especially if no organic modifier is present in the mobile phase.

It is also understood that in SFC, the set and true volumetric flow rates differ from each other, that can become an important issue when trying to translate retention times and hold-up times into retention volumes and hold-up volumes, respectively. There are two requirements to do this conversion. One is the mass flow rate, that is the only flow parameter considered to remain constant throughout an SFC system. Therefore, it can be utilized very well to determine actual volumetric flow rates by accurate, but careful measurements. The other would be the accurate knowledge of the hold-up time, another often overlooked parameter, whose determination has always been problematic in SFC, since the methods available are not as universal as in liquid chromatography.

2. Research objectives

The aim of the dissertation is to delve into the fundamentals of SFC, more specifically topics related to uncommon retention behavior observed in the case of neat carbon dioxide mobile phases, then the challenges of accurate mass flow rate measurements and lastly, exploring the options and limitations of hold-up time measurements. Our research objectives are described in detail as follows:

1. Studying the uncommon retention behavior of n-alkylbenzene homologues and the effect of different sample solvents on chromatographic efficiency:
    a) screening of different stationary phases and sample solvents (acetonitrile, heptane and methanol) to find anomalous retention behavior;
    b) determination of the single-component adsorption isotherms using the bi-Langmuir model and the inverse method (IM);
    c) determination of the competitive bi-Langmuir adsorption isotherms to understand the competition of the sample solvents and solutes;
    d) construction of a numerical model in order to simulate the competitive adsorption in the case of the real and two hypothetical compounds.
2. Scouting the effects of placement, experimental conditions and injections on mass flow measurements:
    a) studying the effect of placing the Coriolis flow meter (CFM) and pressure gauge at different positions in the SFC system;
    b) studying the effect of pressure and temperature on mass flow rate;
    c) studying the effect of injections on the mass flow rate at equilibrium.
3. Exploring the limitations of hold-up time measurements including the study of nitrous oxide as a new unretained marker:
    a) comparison of different compounds previously used as unretained markers in SFC;
    b) studying the effect of the amount of organic modifier in the mobile phase;
    c) surveying whether the stationary phase has any effect on the detection of hold-up time markers.

3. Materials and methods

Instruments

The experiments were performed using a Waters ACQUITY UPC^2 system (Milford, MA, USA). The instrument was equipped with a binary solvent delivery pump, an autosampler with a 10 μL sample loop, a column thermostat, a photodiode array (PDA) detector and a back pressure regulator. The instrument was controlled by the Empower 3 chromatography data software. The extra-column volume of the instrument was 60 μL from the loop to the detector cell and was measured by replacing the column with a zero-volume connector. All retention volumes were corrected for this contribution. Multiple dynamic leak tests were performed for the CO_2 and solvent pumps to verify they are not leaking. Both the accumulator and primary heads passed the tests.

Some experiments were performed using an Agilent 1260 Infinity II SFC System equipped with a binary pump, multisampler, multicolumn thermostat, diode array detector (DAD) and SFC control module. The instrument was controlled by the Agilent ChemStation software.

Mass flow rate of the mobile phase was measured with a mini CORI-FLOW mass flow meter from Bronkhorst High-Tech B.V. (Ruurlo, Netherlands), Model No. M13-ABD-11-0-S, Serial No. B11200776A. This model provides an accuracy of ± (0.2% of the read value + 0.5 g/h), expressed as a sensitivity of 0.01 g/min of CO_2.

The calibration of the mass flow meter was verified by disconnecting CO_2 from the binary solvent delivery system, then pumping water through the CFM at 0.50, 1.00 and 1.50 mL/min set flow rates for 5 minutes at room temperature and collecting the water in a pre-weighed container. In each case, the CFM readings were within the acceptable range of the expected mass flow rates.

Pressure values at the inlet and outlet of the column were recorded using a DPG4000 external pressure gauge from OMEGA Engineering (Norwalk, CT, USA).

Experiments

Competitive adsorption studies

In the early stages of the competition studies, several stationary phases were tested for uncommon retention behavior. Eventually, a 4.6 × 150 mm Supelcosil ABZ+Plus alkylamide column packed with 3 μm particles from Sigma-Aldrich was chosen for further studies. The total volume of the column was V_{tot} = 2.492 cm^3. The void volume was estimated by two methods, the weight-difference method, that usually gives an underestimation, and with the help of heptane used as

an unretained marker. Both approaches gave very similar results so V_0 = 1.590 cm^3 was used in the end. The total porosity was calculated to be ε_t = 0.638.

Calculation of the mobile phase densities was based on the column thermostat temperature and the inlet and outlet pressures of the column measured directly using the external pressure gauge. The densities were calculated using the National Institute of Standards and Technology (NIST) REFPROP database. The mean volumetric flow rate ($\bar{F}_v$) was estimated from the measured mass flow rate of the mobile phase recorded downstream the mixer and the average density ($\bar{\rho}$), calculated as the arithmetic mean of the densities at the inlet and outlet of the column.

The chromatographic measurements were carried out with 100% CO_2 mobile phase. The volumetric flow rate was set at 1.00 mL/min, the actual flow rate was calculated to be 1.18 mL/min at 60 °C and 150 bar back pressure. The column temperature was varied between 35 and 60 °C, the back pressure regulator was set at either 105, 150 or 200 bar. The injection volume was 2.0 μL. The detector signal of the alkylbenzenes was recorded between 190 and 400 nm, the optimal channels were 260 and 273 nm.

The samples contained benzene, ethylbenzene, butylbenzene, hexylbenzene, octylbenzene, decylbenzene, dodecylbenzene, tetradecylbenzene and octadecyl-benzene dissolved in either acetonitrile, methanol or heptane. The concentrations were set at 0.5, 0.7, 1.1, 1.8, 2.2, 3.4, 4.5, 5.0 and 5.4 g/L, respectively.

During the evaluation of the results, column efficiency was characterized by the number of theoretical plates or plate count, acquired by fitting exponentially modified Gaussian functions (EMG) to the experimental data. The fitting was performed in PeakFit v4.12 software. After fitting EMG functions to the experimental data, the plate count values were calculated using the first absolute moment (μ_1), that refers to the mean or average retention time, and the second central moment (μ'_2), that refers to the variance of the peak. Linear detector calibration was performed in order to convert absorbances into concentrations, that is necessary to use the equilibrium-dispersive (ED) model of chromatography.

Mass flow studies

During the mass flow studies, two columns were utilized, a Spherisorb Silica column (5 μm, 4.6 × 100 mm) from Waters and the Supelcosil ABZ+Plus alkylamide column (3 μm, 4.6 × 150 mm) from Sigma-Aldrich. All experiments were performed with a 100% CO_2 mobile phase with a set volumetric flow rate of 1 mL/min. The injection volume was 2.0 μL, the detector signal was recorded between 190 and 400 nm. The column thermostat was set to either 20 or 40 °C, while the back pressure regulator was to either 104 or 150 bar.

Total mass flow rates and pressures were measured directly at the inlet and outlet of the column in four different configurations. During the data acquisition, all instruments were brought to an equilibrium, then an injection of hexane was made. The chromatograms were recorded for 3 minutes, during which the CFM signal was recorded as well, consisting of the mass flow, density and temperature profiles of the eluent passing through the CFM cell. Three replicate

measurements were performed for all temperature and pressure settings, all configurations and the two columns as well as a zero-volume union.

Hold-up time measurements were performed with the same experimental conditions and columns but without the CFM and pressure gauge installed. Nitrous oxide was selected as the hold-up time marker. The gas was bubbled through methanol for one minute then the solution was injected in three replicate measurements. Detection wavelengths were 195 and 200 nm.

Extra-column volumes and variances with and without the CFM installed were determined by disconnecting the column, the CO_2 pump and the back pressure regulator. Then three replicate injections were performed using 70/30 MeOH/H_2O mobile phase with a volumetric flow rate of 0.25 mL/min. EMG functions were fitted to the experimental profiles and extra-column volumes and variances were calculated using the first absolute moment and the second central moment, respectively. The volume with no CFM (and pressure gauge) installed was 60 μL and the variance was 406 μL^2. With the CFM connected, the volume was 2.06 mL and the variance was 1.67 mL^2, so the volumetric contribution of the CFM was 2.00 mL.

Hold-up time studies

The columns employed in the hold-up time studies were two Spherisorb Silica columns (5 and 10 μm, 4.6 × 100 mm), a Symmetry C18 (3 μm, 4.6 × 150 mm), a Viridis BEH (1.7 μm, 3.0 × 50 mm) and a Torus Diol (1.7 μm, 3.0 × 50 mm), all from Waters along with the Supelcosil ABZ+Plus alkylamide column (3 μm, 4.6 × 150 mm) from Sigma-Aldrich and a (S,S) Whelk-O1 (1.8 μm, 4.6 × 100 mm) packed with fully porous, 1.8 μm particles synthesized at the Department of Chemical, Pharmaceutical and Agricultural Sciences (University of Ferrara, Italy).

The experiments were performed with a set volumetric flow rate of 1 mL/min. The samples were hexane, acetone (10 V/V% in MeOH), nitrous oxide (1 min in MeOH) and 1,3,5-tri-*tert*-butylbenzene (TTBB, 0.01 mg/mL), while the mobile phases were neat carbon dioxide or mixed with either 5, 10, 15 or 20% methanol. The injection volume was 2.0 μL. The detector signal was recorded between 190 and 400 nm. Column temperature was either 20 or 40 °C, while the back pressure was set to either 104 or 150 bar.

4. Results

The competitive adsorption of the sample solvent and solutes was investigated in supercritical fluid chromatography. A series of n-alkylbenzene homologues were chosen as model compounds along with acetonitrile, methanol and heptane as sample solvents. After a series of preliminary experiments, the phenomenon was successfully detected with an alkylamide column, at 60 °C temperature, 150 bar back pressure and neat carbon dioxide mobile phase. In the case of methanol, the competition was easily identified based on the decreased column efficiency, shifts in retention times and changes in peak widths, since the variation of these properties was highest around the methanol band.

Single-component isotherms were determined for methanol and two alkylbenzenes surrounding the solvent band. To account for the adsorption energy distribution of the heterogeneous surface of the stationary phase, the bi-Langmuir isotherm was selected and the parameters were determined by the inverse method using a numerical method where the differential mass balance equation given by the equilibrium-dispersive model was integrated by a modified Rouchon algorithm. The results showed a very good agreement between the experimental and calculated band profiles and the behavior of the two different adsorption sites were also explored, all compounds favored site 1 by around two orders of magnitude in terms of the saturation capacity.

The competitive bi-Langmuir isotherm was chosen to model the competition. The model employed the determined parameters and a similar numerical approach as before. A series of *in silico* experiments were performed where all solute concentrations were set in the analytical range and the amount of the solvent was increased step by step to imitate the real injections. Besides the alkylbenzenes, two hypothetical solutes (H1 and H2) were also investigated with varying retentions compared to methanol. Octylbenzene, decylbenzene and compound H1 were all affected by the displacement effect caused by the strongly adsorbing methanol acting as a displacing agent, resulting in distorted, compressed band profiles and anomalous efficiency. Compound H2 was affected by the tag-along effect caused by the abundance of methanol acting as an inhibitor, resulting in elongated peak shapes and decreased efficiency.

Our work regarding the behavior of mass flow demonstrated that even though mass flow rate is the only flow parameter considered constant in SFC, some variation can be still expected when taken at different parts of the chromatographic instrument, since the CFM alters the system configuration. Comparing mass flow rates between the inlet and outlet of the columns showed diverse tendencies in differences ranging from 0.6% to 4.2%. Considering that only neat CO_2 was used as mobile phase in the study, deviations were not too severe. In the case of mobile phases containing organic modifier and additives as well, even lower differences should be expected.

Additional precision studies revealed that measuring accurate, reproducible mass flow rates in a low-flow, low-viscosity environment is problematic in a standard laboratory setup even if the built-in self-diagnostics of the SFC system show no leaks, the CFM calibration is correct and all instructions are strictly followed.

Pressure measurements complementing the work showed varied pressure drops on the columns depending on their length and particle size. Interestingly, significant pressure drops were found on the mass flow meter, more pronounced at the inlet side (1.5–2 bar), that suggest a slight effect on mobile phase flow.

Studying the effect of pressure and temperature on mass flow rate showed that the former had a larger influence while changing temperature only had minimal effects. Accounting for injections showed that although the initial drop in mass flow is severe compared to the equilibrium, taking the average from the injection time until the hold-up time reduced this effect significantly. The use of well-retained compounds should further minimize the adverse effect of injections.

In terms of hold-up time markers, nitrous oxide can be a good option for supercritical fluid chromatography. It has good solubility in alcohols, sample preparation is simple and the sample is stable enough for a day. In addition, the properties of the gas guarantee that no adsorption or competition should take place on the stationary phase during an SFC run. The preliminary studies showed that out of all tested markers, nitrous oxide demonstrated the lowest elution times, suggesting that it was unretained, while the other probes showed some amount of retention or distorted, noisy peak shapes.

However, detection was problematic, because the signal of nitrous oxide had very low intensity due to the low concentration in the sample. Its UV spectrum is also unfortunate, because the compound can be detected only in the range of 190–220 nm, where most of the organic modifiers employed in SFC have their cutoff wavelengths. Thus, the signal is masked as seen on many occasions during our experiments, especially if the mobile phase contains 10% of methanol or more.

The comparison of different columns implied that the stationary phase did have a slight effect on the detection of nitrous oxide, however, this would contradict the theory behind the use of the compound as unretained marker. With a more comprehensive study, the limit of application could be further extended.

5. Thesis points

1. I found the sample solvent methanol competing for adsorption with n-alkylbenzene homologues in the case of neat carbon dioxide mobile phases in supercritical fluid chromatography. I identified the region where competition occurred marked by decreased column efficiencies, shifts in retention times and changes in peak widths, all induced by the displacement and tag-along effects.

2. I determined the single-component adsorption isotherms of methanol and two alkylbenzenes closest to the competing methanol band using the inverse method. I confirmed the bi-Langmuirian behavior of the compounds and then constructed a numerical method using the competitive bi-Langmuir isotherm. I modeled the competition of the real and hypothetical solutes with the solvent, and proved that the sources behind the anomalous retention behavior were the displacement and tag-along effects.

3. I have shown that accurate and precise mass flow measurements in a low-flow, low-viscosity environment are problematic when recorded at different parts of the chromatographic instrument, since the flow meter alters the system configuration. The discrepancies were as high as 4.2%, although using organic modifiers in the mobile phase mitigates the severity of the differences. I have shown that the flow meter affects the mobile phase flow and adds significant extra-column volume and variance to the system.

4. I have demonstrated that pressure had a larger effect on mass flow rate than temperature did. I have also shown that injections introduce a severe drop to the equilibrium mass flow rate, but successfully reduced this adverse effect by using the averages taken from the injection time until the elution times of the compounds instead.

5. I have concluded that nitrous oxide is the best unretained marker for accurate hold-up time measurements out of the four potential markers, since this compound gave the lowest elution times. However, detection was difficult in mobile phases containing 10% or more of organic modifier, because most solvents mask the signal of nitrous oxide. Interestingly, the comparison of multiple columns showed that the stationary phase also had a slight effect on detection.

Publications

Publications related to the thesis

1. **Rédei, Cs.**, Felinger, A. Modeling the competitive adsorption of sample solvent and solute in supercritical fluid chromatography, *J. Chromatogr. A*, **2019**, *1603*, 348–354. **IF: 4.049**

2. **Rédei, Cs.**, Felinger, A. The impact of placement, experimental conditions, and injections on mass flow measurements in supercritical fluid chromatography, *J. Chromatogr. A*, **2022**, *1668*, 462919 **IF: 4.1**

3. **Rédei, Cs.**, Buratti, A., Catani, M., Felinger, A. Exploring the limits of application of nitrous oxide as a hold-up time marker in supercritical fluid chromatography, *Anal. Bioanal. Chem.*, under review **IF: 4.3**

Posters and presentations related to the thesis

1. **Rédei, Cs.**, Felinger, A. Investigation of the retention behaviour of alkylbenzenes on a C18 stationary phase in supercritical fluid chromatography, 11^{th} Balaton Symposium on High-Performance Separation Methods, September 6–8, 2017, Siófok, Hungary

2. **Rédei, Cs.**, Felinger, A. Studying the competitive adsorption of alkylbenzenes and methanol in supercritical fluid chromatography, Applications of Supercritical Fluids 2018, May 17, 2018, Budapest, Hungary

3. **Rédei, Cs.**, Felinger, A. Competitive adsorption in supercritical fluid chromatography: A model, 32^{nd} International Symposium on Chromatography (ISC 2018), September 23–27, 2018, Cannes-Mandelieu, France

4. **Rédei, Cs.**, Felinger, A. Competitive adsorption of sample solvent and analyte in supercritical fluid chromatography, 24^{th} International Symposium on Analytical and Environmental Problems, October 8–9, 2018, Szeged, Hungary

5. **Rédei, Cs.**, Felinger, A. A minta és a minta oldószerének kompetitív adszorpciója szuperkritikusfluidum-kromatográfiában, Elválasztástudományi Vándorgyűlés 2018, November 8–10, 2018, Tapolca, Hungary

6. **Rédei, Cs.**, Felinger, A. Modeling the solute-solvent competition in supercritical fluid chromatography, 8^{th} Interdisciplinary Doctoral Conference (IDK 2019), May 24–25, 2019, Pécs, Hungary

7. **Rédei, Cs.**, Felinger, A. Exploring the limits of nitrous oxide as a tracer in supercritical fluid chromatography, 48$^{\text{th}}$ International Symposium on High-Performance Liquid Phase Separations and Related Techniques (HPLC 2019), June 16–20, 2019, Milan, Italy

8. **Rédei, Cs.**, Felinger, A. Comparison of nitrous oxide and other hold-up time markers in supercritical fluid chromatography, 12$^{\text{th}}$ Balaton Symposium on High-Performance Separation Methods, September 11–13, 2019, Siófok, Hungary

9. **Rédei, Cs.**, Felinger, A. A tömegárammérés rejtélyei szuperkritikusfluidum-kromatográfiában, METT25, October 18–20, 2021, Egerszalók, Hungary

10. **Rédei, Cs.**, Felinger, A. Exploring the difficulties of mass fow measurements in supercritical fluid chromatography, 33$^{\text{rd}}$ International Symposium on Chromatography (ISC 2022), September 18–22, 2022, Budapest, Hungary

11. **Rédei, Cs.**, Felinger, A. Exploring the difficulties of neat carbon dioxide as the mobile phase in supercritical fluid chromatography, 33$^{\text{rd}}$ International Symposium on Chromatography (ISC 2022), September 18–22, 2022, Budapest, Hungary

Publications not related to the thesis

1. Simon, J., **Rédei, Cs.**, Felinger, A. The use of alteration analysis in supercritical fluid chromatography to monitor changes in a series of chromatograms, *J. Chromatogr. A*, **2019**, *1596*, 217–225. **IF: 4.049**

2. Kazmouz, M. Y., **Rédei, Cs.**, Felinger, A. The adsorption of methanol on reversed phase stationary phases in supercritical fluid chromatography, *J. Chromatogr. A*, **2021**, *1653*, 462386 **IF: 4.601**

Posters and presentations not related to the thesis

1. Kazmouz, M. Y.*, László, Sz.*, **Rédei, Cs.**, Bacskay, I., Felinger, A. Surface excess isotherms of organic modifier and carbon dioxide mixture in sub- or supercritical fluid chromatography, Applications of Supercritical Fluids 2018, May 17, 2018, Budapest, Hungary

2. Horváth, É., **Rédei, Cs.**, Boros, B., Felinger, A. Investigation of interactions on chiral zwitterionic stationary phases in enantiomer separation using HPLC and SFC methods, 24$^{\text{th}}$ International Symposium on Analytical and Environmental Problems, October 8–9, 2018, Szeged, Hungary

3. Horváth, É., **Rédei, Cs.**, Boros, B., Felinger, A. Királis vegyületek elválasztása során kialakuló kölcsönhatások elméleti hátterének vizsgálata HPLC és SFC módszerek alkalmazásával, XXIV. Nemzetközi Vegyészkonferencia, October 24–27, 2018, Szovátafürdő, Romania

4. Simon, J., **Rédei, Cs.**, Zelenyánszki, D., Felinger, A. Komplex mintakeverékből származó komponensek eredetének meghatározás és vizsgálata alterációs analízissel, Elválasztástudományi Vándorgyűlés 2018, November 8–10, 2018, Tapolca, Hungary

5. Horváth, É., **Rédei, Cs.**, Boros, B., Felinger, A. Királis kromatográfiás mérési módszerek összevetése az elválasztás során kialakuló kölcsönhatások vizsgálata alapján, Elválasztástudományi Vándorgyűlés 2018, November 8–10, 2018, Tapolca, Hungary

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9. Kulágin, R., **Rédei, Cs.**, Lindner, W., Felinger, A. Kinidin alapú királis, ikerionos típusú állófázis öregedésének vizsgálata SFC alkalmazásával, METT25, October 18–20, 2021, Egerszalók, Hungary