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Cover letter

Monday, February 16, 2015
Editorial Office of Journal of Chromatography A

Dear Editor,

We are pleased to submit the revised manuscript Review JCA-14-2591 for publication in Journal of Chromatography A:

Method transfer from high-pressure liquid chromatography to ultra-high-pressure liquid chromatography. II. Temperature and pressure effects

by

Dennis Åsberg, Jörgen Samuelsson[*], Marek Leśko, Alberto Cavazzini, Krzysztof Kaczmarski[*] and Torgny Fornstedt

D. Åsberg, M. Leśko, J. Samuelsson, K. Kaczmarski, T. Fornstedt, Method transfer from high-pressure liquid chromatography to ultra-high-pressure liquid chromatography. I. A thermodynamic perspective, J. Chromatogr. A. 1362 (2014) 206–217.

Corresponding authors: Dr. Jörgen Samuelsson and Professor Kaczmarski

We are grateful for the thoroughly review of our papers by four different reviewers. We have revised the MS carefully according to all opinions of the four experts which can be seen in the attached response file attached and in the revised manuscript attached where all changes according the reviewers options are marked. We are convinced the manuscript is now suitable for publication in Journal of Chromatography A. In this context we also want to confirm that the work is new and is not under consideration elsewhere and that the institutions where the authors work agree to the submission of this paper to Journal of Chromatography A.

Please observe that we loaded Figure 3 as two files, one of them in colour for the web.

Best Regards,
Jörgen Samuelsson, one of two corresponding's authors*

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Response to reviewer's notes

First we would like to thank all reviewers for their valuable opinions. We strongly believe their opinions have substantially improved the manuscript. Below we first summary the major changes done thereafter follows detailed, point-by-point response to each comment raised in the decision letter, including the actual changes made and their location in the revised manuscript (with marked changes).

Summary of major changes that have been done on the manuscript (all numbers refer to the original MS):

- The *Introduction* has been condensed by removing unnecessary references and discussions. The novelty of the work has been more clearly stated and the scope rewritten.

- Unnecessary details have been removed from *Theory* and *Materials and Method* to make them clearer and more condensed.

- The *Result and Discussion* section has been completely revised:

  - The disposition have been changed to make the MS clearer and easier to follow

  - Fig. 3 has been removed and replaced by a much reduced figure showing only the results necessary for the subsequent calculations

  - Table 2 has been removed

  - Table 3 has been removed

  - Sec. 4.2.1 discussing the van't Hoff plots has been removed

  - Fig. 4 has been removed (1-site model temperature dependence)

  - Parts of Sec. 4.2.2 concerning 1-site model have been removed

  - Eq. 8 has been removed and the discussion in 4.3.1 rewritten

  - Sec. 4.3.1 has been condensed

- *Abstract* and *Conclusions* have been revised to reflect the changes made to the MS

Reviewer #1: The manuscript "Method transfer from high-pressure liquid chromatography" by Asberg et al. is an interesting piece of work and definitely worth to be published. The MS is well written, conclusions are well vindicated. I have one question concerning the measurements and a few minor comments, mostly typos.

Question:

The authors used restriction capillaries installed between the column outlet and a detector. It means that a capillary contributes to the retention time and peak broadening. I recognized that the authors measured the extracolumn contribution in a system without a restriction capillary. Did they measure those for each restriction capillary used?

Our Reply: Yes, all extra column contributions from the restriction capillaries were measured and accounted for in the calculations and the data was presented in the MS. Thanks for noticing we did not mention anything about it. As consequence of the reviewers question we have clarifying this in Section 3.5 (p11): "The extra column contributions from the restriction capillaries were measured and accounted for in the calculations, by lifting out the column and injecting the sample at each restriction capillary set up."

Minor comments:

P. 1, L. 18-19: The first sentence of the abstract is not really necessary. It adds no essential information to the abstract.

Our Reply: We agree with the Reviewer and have removed the sentence.

P. 9, L. 198: Write refs. [9,10].

Our Reply: Thanks, it is fixed

P. 11, L. 233: It should be Eq. 6, not Eq. 7, should not it?

Our Reply: Yes correct, fixed (eq. removed due to stream lining of the MS)

P. 11, L. 249: This sentence does not seem grammatically correct, "systems contributing ….were removed". Please revise.

Our Reply: Thanks, the section has been removed due to streamlining. This information is now instead present in the end of Section 2 (bottom of p7 /top of p8).

"To obtain pure data for calculations of adsorption/desorption kinetics, the extra column contribution to the elution zone must be removed. This was done by fitting the peak of the void volume marker to an exponentially modified Gaussian distribution and by deconvolution of the elution peak of the void volume contribution"

P. 17, L. 384: Write "with increasing (data not shown) pressure."

Our Reply: Thanks, fixed. (However, due to streamlining, section 4.3.2 do not exist instead the sentence is the second line under Section 4.2.2)

Reviewer #2: In this manuscript, the authors study the influence of temperature and pressure effects on method transfer between HPLC and UHPLC. The experiments are complemented by various calculations to model the detailed processes.

The presentation of the results is somewhat complex; a more clear strategy should be followed.

Some statements are contradictory, or they call for clarification, better explanation.

On the other hand, a number of studies have been published on the temperature and pressure effects. The authors should better focus on the novelty of their presentation.

Our Comments/Replies: The following major changes have been done in accordance with Reviewer's suggestions:

- The introduction has been condensed and more clearly focused on the novelty of the work. Unnecessary background information and references regarding HPLC pressures and stochastic modelling has been removed. The aim has been rewritten to highlight what makes this publication unique and reflect the revised form of the Results.

- The Material and Method Section has been condensed.

- The Result section has been completely revised in order to present the result with a new strategy to make them clearer and remove any results of minor importance or questionable physical relevance. The section has also been reduced. For example:

  o The former Section 4.2.1 "Van´t Hoff plots" has been removed since the discussion about the delta S and delta H lacks novelty and it is not essential for the conclusions presented in the MS. Hence Table 2 and Fig. 3 have been also removed.

  o The discussion about stochastic modelling of one-site model in Section 4.2.2 and Section 4.3.2 has been much reduced and the discussion has been revised. The former Fig. 4 has been removed. This since the Reviewers 2 and 4 find these findings doubtful.

  o Table 3 has been removed and these results are now only mentioned in the text.

- Abstract and conclusions have been modified to reflect the changes in the MS.

For details see the answers given below.

When the temperature effect was studied, why was the temperature range different for the HPLC or the UHPLC column?

Our Reply: This is due to technical limitations of the column thermostat used in this study. The HPLC column thermostat was unable to go above 40°C without significant heat losses and unstable temperature inside the column thermostat. The UHPLC thermostat could not operate at temperatures below ambient one. As consequence of the reviewers question we have clarifying this in Section 3.5, bottom of 2nd paragraph "The intervals were different due to different technical limitations: the HPLC

The calculated temperature profile along the column is presented in Figure 2. Why is the outer wall of the column tubing warmer at 50 mm (about 55 C) than the temperature inside the column? I assume that heat propagates from inside to outside.

Why is the radial temperature gradient inside the column negligible?

Is the calculated 16 C temperature difference supported by the experimental data? These points should be addressed.

Lines 300, 305: reference should be to eq 7 rather than 6.

Eq 8 is incorrect. The enthalphy and entropy parts should be included when ln(k) is expressed.

The absolute values of the enthalpies presented in Table 2 are systematically larger on the UHPLC column than on the HPLC column. This should be discussed and explained properly.

Our Reply: The calculated values of enthalpies – Table 2 – have been removed from the manuscript following Reviewer's opinions that the MS should be condensed and more focused on the novelty of the work. The primary reason for the van't Hoff plots was to use them to calculate the contribution to the retention factor due to temperature gradients in the original Section 4.4 so that the interpretations of the enthalpies and the comparison between HPLC and UHPLC did not contribute to the new aim of the study. The van't Hoff plots are now used only as an empirical equation for $k(T)$ and no physical interpretations are done.

With a properly chosen void volume marker the accurate determination could have been possible. No real explanation is given why this point was not pursued.

Our Reply: Thiourea was tested and it gave significantly different values compared to $NaNO_3$. It was therefore deemed that the true void volume was hard to estimate. Also, the entropy was not interesting in regard to the aim of this study. In this study we are only interested in how fast the retention factor changed with temperature. See also the comment above.

When the stochastic model is applied, one would expect that the sojourn times decrease with increasing temperature. At higher temperature, the thermal energy of the molecules can facilitate desorption, interactions are weaker. The results should be interpreted accordingly.

Our Reply: The response given here is the same as comment 5 by Reviewer 4:

Yes, the Reviewer's opinion is correct. After examining our calculations, we conclude that the results that we obtained for the one-site model (SNS, OM, C7), where the abovementioned trend is present, are due to:

(i) The elution peaks for these three solutes are very symmetrical and it is therefore very difficult for the optimization routine to differentiate between contributions from n and tau_s. This means that a decrease in n could be manifested as a decrease in tau_s and vice versa. For BTEAC, which is described by a two-site model, and has elution peaks that are tailing, it is mathematically easier to differentiate between the individual contributions from tau_s and n. So BTEAC gives the theoretically expected trend i.e. a decrease in the sojourn time and an increase in tau_s with increasing temperature.

(ii) The temperature interval studied ($20^{o}C$) is relatively small compared to the ones used in other studies, e.g. ref [25], which could make the general trends predicted by theory hard to detect.

In response to the Reviewer's comment we have removed Fig. 4 and any discussion in Section 4 concerning the trend in n and tau_s with temperature or pressure for the 1-site model. The discussion above has also been added to this section as an explanation. Se marked manuscript Sections 4.2 and 4.2.1, respectively.

The results in Figure 7 are rather suspicious. Why is the number of steps and time constant so much

changing with pressure? The authors recognize this problem and refrain from any concluding remarks. This should be amended. That figure should be omitted or a clear explanation should be given to the results.

Our Reply: We have rechecking all calculations regarding Fig. 7 and we have found that the calculations are correct. It is therefore our firm belief that these Results are correct and physically relevant and that the conclusions made are sound. To the best of our knowledge, this is the first time where time constant and sojourn time have been studied as a function of pressure, why only because of this, the observation is interesting to report. We have searched the literature again and we have not found any data available to compare our results with or any theoretical calculations.

We believe that an important conclusion *per se* is that the time constant and sojourn time **do** change with pressure for certain compounds. A deeper theoretical explanation for this observation is outside the scope of this paper because more basic compounds need to be tested.

However, as a consequence of the opinion of the reviewer we have elaborated and streamlined the text and also added a few lines about the need to have more components with more data for a better understanding of the phenomenon (see new Section 4.2.2).

Reviewer #3: This article describes issues dealing the pressure and temperature effects on retention in the context of method transfer between HPLC and UHPLC. Overall I found it to be well organized and easy to read. The authors only compare one column set, but for demonstration purposes, this is sufficient. However, it should be noted that method transfer on other column pairs may behave differently.

My comments are minor, as follows:

1. On line 217 the UHPLC flow rate is listed as 0.13 mL/min, but later on lines 261 and 276 it is listed at 1.2 mL/min. Which is correct?

Our reply: Both values are correct. For fundamental studies in this the flow rate was 0.13 mL/min to avoid any pressure and temperature gradients. The reason is that we wanted to study all effects as unbiased as possible. But for experiments where we want to show how optimized separations perform (for example those presented in lines 261 and 276) a flow rate of 1.2 mL/min was used. In these cases we will have pressure and temperature gradients.

As a consequence of the point raised by the reviewer, we have added some clarifying lines at the bottom of the Section "3.2 Chromatographic Equipment".

2. In the section on van't Hoff analysis, line 300: linearity of van't Hoff plots does not necessarily confirm constant heat capacity, it only suggests it, and only over the temperature range studied. If there are changes in both delta H and F over the temperature range examined, they can "cancel out" and result in a linear van't Hoff plot.

Our Reply: The Reviewer is correct. Thank you for the clarification so we know it for the next time. The sentence with this expression has anyway been removed now, since the whole section has been lifted out as a consequence of the opinion of Reviewer #2.

3. If the authors wish to attempt to calculate the phase ratio, there are methodologies in the literature to do so. Vs can be calculated from stationary phase physicochemical parameters, and Vm from a hold-up time marker like nitrate or thiourea. That should provide a reasonable estimate. Alternatively, the authors could discuss the combined entropy and phase ratio term as a single parameter, since it's the combination of the two that provide the van't Hoff intercept.

Our Reply: We are aware of this, and Vm was actually measured with nitrate and thiourea (not discussed in the MS) at all temperatures. They yielded significantly different results and we decided that it would give a large uncertainty in the delta S values. We believe that the delta S values are of minor importance for the conclusions and novelty of this MS and it has been decided to remove these data from the MS.

4. The two columns used are slightly different in column chemistry (bonding density, % carbon). I realize they are probably as close to identical as one could find, but is there any concern that results may be contaminated by differences in the stationary phase chemistry?

Reviewer #4: This paper investigates from a theoretical viewpoint the transfer of LC methods from conventional pressures (< 400 bar) to ultra-high-pressures (400 < P < 1000 bar). They measured the impact of temperature and pressure changes on retention and peak width. The ultimate goal and motivations of this work are very legitimate because this should benefit the practitioners who aim at replacing "conventional" with "very high pressure" LC systems (for faster and more resolution power). However, the different experimental contents presented in this work appear to be already well established in the literature (pressure and temperature effects, frictional heating, etc...). Additionally, some interpretation are quite unexpected. Revision and condensation is then needed before final acceptance for publication.

Our general reply to what we have done as a consequence of the opinion by Reviewer #4:

- Work that is already established, e.g. calculation of enthalpies, partial molar volumes etc., have been removed or severely condensed. E.g. Fig. 3, Fig. 4 and Tables 2 and 3 have been removed.

- The interpretation of the stochastic analysis has either been revised or removed. The presentations of delta H have been lifted out.

- The Result Section has been completely revised and condensed. All other sections have been condensed and the Introduction has been streamlined.

See also the list of major changes at the beginning of the Response Letter.

General remarks:

1) The main question is whether the common LC practitioner (such as in pharmaceutical industries) will have the expertise to (1) measure the eluent compressibility (affecting the local flow), its expansion coefficient (absorbing heat), its heat capacity (on the bleeding edge of the qbd imposing axial temperature gradients), and the effective thermal conductivity of the packed bed (imposing radial temperature gradients), the solute enthalpy, entropy, and molar volume changes from the bulk to the stationary phases AND (2) to predict quantitatively the shift in retention and change in peakwidth from these data. This appears challenging in practice for a basic LC technician.

Our reply: We want to clarify that this is primarily a research study and the main goal is to present new research of importance for the very forefront of theoretical and technological development of the quality by design (QbD) concept. More particular, the intention was to demonstrate how information coming from different theoretical and experimental considerations including stochastic modelling the study of temperature and pressure dependencies of retention factors of solutes and the theoretical calculations of the temperature profile along the column could be used to get a deeper understanding of how and why HPLC and UHPLC conditions differ. .

However, this does not contradict that the finding are valuable also as guidelines for the LC practitioner. On contrary, our intention is that the work should be of value for both advanced academic theoreticians as well as by the industrial community including the common LC practitioner and that the

latter community- could get further insight from the results – especially if he/she works with small molecules. We cannot see why it should be that difficult for the common LC practitioner for as example using external sensors and mass flow devices if necessary.

As a consequence of the point raised by the reviewer, we have added some lines to clarify further the purpose of the article, see bottom of the introduction, in the context where the aim of the study is presented: "Even if this is primarily a research study focusing on the very forefront of technological development necessary to push forward the quality by design concept our intention is also that the findings should be of importance for the common LC practitioner."

2) The data in Figure 3 are surprising. Since the pressure drop was maintained below 200 bar and heat effects were negligible (the temperature is uniform in the whole column, section 4.2.1), the van't Hoff plots should be rigorously the same regardless of the LC system used (HPLC or vHPLC). If the chemical natures of the stationary phase are the same (same batch of BEH-C18 or at least similar surface coverages), the plots should be nearly the same.

Our reply: Yes, we agree with the Reviewer that these results are surprising. We were also expecting to obtain almost identical van't Hoff curves. The experiments were actually performed a second time just to confirm that no experimental errors were present. We can only think of the following reason for the disagreement: (1) that the temperature intervals are slightly different and the van't Hoff plots may be nonlinear in a larger temperature interval so that the curves for HPLC and UHPLC describe different portions of a nonlinear curve and (ii) that the stationary phases are different.

As a consequence to this Reviewer and also to Reviewer #2 , we have decided to remove the section 4.2.1 discussing the van't Hoff plots including removing also the above mentioned Figure (former Figure 3) which can easily be seen in the marked revised MS.

The van't Hoff plots are now instead treated merely as an empirical relationship between k and T used to model how the retention factor changes with temperature in a limited interval.

3) Do the values of Delta H and Delta S derived in this work make physical sense in terms of the transfer of one mole of analyte molecules from a polar aqueous/organic eluent to liquid octadecane? Or are they just empirical parameters given the complexity of the adsorption process at the interface between silica-C18 and bulk eluents?

Our reply: The Reviewer may be right that van't Hoff plots only capture a simplified version of the complex phenomena present. The discussion about delta_H is therefore completely removed from the MS. For more details see also our answer to the comment nr 2, just above).

4) Is the band broadening of the four compounds (BTEAC, C7, SNS, and OM) studied in this work truly governed by the model Eq. (4) (dispersion for a reference non-reteained marker + dispersion due

to a slow adsorption-desorption process)?

Our reply: We believe that Eq. (4) is a sound model that could be applied to the experimental data presented here for BTEAC, which exhibits asymmetric peaks that can be accurately modeled (see also answer to comment 5, below). There is a number of peer-reviewed papers, e.g. ref [29; in the revised MS ref 21] which justifies the stochastic model for liquid chromatography. Although one should note that the main objective for the stochastic models were to correlate the changes in peak asymmetry (tailing) with pressure and temperature – not to study band broadening. To study band broadening itself for more or less symmetrical peaks there are other more convenient models/approaches accounting for the physical parameters, e.g. the approach presented by F. Gritti in J. Chromatogr. A (2014), 1332, 35-45.

We have clarified that the main objective of the stochastic modelling was to study peak shape and not band broadening and also inserted the reference mentioned above, see bottom of the Section "2. Theory".

5) Regarding the stochastic analysis, it is not clear why the number of adsorption (or desorption) events (n) decrease with increasing T (it is a priori expected that n increases because the average molecular speed is increasing). Also, why would the average residence time remain constant (it should a priori decrease according to an "Arrhenius-like" law tau_s=tau_0 Exp(E_a/RT))? In the end, the reader wonder about the physical relevance of Eq. (4) for the different compounds tested in this work.

to a slow adsorption-desorption process)?

Our reply: The Reviewer's opinion is correct. After examining our calculations we conclude that the results that we obtained for the one-site model (SNS, OM, C7), where the abovementioned trend is present, are due to:

(i) The elution peaks for these three solutes are very symmetrical and it is therefore very difficult for the optimization routine to differentiate between contributions from n and tau_s. This means that a decrease in n could be manifested as a decrease in tau_s and vice versa. For BTEAC, which is described by a two-site model, and it has elution peaks that are tailing, it is mathematically easier to differentiate between the individual contributions from tau_s and n. So BTEAC gives the theoretically expected trend i.e. a decrease in the sojourn time and an increase in tau_s with increasing temperature.

(ii) The temperature interval studied (20°C) is relatively small compared to the ones used in other studies, e.g. ref [25], which could make the general trend predicted by theory hard to detect.

In response to the Reviewer's opinion we have removed Fig. 4 and any discussion in Sec. 4 about the trend in n and tau_s with temperature or pressure for the 1-site models. The discussions above have also been added to section 4.2 (l. 330-338) as an explanation.

Highlights

- Temperature and pressure gradients were studied in UHPLC using HPLC as reference
- Axial temperature gradients reached 16°C and the radial gradient 2°C in UHPLC
- The stochastic model was used to evaluate temperature and pressure effects
- Pressure effects are more pronounced and has larger impact than temperature effects
- Pressure effects has much more impact on charged compounds as compared to neutrals

1 **Method transfer from high-pressure liquid chromatography to ultra-**

2 **high-pressure liquid chromatography. II. Temperature and pressure**

3 **effects**

4 Dennis Åsberg[1], Jörgen Samuelsson[1*], Marek Leśko[2], Alberto Cavazzini[3], Krzysztof

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17 **Abstract**

18 ~~This is the second investigation in a series that aims to enhance the scientific knowledge~~

19 ~~needed for reliable analytical method transfer between HPLC and UHPLC. Here,~~ ~~T~~the

20 importance of the generated temperature and pressure gradients in ultra-high-pressure

21 liquid chromatography (UHPLC) are investigated and compared to high-pressure liquid

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22   chromatography (HPLC). The drug Omeprazole, together with three other model compounds

23   (with different chemical characteristics, namely ~~neutral~~uncharged, positively and negatively

24   charged) were used. Calculations of the complete temperature profile in the column at

25   UHPLC conditions showed, in our experiments, a temperature difference between the inlet

26   and outlet of 16°C and a difference of 2°C between the column center and the wall. Through

27   van't Hoff plots, this information was used to single out the decrease in retention factor (k)

28   solely due to the temperature gradient. The uncharged solute was least affected by

29   temperature with a decrease in k of about 5% while for charged solutes the effect was more

30   pronounced, with k decreases up to 14%. A pressure increase of 500 bar gave roughly 5%

31   increase in k for the uncharged solute, while omeprazole and the other two charged solutes

32   gave about 25, 20 and 15% increases in k, respectively. The stochastic model of

33   chromatography was applied to estimate the dependence of the average number of

34   adsorption/desorption events (n) and the average time spent by a molecule in the stationary

35   phase (τ_s) on temperature and pressure on peak shape for the tailing, basic solute.

36   Increasing the temperature yielded an increase ~~decrease~~ in n and decrease in τ_s ~~was nearly~~

37   ~~constant~~which resulted in less skew at high temperatures. With increasing pressure, the

38   stochastic modelling gave interesting results for the basic ~~modelling compound~~solute

39   showing that the skew of the peak increased with pressure. The conclusion is that pressure

40   effects are more pronounced for both retention and peak shape than the temperature

41   effects for the polar or charged compounds in our study.

1 Introduction

The interest from the industry to move analytical methods from high-pressure liquid chromatography (HPLC) to ultra-high-pressure liquid chromatography (UHPLC) has grown in the last five years [1]. ~~This is especially true in the pharmaceutical industry [2].~~ UHPLC provides faster separations and lower solvent consumption compared to HPLC, with preserved column efficiency [2,3]~~[3,4]~~. This is achieved by decreasing the particle size of the stationary phase and increasing the linear velocity of the mobile phase. As a consequence, the pressure drop over the column is much larger in UHPLC compared to HPLC, which leads to significant pressure and temperature (due to ~~frictional~~viscous heating and solvent compression) gradients in the column. These gradients have been shown to affect chromatographic performance and predictability [4]~~[5]~~.

Temperature gradient depends strongly on the method employed to thermostat the column and ~~have~~has been calculated for different conditions in UHPLC [5–9]~~[6–10]~~. Longitudinal temperature gradients prevail when the column compartment is close to adiabatic (e.g. in still-air conditions); they essentially affect only retention time, without compromising column efficiency. Radial temperature gradients, on the other hand, arise in well-thermostated conditions (e.g., with water thermostating), where the center of the column has a different temperature than the wall. Radial temperature gradients result in decreased column efficiency and should therefore be avoided ~~[8,10]~~.

~~Early work on how pressure affects retention in HPLC reported of increases in the retention factor of small neutral compounds up to 25% when the pressure was increased from 100 to 350 bar [11,12]. It was also observed that, for homologues series, the effect of pressure on retention increases with molecular weight [13]. Large molecules, such as proteins and peptides, on the other hand, exhibit much larger pressure dependencies. The retention factor for insulin, for instance, increased nearly 3 times when the pressure increased from~~

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47 to 147 bar [14]. At these pressure, the effect of frictional heating and solvent compression is practically negligible [9,15]. The effect of typical pressures found in modern UHPLC systems (up toca 1200 1 000 bar) on retention has been investigated for a number of small compounds and large biomolecules [4,10,11][5,16,17]. For neutraluncharged species, relatively small changes (of up to 12%) in retention factor were observed for a pressure increase of 500 bar. For polar or ionic solutes much larger increases, up to 50%, were notedreported. The different behavior of non polar and polar/ionic compounds was explained in terms of the larger variation in partial molar volume of polar solutes when they are transferred from the mobile to the stationary phase.

In this study we are going to use the stochastic theory of chromatography to investigate, from a microscopic point of view, the effect of pressure and temperature on the chromatographic behavior of small molecules. Stochastic models of chromatography, first introduced by Giddings and Eyring [18], describe the chromatographic processes at a molecular level [12]. In these models, the chromatographic migration is represented as a random process in which each molecule, while migrating along the column, performs a random number of adsorption/desorption steps of random duration. Using the Characteristic Function formalism in the Fourier domain [13] the stochastic model have been used tofor studying solve the case of heterogeneous adsorption, and to extend the model to includeing the effect of mobile phase dispersion [14,15][22,23]. Stochastic models have been applied tofor reversed phase [16][24], chiral [17][25], size-exclusion [18][26], and ion-
exchange separations [19][27]. The time-based solution of these models, given by Giddings and Eyring in 1955, led to complex mathematics already for homogeneous adsorption and was unsolvable for adsorption onto heterogeneous surfaces [18–20]. Dondi and Remelli solved the original stochastic model of chromatography through the Characteristic Function formalism, in the Fourier domain [21]. More recently, this approach has been used to solve the case of heterogeneous adsorption and to extend the model to include the effect of

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95 ~~mobile phase dispersion [22,23]. Stochastic models have been applied to reversed phase~~

96 ~~[24], chiral [25], size-exclusion [26], and ion-exchange separations [27].~~

97 Even though much work has been done to investigate the impact of pressure and

98 temperature gradients in UHPLC, there is a lack of studies combining the different models to

99 give a holistic view of the ~~both~~ effects of both temperature and of pressure. ~~effects.~~

100 ~~H~~Hence, the relative importance of these effects on retention and peak shape is still rather

101 unclear.

102 ~~In the first study we investigated how the modifier content affects the adsorption in UHPLC~~

103 ~~and HPLC, by using model compounds with different properties at the studied conditions: a~~

104 ~~neutral (cycloheptanone), a positively (benzyltriethylammonium chloride) and a negatively~~

105 ~~charged (sodium 2-naphtalene sulphonate) and the drug omeprazole [28].~~ The aim of this

106 study is to investigate the effect of pressure and temperature ~~effects~~ gradients ~~arising when~~

107 ~~switching from HPLC to UHPLC~~ by calculating the~~ir~~ gradients and determining the~~ir~~ individual

108 contributions to retention and peak shape. ~~Temperature and pressure effects are first

109 investigated separately and then the combined effect is studied using the same ~~system~~

110 ~~as~~model compounds as in ~~the previous study~~part I [20]~~[28]~~ (a ~~neutral~~uncharged, a positively

111 and a negatively charged and the drug omeprazole). The peak shape of the positively

112 charged, tailing compound is modelled using the stochastic theory at different temperatures

113 and pressure. Even if this is primarily a research study focusing on the very forefront of

114 technological development necessary to push forward the quality by design concept our

115 intention is also that the findings should be of importance for the common LC practitioner.

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Field Code Changed

2 Theory

The stochastic theory [15,21,22][19,23,29] describes the chromatographic process in terms of random variables, namely the time spent by a molecule on the stationary phase (sojourn time) and that elapsed between two successive adsorption/desorption events (flying time). The "history" of a molecule traveling through a ~~chromatographic~~ column can be interpreted as the sum of a random number of adsorption/desorption steps performed by that molecule inside the column~~, traditionally described as a Poisson process~~. Mathematically, for each molecule, this history will be the convolution integral of the density functions of the time spent on the site. By means of the properties of the Characteristic Function (i.e., the inverse Fourier transform of the probability density function) it is possible to substitute the convolution integral with the product of the elementary characteristic functions and to obtain both the fundamental peak shape parameters (mean, variance, skew, etc.) and the chromatogram itself~~, when the inversion of the characteristic function is possible~~. In the language of stochastic models, the chromatographic process is a compound Poisson process, the chromatographic peak being the probability density function of time spent in the column by molecules. Let us define the average number of adsorption/desorption events by n and the average sojourn and flying times by τ_s and τ_m, respectively. Under the previous hypotheses, it can be demonstrated that, for a homogeneous surface (that is one characterized by a single sorption site type), the average retention time is given by:

$$t_R = n\tau_m + n\tau_s \qquad (1)$$

where $n\tau_m$ is the time spent, on average, by a molecule in the mobile phase (i.e., the column hold-up time) and $n\tau_s$ is the average time a molecule spends in the stationary phase.

For a heterogeneous surface, on the other hand, the corrected retention time will depend on both the characteristics of the different adsorption sites and their relative abundance on the phase. In the simple case of a surface paved with only two types of adsorption sites (2-

143   site heterogeneous model [15][23]), accordingly, the average retention time can be

144   expressed as:

145

$$t_{\mathrm{R}} = n\tau_{\mathrm{m}} + n\left(p_1\tau_{\mathrm{s},1} + p_2\tau_{\mathrm{s},2}\right) \tag{2}$$

146   where p_i is the relative amount of the i:th site (i = 1, 2) and $n = n_1 + n_2$.

147   Through the characteristic function method, the calculation of statistical peak moments is

148   straightforward [13,15][21,23]. Using this statistical moments Through them, then, it is

149   possible to calculate the parameters traditionally used to describe peak asymmetry, such as

150   peak skew or peak excess, and to obtain expressions for the height equivalent to a

151   theoretical plate (H) or the number of theoretical plates (N). The skew (S) for the 1-site and

152   the 2-site models are given by Eq. 3a and Eq. 3b, respectively [15][23]:

153

$$S = \frac{3}{2\sqrt{n}} \tag{3a}$$

154

$$S = \frac{3}{2\sqrt{n}} \frac{p_1\tau_{\mathrm{s},1}^3 + p_2\tau_{\mathrm{s},2}^3}{\left(p_1\tau_{\mathrm{s},1}^2 + p_2\tau_{\mathrm{s},2}^2\right)^{3/2}} \tag{3b}$$

155   Column efficiency is determined according to [15][23]:

156

$$\frac{1}{N} = \frac{1}{N_{\mathrm{D}}} + \frac{2}{N_{\mathrm{M}}}\left(\frac{k}{k+1}\right)^2 \tag{4}$$

157   where, k is the retention factor and N_{D} is the dispersion effect from the mobile phase

158   estimated from an unretained marker fitted to the exponentially modified Gaussian

159   distribution (EMG) and calculated from the distribution's mean and variance. N_{M} is defined

160   for the 1-site model by:

161

$$N_{\mathrm{M}} = n \tag{5a}$$

162   and for the 2-site model as:

7

163

$$N_\text{M} = n \frac{\left(p_1 + p_2\left(\tau_{s,2}/\tau_{s,1}\right)\right)^2}{p_1 + p_2\left(\tau_{s,2}/\tau_{s,1}\right)^2} \ .$$

(5b)

164 To getobtain pure data for calculations of pure adsorption/desorption kinetics, the extra

165 column systems contributionng to the elution zone must be removed. This was done by

166 fitting the peak of the void volume marker to an exponentially modified Gaussian

167 distribution and by deconvolut deconvolutingdeconvolution of ed it from the elution

168 zonepeak of the void volume contribution. In this work the stochastic model parameters

169 were estimated using a super modified sequential simplex optimization by minimization of

170 the least-squares errors. In this context it is worth mentioning that the main objective for

171 the stochastic models are to correlate the changes in peak asymmetry (tailing) with pressure

172 and temperature - not to study band broadening itself. For the latter, if the band broadening

173 is not tailed so much, general models/approaches that is sufficient [23].

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3 Material and methods

3.1 Chemicals

176 Mobile phase were acetonitrile/aqueous-buffer (15 mM phosphate buffer, pH 8.00 prepared

177 and measured at 22°C) mixtures. Gradient grade acetonitrile was purchased from VWR

178 International (Radnor, PA, USA). The buffer was prepared from water with conductivity 5.5

179 µS/m delivered from a Milli-Q Plus 185 water purification system from Merck Millipore

180 (Billerica, MA, USA) and from analytical grade sodium phosphate dibasic dihydrate and

181 sodium phosphate monobasic dihydrate purchased from Sigma-Aldrich (St. Louis, MO, USA).

182 The phosphate buffer was filtered through a 0.2 µm nylon filter membrane purchased from

183 Whatman (Maidstone, UK) before it was mixed with acetonitrile. The amount of acetonitrile

184 varied from 7 to 25% v/v, depending on the solute. Benzyltriethylammonium chloride,

185 BTEAC, (99%), cycloheptanone, C7, (99%), sodium 2-naphtalenesulfonate, SNS, (≥95%), all

8

186   from Sigma-Aldrich, and omeprazole sodium monohydrate, OM, (>99%), kindly gifted by

187   AstraZeneca (Mölndal, Sweden), were used as solutes. BTEAC is positively charged, SNS is

188   negatively charged and C7 and OM are ~~neutral~~uncharged at pH 8. The column hold-up

189   volume was determined with sodium nitrate (≥99.0%) purchased from Sigma-Aldrich.

190   **3.2 Chromatographic equipment**

191   The HPLC system was an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA,

192   USA) equipped with a binary pump, an auto sampler, a diode-array UV-detector and a

193   thermostated still air column oven. The extra column volume from the auto sampler to the

194   detector was 0.037 mL and has been subtracted from the experimental data. The HPLC

195   column was a 100 × 4.6 mm XBridge BEH C_{18} column with an average particle diameter of 3.5

196   μm and column hold-up volume 0.97 mL. The physicochemical properties of the column are

197   reported in Table 1. As the column was thermostated in a still air compartment, it can be

198   assumed that it is under adiabatic conditions [9]~~[10]~~.

199   The UHPLC system was a Waters Acquity UPLC H-class (Waters Corporation, Milford, MA,

200   USA) equipped with quaternary pump system, an auto sampler, a diode-array UV-detector

201   and a thermostated column oven. Also in this case, one may assume the column to be under

202   adiabatic conditions. The extra column volume from the auto sampler to the detector was

203   0.027 mL and has been subtracted from the experimental data. The UHPLC column was a 50

204   × 2.1 mm Acquity UPLC BEH C_{18} with an average particle diameter of 1.7 μm. The physico-

205   chemical properties of the column are given in Table 1. The flow rates of the respective

206   systems used depended on the goals of the experiments and are mentioned in connection to

207   the actual experiment, below. As example, for fundamental studies in UHPLC we used a very

208   low flow rate to avoid pressure and temperature gradients whereas for optimized UHPLC

209   experiments we used a high flow rate.

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3.3 Temperature, mass flow and pressure measurements

The temperature of the column wall was measured by attaching three PT-100 (4-wire)

resistance temperature detectors from Pentronic AB (Gunnebo, Sweden) directly on the

column surface. For the UHPLC column, they were attached on the column wall at 10.7, 21.9

and 35.0 mm from the column inlet and for HPLC they were placed at 21 and 81 mm from

the inlet. A thermal adhesive from Arctic Silver Inc. (Visalia, CA, USA) was used to attach

them. The PT-100 elements had the accuracy ±0.2°C and were verified in house against a

reference thermometer. ~~The PT-100 elements were connected to a PT-104 data logger and~~

~~recorded with the PicoLog from Pico Technology Ltd. (St. Neots, UK).~~

The total mass flow was measured by connecting a mini CORI-FLOW Coriolis mass flow

meter after the detector which was purchased from Bronkhorst High-Tech B.V. (Ruurlo,

Netherlands) and had accuracy equal to ±0.2% of the mass flow.

The pressure at the column inlet and outlet was determined by repeating the experiments

first with the capillary going to the column inlet reconnected directly to waste and then with

the column replaced by a zero-volume union. The temperature was measured at the flow

rates 0.25, 0.50, 1.00 and 1.20 mL/min for UHPLC and at 0.40 and 1.00 mL/min for HPLC. The

mobile phase was 25% acetonitrile as this composition corresponds to the largest viscosity

of the mobile phase [24][23][30].

3.4 Calculating temperature profiles

The experimentally measured axial temperature difference between column inlet and outlet

was less than 0.5°C in HPLC at flow rate ≤ 1 mL/min and in UHPLC at flow rate ≤ 0.25

mL/min. This temperature difference is deemed negligible so these conditions were not

modelled.

233 The modelling of temperature profiles in chromatographic columns was done with the same

234 method as described in refs. [8,9][9,10]. This method combines models of heat and mass

235 transfer and mobile phase velocity distribution. In these calculations, the mass flow

236 measured externally was used in place of the set up volumetric flow rate. The external heat

237 transfer coefficient and the parameter in the Blake-Kozeny-Carman correlation were

238 estimated by minimizing the differences between calculated and experimental values of

239 column outlet pressure and temperature (measured at the third temperature sensor). The

240 external heat transfer coefficient was equal to 30 W/(m^2 K) and the Blake-Kozeny-Carman

241 parameter was 146. At the different flow rates, calculations were validated by comparing

242 the estimated temperature at the column wall (at the positions of the temperature sensors)

243 with the experimental temperatures. The agreement between calculated and experimental

244 data was very satisfactory, with relative errors smaller than 0.5%.

245 **3.5 Chromatographic experiments**

246 Triplicate analytical injections of the four compounds were done at different temperatures

247 in HPLC and UHPLC and for different pressures for UHPLC. BTEAC (0.01 g/L) was studied at

248 7% acetonitrile in the eluent, SNS (0.001 g/L) at 15% and C7 (1 g/L) and OM (0.025 g/L) at

249 25%. The column hold-up volume was measured with NaNO$_3$ (0.005 g/L) before each

250 injection. In HPLC it was equal to 1.00, 0.96 and 0.93 mL and in UHPLC it was 0.11, 0.10, 0.10

251 mL for 7%, 15% and 25%, respectively. Injection volumes were 5 µL in HPLC and 2 µL in

252 UHPLC while all compounds were detected at 220 nm except C7, which was detected at 280

253 nm. The flow rates were 1.00 and 0.13 mL/min in HPLC and UHPLC, respectively, which

254 resulted in maximum pressure drops of 150 and 100 bar over the columns (negligible

255 pressure and temperature gradients). At these pressure drops there are neither significant

256 temperature gradients along the columns, nor does the pressure has any significant effect

257 on the retention mechanism.

258   When investigating the effect of temperature, the temperature was changed in 5°C

259   increments and analytical peaks were recorded at each temperature. The interval for HPLC

260   was 20-40°C and for UHPLC 30-50°C~~.~~

261   . The intervals were different due to different technical limitations: the HPLC column

262   thermostat was unable to set for stable temperature > 40°C while the UHPLC thermostat

263   could not operate in a reliable way at temperature < 25°C. The pressure was studied by

264   placing a restriction capillary between the column outlet and the detector. The extra column

265   contributions from the restriction capillaries ~~was~~were measured and accounted for in the

266   calculations, by lifting out the column and injecting the sample at each restriction capillary

267   set up. ~~-~~As restriction capillaries LC PEEKsil tubing with inner diameter 25 ± 1 µm from SGE

268   Analytical Science (Milton Keynes, U.K.) was used with lengths 5, 10, 15 and 20 cm. This

269   approach allows to ~~kept the~~minimize ~~pressure~~minimizing pressure and temperature

270   gradients over the column. ~~to a minimum.~~

271   The pressure used in the calculations is taken as the average pressure in the column when

272   assuming a linear pressure drop over the column [25]~~[24][15]~~ and ~~it~~is denoted P_{avg}~~.~~ It is

273   calculated as:

$$P_{avg} = \frac{P_{col.\ inlet} - P_{col.\ outlet}}{2} + \Delta P_{after\ col.} \qquad (6)$$

275   where $P_{col.\ inlet}$ and $P_{col.\ outlet}$ is the pressure at the column inlet and outlet while $\Delta P_{after\ col.}$ is the

276   total pressure drop from the column outlet to atmosphere including the restriction capillary.

277   The pressures in Eq. 6~~7~~ were determined separately for all experimental systems. Five

278   different column pressures were investigated for each solute; $P_{avg,\ 25\%}$ = 53, 174, 302, 420,

279   550 bar, $P_{avg,\ 15\%}$ = 53, 175, 314, 419, 552 bar and $P_{avg,\ 7\%}$ = 51, 167, 301, 398, 524 bar, where

280   the "%" denotes the ~~volumetric~~fraction of acetonitrile in the mobile phase. ~~It should be~~

281   ~~noted that without a restriction capillary, i.e. with the unmodified UHPLC system, an~~

282 average column pressure, P_{avg}, of 550 bar would give a system pressure of ca 1000 bar.

283 Therefore the pressure range available in most modern UHPLC system was covered.

284 This approach had the advantage of keeping the pressure and temperature gradients over

285 the column to a minimum compared to adjusting the pressure by changing the volumetric

286 flow rate. The experiments involving pressure effects were only performed on the UHPLC

287 system due to the limited pressures limits in the HPLC systems. One mobile phase

288 composition was investigated for each solute because the mobile phase composition have

289 been shown to have very low influence on the pressure dependence of the retention factor

290 [17].

291 **3.6 Stochastic calculations**

292 For the analytical injection presented in section 3.5, stochastic calculations were conducted.

293 The chromatographic systems contributing to the elution peak shape were removed by

294 fitting the void volume marker to an exponentially modified Gaussian distribution and later

295 deconvoluted by division in the frequency domain. The elution profiles were transformed

296 into the frequency domain using fast Fourier transforms and fitted to characteristic

297 functions [29].

298 All numerical calculations were performed with in-house-written Fortran 77 program using a

299 python interface to randomly generate starting guesses that fulfills Eq. 1 or 2 depending on

300 if a one site or two site models are used in the fitting. The stochastic model parameters

301 were estimated with a nonlinear simplex minimization of the least-squares errors.

302 4 Results and discussion

303 To demonstrate that a direct method transfer from HPLC to UHPLC is not always

304 straightforward, the four compounds employed in this work have been eluted on the two

305 columns under isocratic conditions and typical flow rates of HPLC and UHPLC (1.0 and 1.2

306 ml/min, respectively). It is worth to mention that these flow rates were not obtained as the

307 optimal flow rate in a van Deemter curve, as the study of column efficiency was not the

308 purpose of this work. Temperature in both cases was set to 30°C. Overlaid chromatograms

309 are presented in Fig. 1 where, for the sake of comparison, retention is expressed as column

310 volumes instead of retention time. It is evident from Fig. 1 that the retention in column

311 volumes is longer for UHPLC (gray lines) compared to HPLC (black lines), especially for the

312 late eluting peaks which also exhibit more tailing in UHPLC. This difference in retention

313 might be caused by factors such as different pressures, temperature gradients andor column

314 chemistry.

315 Through the aid of the stochastic modelling and by traditional ln-(k) vs. 1/T and ln-(k) vs. P

316 plots we will try to investigate the individual contributions of temperature and pressure to

317 retention and the effect of column chemistrypeak shape.

4.1 Temperature and pressure effects on retention

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~~4.1 The column temperature profile~~

~~To better understand the effect of frictional heating, the temperature gradients in the column were quantified. The 2-dimensional temperature profile corresponding to UHPLC conditions of 25% acetonitrile and flow rate 1.2 mL/min (same as Fig. 1) has been calculated and shown in Fig. 2. The dotted line at 1.05 mm represents the inner column wall. Because the column temperature profile was assumed to be radially symmetrical, only half of the temperature contour plot is shown. As can be seen from Fig. 2 the temperature along the column, i.e. longitudinally, increased ~16°C from the inlet to the outlet and the temperature inside the column from centrum to wall, i.e. radially, decreased ~2°C. The values calculated for this specific system are close to those reported in the literature [7,10] for similar pressure drops over the column.~~4.1.1 Pressure dependence

~~When plotting ln(k) vs. P_{avg}, T~~the slope of ~~When plotting~~ ln(k) vs. P_{avg} plots, is equal to ΔV_m = $V_{stat.} - V_{mob.}$, w~~i.e. hich is~~ the change in solute molar volume associated with the transition between the stationary and mobile phase [26]~~[25]~~. Generally the solute~~s~~ molar volume is larger in the mobile phase, i.e. $\Delta V_m < 0$. According to Le Chatelier principle, when the pressure is increased the equilibrium between solute molecules in the mobile and stationary phase will be pushed toward the stationary phase, resulting in increasing retention time.~~ If the solute molecules spend longer time in the stationary phase, the retention time will increase~~ [26,27]~~[25,26]~~.

The ~~experimental data~~retention times for different pressures w~~ere~~as fitted with linear regression and the result is presented in Fig. 2. The relationship between ln(k) and P_{avg} is linear with a R^2 value larger than 0.980. Calculated ΔV_m values were -11.9 ± 1.0 cm^3/mol for BTEAC, -15.5 ± 0.3 cm^3/mol for SNS, -3.4 ± 0.3 cm^3/mol for C7 and -18.4 ± 0.5 cm^3/mol for OM given with a 95% confidence interval. These observations are in good agreement with

those reported by Fallas et al. [10,11] for a similar system. The negative ΔV_m is attributed to the partial loss of the solvation layer of the solutes when they move from the mobile to the hydrophobic stationary phase. The difference in ΔV_m between ~~neutral~~uncharged and ionized solutes is believed to be due to the hydration of the ions which are partially lost when entering the stationary phase. However, this clearly shows that pressure is a factor that needs to be considered because it could affect the selectivity as well as retention.

4.1.2 Temperature dependence

To better understand the effect of viscous heating, the temperature gradients in the column were quantified. The 2-dimensional temperature profile corresponding to UHPLC conditions of 25% acetonitrile and flow rate 1.2 mL/min (same as Fig. 1) has been calculated and shown in Fig. 3. The dotted line at 1.05 mm represents the inner column wall. Because the column temperature profile was assumed to be radially symmetrical, only half of the temperature contour plot ~~is~~ has been shown. As can be seen from Fig. 3, the temperature along the column, i.e. longitudinally, increased ≃16°C from the inlet to the outlet and the temperature inside the column from centrum to wall, i.e. radially, decreased ≃2°C at most. The values calculated for this specific system are close to those reported in the literature [6,9] for similar pressure drops over the column.

The effect of the temperature gradient on retention was estimated by calculating the local propagation speeds along the column by first calculating the geometric radial average temperature using the temperature profile in Fig. 3. Then the temperature dependence of the retention factor was determined by fitting the logarithm of retention factors to the reciprocal temperature, Fig. 4. Linearity of ln (k) vs. 1/T was observed in all cases with R^2-values above 0.990, except for BTEAC for which a slight nonlinearity was observed with R^2-value 0.960. The retention factor of all solutes decreases with temperature, which is the common behavior in RP-LC for moderate temperature variations. Combining this

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information the retention time was estimated by integrating the solutes local propagation speed along the UHPLC column. The ~~neutral~~uncharged solute, C7, is least affected by the temperature gradient with a decrease in k of roughly 5%. The positively charged solute, BTEAC, is somewhat more sensitive and k decreases about 10% while the negatively charged solute, SNS, is most affected by temperature and k decreases almost 14%. The retention factor of OM decreases approximately 9%, which places it in the same region as the other two charged solutes.

4.1.3 The relative importance of temperature and pressure gradients

In an attempt to compare the relative importance of pressure and temperature on retention in UHPLC, the results from Sec. 4.1.1 and 4.1.2 have been combined in Fig. 5, where the retention factors of the four investigated molecules have been normalized against the respective retention factor at 0.13 mL/min (where pressure and temperature gradients are negligible). In Fig. 5, white bars show the estimated contribution for temperature, while gray bars show the pressure contribution, which has been estimated by assuming a linear pressure gradient over the column. Finally, black bars represent the observed, experimental retention factors found in UHPLC.

The pressure and temperature have opposite effects on retention and will therefore, to a certain degree, cancel each other ~~out~~. Under typical UHPLC conditions, t~~T~~he pressure effect is always larger ~~for all solutes, except C7,~~ than the temperature effect, except for C7~~, for typical UHPLC conditions~~. For C7, which is ~~neutral~~uncharged, the pressure effect is very small. Both the pressure and temperature effects are solute dependent. In particular, our data show that ~~and in this case it is evident that~~ charge~~d~~d solutes and those with larger molecular weight ~~solutes and that with larger molecular weight~~ are most affected by pressure. These results also suggest that in the method transfer from HPLC to UHPLC, especially if charged solutes are considered, the effect of pressure gradient along the

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column more than that of temperature gradient should be taken into account, as it is the

dominating one.

4.2 Stochastic Modelling

The effect of temperature on retention has been evaluated by using both the traditional

van't Hoff plots and the stochastic theory of chromatography. The investigation has been

performed at flow rates of 1.0 (HPLC) and 0.13 (UHPLC) mL/min corresponding to pressure

drops over the columns of less than 200 bar, so to avoid any temperature gradient along the

column due to frictional heating [9,10].

4.2.1 Van't Hoff plots

Van't Hoff equation correlates the logarithm of retention factor to the standard enthalpy

($\Delta H°$) and entropy ($\Delta S°$) of solute transfer from the mobile phase to the stationary phase. By

assuming constant heat capacity, it is written as [31]:

$$\ln(k) = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} + \ln(F) \qquad (7)$$

where F is the phase ratio (i.e., the ratio between stationary and mobile phase volume) and

R the universal gas constant.

Van't Hoff plots are shown in Fig. 3 for the four solutes. Linearity of ln (k) vs. 1/T has been

observed in all cases with R^2-values above 0.99 except for BTEAC in UHPLC, Fig 3a, for which

a slight nonlinearity was observed with R^2-value 0.96. Linearity confirms that the assumption

of constant heat capacity is acceptable in this region. The phase ratio used in Eq. 6 was

estimated by using NaNO$_3$ as hold-up time marker and it was found to be constant within

the limits of experimental error. The retention factor of all solutes decreases with

temperature, which is the common behavior in RP-LC for moderate temperature variations.

18

As expected, the slopes of the curves were similar for the HPLC and the UHPLC columns. The corresponding $\Delta H°$ values were calculated from Eq. 6 and are listed in Table 2. $\Delta H°$ values are all included in the range -3 to -14 kJ/mol. SNS, Fig. 3b, exhibited the largest absolute value of $\Delta H°$, i.e. is the one most sensitive to temperature variations. The $\Delta H°$ values are similar to those previously reported in RP-LC [32,33]. The differences seen between HPLC and UHPLC in $\Delta H°$ may be due to the different temperature intervals studied for HPLC and UHPLC, but differences in the stationary phase properties such as binding density and porosity [32] may also affect enthalpy and entropy changes.

The $\Delta S°$ values are not reported here because to determine the entropy change the true phase ratio must be known, Eq. (7), and we have not enough evidence for that $NaNO_3$ in this case estimates a the sufficiently good phase ratio, and therefore the $\Delta S°$ values that we calculate are not meaningful .

4.2.2 Stochastic analysis

The dependence of k on T was also interpreted in terms of the stochastic model of chromatography. The adsorption thermodynamics isotherms for the modeling compounds has previously been investigated and it was found that C7 and OM exhibited homogeneous adsorption while SNS and BTEAC had heterogeneous adsorption [20][28]. Since the peak shape was also very symmetrical for C7, OM and SNS, they were described by 1-site stochastic models. One must stress that heterogeneous adsorption not necessarily results in measurable heterogeneous kinetics.

For very symmetrical peak shapes as those observed under our conditions, the number of adsorption/desorption events (n) and adsorption sojourn time (τ_s) are strictly correlated, so that nonlinear fitting cannot differentiate between them. Therefore, any trends with temperature or pressure seen for this kind of peaks may be an artifact from theof nonlinear fitting. As a consequence of this, only the range of these parameters areis given here. The

conclusion is that ~~that~~ for solutes with very symmetrical peaks, the stochastic approach is unable to single out pressure and temperature effects.

For BTEAC, the elution peaks are asymmetrical so the nonlinear fitting could differentiate between contributions of n and τ_s.

4.2.1 Temperature dependence

~~Calculations of the adsorption sojourn time (τ_s) and the number of adsorption/desorption events (n) are shown for C7, SNS and OM, in Fig. 4. Note the different temperature scales for HPLC and UHPLC experiments.~~ For OM, C7 and SNS (1-site model~~s~~) ~~The observed n and τ_s are similar to previous observations of roughly 12 000 to 20 000 and 10 to 50 ms for n and τ_s, respectively, for a 150 mm column [24].~~ n is similar for all three solutes (10 000 to 20 000) in HPLC and UHPLC with ~~a trend of decreasing n with increasing temperature.~~the values for UHPLC being slightly lower. τ_s is between 10 and 20 ms and the values are slightly higher for UHPLC than for HPLC. These observations reflect~~s~~ the fact that the measured column efficiency is higher in HPLC.

~~The decrease in n with temperature can be understood by considering that the solubility in the mobile phase increases with temperature and thereby making it more favorable for the molecule to stay in the mobile phase. The average sojourn time is almost constant in the studied temperature intervals for both HPLC and UHPLC, which means that the time the molecule on average spend not in the mobile phase at each adsorption/desorption event is almost unaffected by temperature in the studied regions. This observation is in agreement with the information gathered by van't Hoff's analysis, as constant τ_s in combination with a decreasing n give decreasing plate numbers (see Eq. 4) and retention times (see Eq. 1).~~

~~As we previously found that~~ BTEAC adsorption is described by a 2-site adsorption isotherm ~~(bi-Langmuir)~~ [20][28] and the elution peaks are tailing so ~~,~~the 2-site stochastic model

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467 [22,23] has been employed to fit the chromatograms of BTEAC. Results of 2-site stochastic

468 model have been compared with those given by the homogeneous stochastic model and

469 found to better describe the peak shape.

470 Site-1 is where the majority of all adsorption/desorption events take place and the sojourn

471 time for these sites are in the millisecond scale, Fig. 56. On the other hand, at site-2 only a

472 few adsorption/desorption events take place. However, the sojourn time found on these

473 sites is roughly one thousand times longer than on site-1. The kinetic peak tailing observed

474 for BTEAC (see Fig. 1) originate from the slow adsorption/desorption events.

475 Interestingly, it was found that for HPLC, n_1 and $\tau_{s,1}$ are approximately constant in the

476 temperature interval while n_2 is increasing and $\tau_{s,2}$ is decreasing; on the other hand, for

477 UHPLC, n_1 is approximately constant and $\tau_{s,1}$ is decreasing in the temperature interval while

478 n_2 is increasing and $\tau_{s,2}$ is decreasing. The skew can be seen as a measure of the peak

479 symmetry where a large positive skew means that the peak is tailing and a negative skew

480 means that the peak is fronting. The skew decreases with temperature for both HPLC and

481 UHPLC which indicates that the peak shape becomes more symmetrical when the

482 temperature is increased. Relating the observation of decreasing skew to Eq. 3b, it is due to

483 the increase in n_2 and the decrease in $\tau_{s,2}$ for the slow site with increasing temperature.

484 **4.2.23 PSolute pressure dependence**

485 As with the temperature effect, the effect of pressure has been evaluated by using both

486 traditional thermodynamic plots and the stochastic theory of chromatography. The

487 experiments involving pressure was only performed for the UHPLC setup.

488 **4.3.1 Thermodynamic study**

489 The effect of pressure on the retention factor at constant temperature is described by the

490 equation:

$$\ln(k) = -\frac{\Delta V_{\mathrm{m}}}{RT} P_{\mathrm{avg}} + \ln(\Gamma) \tag{8}$$

which is derived from classical thermodynamics [13]. $\Delta V_{\mathrm{m}} = V_{\mathrm{stat.}} - V_{\mathrm{mob}}$ is the change in solute molar volume associated with the transition between the stationary and mobile phase and P_{avg} is the average pressure, Eq. 6. Generally the solute volume is larger in the mobile phase, i.e. $\Delta V_{\mathrm{m}} < 0$. According to Le Chatelier principle, when the pressure is increased the equilibrium between solute molecules in the mobile and stationary phase will be pushed toward the stationary phase. If the solute molecules spend longer time in the stationary phase, the retention time will increase [13,34].

The experimental data have been fitted to Eq.8 with linear regression and the result is presented in Fig. 6. The relationship between k and P_{avg} is described well with Eq. 8 with a R^2 value larger than 0.980. Calculated ΔV_{m} values and the change in retention factor for 500 bar pressure difference are shown in Table 3. These observations are in good agreement with those reported by Fallas et al. [16,17] for a similar system. Small neutral solutes were reported to have ΔV_{m} in the range 1-5 cm³/mol while small ionized solutes had ΔV_{m} in the range 10-20 cm³/mol. The negative ΔV_{m} is attributed to the partial loss of the solvation layer of the solutes when they move from the mobile to the hydrophobic stationary phase. The difference in ΔV_{m} between neutral and ionized solutes is believed to be due to the hydration of the ions which are partially lost when entering the stationary phase.

4.3.2 Stochastic analysis

For C7, OM and SNS, that is the compounds described by 1-site stochastic model, ~~the number of adsorption/desorption events~~n has been found to be nearly constant while τ_s ~~increases~~increase slightly with increasing ~~(data not shown)~~pressure. In particular, n ~~has roughly been~~was between 8000 and 14000 and τ_s between 10 and 22 ms. ~~The conclusion is that that for solutes described by 1-site model the stochastic approach is unable to single~~

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out pressure effects. This is most likely due to the fact that with very symmetrical peak

shapes as those observed under these circumstances n and τ_s are strictly correlated, so that

nonlinear fitting cannot differentiate between them.

On the other hand, for BTEAC (described by a 2-site stochastic model) the results are more

interesting. Even though, more experimental data have to be measured and different kinds

of basic compounds must be investigated tThe results of calculations for BTEAC are

presented in Fig. 7 and show that on the "fast" adsorption site there is an increase of n_1 and

a decrease in $\tau_{s,1}$. On the other handWhile, the "slow", second site, presents a nearly 50%

increase in $\tau_{s,2}$ with a 500 bar pressure increase. As a consequence, the peak skew is

increased when the pressure is increased. This increase in peak skew is due to the fact that

the the can be understood as the solute molecules spend on average a longer time adsorbed

on the slow, second site when the pressure increases which makes the tailing more

pronounced. and hence the skew more pronounced. To understand better the underlying

physical reason for this interesting more experimental observation data it should be

necessary to include more experiments with focus on several have to be obtainedmeasured

for ddifferent kinds of basic compounds, however is not the scope of this study.

From Fig. 5 6 and Fig. 7, one may conclude that high temperature and low pressure improve

peak shape for BTEAC. We believe that, even though more information must be gathered to

draw any general conclusions, this is an interesting finding to be reported.

4.4 Practical implications

First we estimated the effect of the temperature gradient in UHPLC on retention. This was

done by calculating the local propagation speeds along the column by first calculating the

geometric radial average temperature using the calculated temperature profile in Fig. 2, and

then combining this information with the van't Hoff plot (Fig. 3). Finally, the retention time

was estimated by integrating the solutes local propagation speed along the UHPLC column.

This study has evidenced that the neutral solute, C7, is least affected by temperature gradient with a decrease in k of only roughly 5%. The positively charged solute, BTEAC, is somewhat more sensitive and k decreases about 10% while the negatively charged solute, SNS, is most affected by temperature and k decreases almost 14%. The retention factor of OM decreases of approximately 9%, which places it in the same region as the other two charged solutes.

In an attempt to compare the relative importance of pressure and temperature on retention time in UHPLC, the results have been combined in Fig. 8, where the retention factors of the four investigated molecules have been normalized against the respective retention factor at 0.13 mL/min (where pressure and temperature gradients are negligible). In this figure, white bars show the estimated contribution for temperature (as described above), while gray bars show the pressure contribution, which has been estimated by assuming a linear pressure gradient over the column (as described in Sec. 4.3). Finally, black bars represent the observed, experimental retention factors found in UHPLC.

The pressure and temperature have opposite effects on retention and will therefore, to a certain degree, cancel each other out. The pressure effect is larger for all solutes, except C7, than the temperature effect, for typical UHPLC conditions. For C7, which is small, neutral molecule, the pressure effect is very small. Both the pressure and temperature effects are solute dependent and in this case it is evident that charged solutes and those with larger molecular weight are most affected by pressure.

These results suggest that in the method transfer from HPLC to UHPLC, especially if charged solutes are considered, the effect of pressure gradient along the column more than that of temperature gradient should be taken into account, as it is the dominating one.

5. Conclusions

The aim of this study has been to investigate how pressure and temperature affect retention and ~~kinetic properties~~peak shape of the solutes in HPLC and UHPLC. To this end, the chromatographic behavior of four model compounds with different physicochemical properties has been modeled from both a thermodynamic and a kinetic (microscopic-stochastic) viewpoint. The thermodynamic models showed that t~~The change~~difference in ~~in~~ solute molar volume for adsorbed and in ~~free~~bulk solution, which determines the pressure dependence of the retention factor, was largest for the polar solute omeprazole which also had the largest molecular weight. When combining the calculated temperature gradient and the linear pressure gradient the individual contributions on retention could be determined~~the enthalpy change of the adsorption process was negative for all solutes and that it was larger for the charged and polar solutes than the neutral with similar trends for HPLC and UHPLC~~. The effect of the pressure gradient was found to be the dominating one and should therefore be taken into account when switching from HPLC to UHPLC.

~~The change in solute molar volume, which determines the pressure dependence of the retention factor, was largest for the polar solute omeprazole which also had the largest molecular weight.~~From the stochastic modelling of the tailing, basic solute it was evident that an increase in temperature yielded a~~n increase~~ ~~decrease~~in average number of adsorption/desorption events while the average time spent by a molecule in the stationary phase was ~~nearly constant~~slightly decreasing. ~~Increased p~~For increased pressure the effect was the opposite. Therefore a high temperature and a low pressure yielded low tailing. ~~resulted in a larger skew of the peak of the basic solute.~~ Even though from different perspectives, the conclusions of these models converge in showing that the effect ~~on retention~~ of pressure gradient along the column is as important as that of ~~frictional~~viscous heating. With charged and polar compounds, we found that the impact of the pressure

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588 gradient is even more important than that of ~~frictional~~viscous heating in UHPLC for the

589 investigated experimental conditions.

590 ## Acknowledgement

600 ## References

601 [1]   P.W. Carr, D.R. Stoll, X. Wang, Perspectives on Recent Advances in the Speed of High-
602   Performance Liquid Chromatography, Anal. Chem. 83 (2011) 1890–1900.
603 [2]   S. Fekete, E. Oláh, J. Fekete, Fast liquid chromatography: The domination of core–shell
604   and very fine particles, J. Chromatogr. A 1228 (2012) 57–71.
605 [3]   M. Szalka, J. Kostka, E. Rokaszewski, K. Kaczmarski, Analysis of related substances in
606   bisoprolol fumarate on sub-2-μm adsorbents, Acta Chromatogr. 24 (2012) 163–183.
607 [4]   S. Fekete, J.-L. Veuthey, D.V. McCalley, D. Guillarme, The effect of pressure and mobile
608   phase velocity on the retention properties of small analytes and large biomolecules in
609   ultra-high pressure liquid chromatography, J. Chromatogr. A 1270 (2012) 127–138.
610 [5]   H. Poppe, J.C. Kraak, Influence of thermal conditions on the efficiency of high-
611   performance liquid chromatographic columns, J. Chromatogr. 282 (1983) 399–412.
612 [6]   F. Gritti, G. Guiochon, Complete Temperature Profiles in Ultra-High-Pressure Liquid
613   Chromatography Columns, Anal. Chem. 80 (2008) 5009–5020.
614 [7]   F. Gritti, M. Martin, G. Guiochon, Influence of Viscous Friction Heating on the
615   Efficiency of Columns Operated under Very High Pressures, Anal. Chem. 81 (2009)
616   3365–3384.
617 [8]   K. Kaczmarski, J. Kostka, W. Zapała, G. Guiochon, Modeling of thermal processes in
618   high pressure liquid chromatography: I. Low pressure onset of thermal heterogeneity,
619   J. Chromatogr. A 1216 (2009) 6560–6574.

620   [9]   K. Kaczmarski, F. Gritti, J. Kostka, G. Guiochon, Modeling of thermal processes in high
621   pressure liquid chromatography: II. Thermal heterogeneity at very high pressures, J.
622   Chromatogr. A. 1216 (2009) 6575–6586.
623   [10]   M.M. Fallas, U.D. Neue, M.R. Hadley, D.V. McCalley, Investigation of the effect of
624   pressure on retention of small molecules using reversed-phase ultra-high-pressure
625   liquid chromatography, J. Chromatogr. A. 1209 (2008) 195–205.
626   [11]   M.M. Fallas, U.D. Neue, M.R. Hadley, D.V. McCalley, Further investigations of the
627   effect of pressure on retention in ultra-high-pressure liquid chromatography, J.
628   Chromatogr. A. 1217 (2010) 276–284.
629   [12]   J.C. Giddings, H. Eyring, A molecular dynamic theory of chromatography, J. Phys.
630   Chem. 59 (1955) 416–421.
631   [13]   F. Dondi, M. Remelli, The characteristic function method in the stochastic theory of
632   chromatography, J. Phys. Chem. 90 (1986) 1885–1891.
633   [14]   A. Cavazzini, M. Remelli, F. Dondi, Stochastic Theory of Two-Site Adsorption
634   Chromatography by the Characteristic Function Method, J. Microcol. Sep. 9 (1997)
635   295–302.
636   [15]   A. Cavazzini, M. Remelli, F. Dondi, A. Felinger, Stochastic Theory of Multiple-Site Linear
637   Adsorption Chromatography, Anal. Chem. 71 (1999) 3453–3462.
638   [16]   A. Felinger, Molecular movement in an HPLC column: A stochastic analysis, LC-GC
639   North America. 22 (2004) 642–647.
640   [17]   M. Enmark, J. Samuelsson, T. Undin, T. Fornstedt, Characterization of an unusual
641   adsorption behavior of racemic methyl-mandelate on a tris-(3,5-dimethylphenyl)
642   carbamoyl cellulose chiral stationary phase, J. Chromatogr. A. 1218 (2011) 6688–6696.
643   [18]   F. Dondi, A. Cavazzini, M. Remelli, A. Felinger, M. Martin, Stochastic theory of size
644   exclusion chromatography by the characteristic function approach, J. Chromatogr. A.
645   943 (2002) 185–207.
646   [19]   K. Horváth, M. Olajos, A. Felinger, P. Hajós, Retention controlling and peak shape
647   simulation in anion chromatography using multiple equilibrium model and stochastic
648   theory, J. Chromatogr. A. 1189 (2008) 42–51.
649   [20]   D. Åsberg, M. Leśko, J. Samuelsson, K. Kaczmarski, T. Fornstedt, Method transfer from
650   high-pressure liquid chromatography to ultra-high-pressure liquid chromatography. I. A
651   thermodynamic perspective, J. Chromatogr. A. 1362 (2014) 206–217.
652   [21]   A. Felinger, Molecular dynamic theories in chromatography, J. Chromatogr. A. 1184
653   (2008) 20–41.
654   [22]   J.C. Giddings, Dynamics of chromatography: principles and theory, Marcel Dekker,
655   New York, 1965.
656   [23]   F. Gritti, G. Guiochon, Mass transfer mechanism in chiral reversed phase liquid
657   chromatography, Journal of Chromatography A. 1332 (2014) 35–45.
658   [24]   J. Billen, K. Broeckhoven, A. Liekens, K. Choikhet, G. Rozing, G. Desmet, Influence of
659   pressure and temperature on the physico-chemical properties of mobile phase
660   mixtures commonly used in high-performance liquid chromatography, J. Chromatogr.
661   A. 1210 (2008) 30–44.
662   [25]   M. Martin, G. Guiochon, Effects of high pressure in liquid chromatography, J.
663   Chromatogr. A. 1090 (2005) 16–38.
664   [26]   V.L. McGuffin, S.-H. Chen, Molar enthalpy and molar volume of methylene and
665   benzene homologues in reversed-phase liquid chromatography, J. Chromatogr. A. 762
666   (1997) 35–46.
667   [27]   P. Atkins, J. De Paula, Physical Chemistry, 9th ed., Oxford University Press, Oxford,
668   2010.
669   [1]   P.W. Carr, D.R. Stoll, X. Wang, Perspectives on Recent Advances in the Speed of High-
670   Performance Liquid Chromatography, Anal. Chem. 83 (2011) 1890–1900.

[2]   S. Fekete, E. Oláh, J. Fekete, Fast liquid chromatography: The domination of core–shell and very fine particles, J. Chromatogr. A. 1228 (2012) 57–71.

[3]   M. Szalka, J. Kostka, E. Rokaszewski, K. Kaczmarski, Analysis of related substances in bisoprolol fumarate on sub-2 µm adsorbents, Acta Chromatogr. 24 (2012) 163–183.

[4]   S. Fekete, J. L. Veuthey, D.V. McCalley, D. Guillarme, The effect of pressure and mobile phase velocity on the retention properties of small analytes and large biomolecules in ultra-high pressure liquid chromatography, J. Chromatogr. A. 1270 (2012) 127–138.

[5]   H. Poppe, J.C. Kraak, Influence of thermal conditions on the efficiency of high-performance liquid chromatographic columns, J. Chromatogr. 282 (1983) 399–412.

[6]   F. Gritti, G. Guiochon, Complete Temperature Profiles in Ultra-High-Pressure Liquid Chromatography Columns, Anal. Chem. 80 (2008) 5009–5020.

[7]   F. Gritti, M. Martin, G. Guiochon, Influence of Viscous Friction Heating on the Efficiency of Columns Operated under Very High Pressures, Anal. Chem. 81 (2009) 3365–3384.

[8]   K. Kaczmarski, J. Kostka, W. Zapała, G. Guiochon, Modeling of thermal processes in high pressure liquid chromatography: I. Low pressure onset of thermal heterogeneity, J. Chromatogr. A. 1216 (2009) 6560–6574.

[9]   K. Kaczmarski, F. Gritti, J. Kostka, G. Guiochon, Modeling of thermal processes in high pressure liquid chromatography: II. Thermal heterogeneity at very high pressures, J. Chromatogr. A. 1216 (2009) 6575–6586.

[10]   M.M. Fallas, U.D. Neue, M.R. Hadley, D.V. McCalley, Investigation of the effect of pressure on retention of small molecules using reversed-phase ultra-high-pressure liquid chromatography, J. Chromatogr. A. 1209 (2008) 195–205.

[11]   M.M. Fallas, U.D. Neue, M.R. Hadley, D.V. McCalley, Further investigations of the effect of pressure on retention in ultra-high-pressure liquid chromatography, J. Chromatogr. A. 1217 (2010) 276–284.

[12]   J.C. Giddings, H. Eyring, A molecular dynamic theory of chromatography, J. Phys. Chem. 59 (1955) 416–421.

[13]   F. Dondi, M. Remelli, The characteristic function method in the stochastic theory of chromatography, J. Phys. Chem. 90 (1986) 1885–1891.

[14]   A. Cavazzini, M. Remelli, F. Dondi, Stochastic Theory of Two-Site Adsorption Chromatography by the Characteristic Function Method, J. Microcol. Sep. 9 (1997) 295–302.

[15]   A. Cavazzini, M. Remelli, F. Dondi, A. Felinger, Stochastic Theory of Multiple-Site Linear Adsorption Chromatography, Anal. Chem. 71 (1999) 3453–3462.

[16]   A. Felinger, Molecular movement in an HPLC column: A stochastic analysis, LC-GC North America. 22 (2004) 642–647.

[17]   M. Enmark, J. Samuelsson, T. Undin, T. Fornstedt, Characterization of an unusual adsorption behavior of racemic methyl-mandelate on a tris-(3,5-dimethylphenyl) carbamoyl cellulose chiral stationary phase, J. Chromatogr. A. 1218 (2011) 6688–6696.

[18]   F. Dondi, A. Cavazzini, M. Remelli, A. Felinger, M. Martin, Stochastic theory of size exclusion chromatography by the characteristic function approach, J. Chromatogr. A. 943 (2002) 185–207.

[19]   K. Horváth, M. Olajos, A. Felinger, P. Hajós, Retention controlling and peak shape simulation in anion chromatography using multiple equilibrium model and stochastic theory, J. Chromatogr. A. 1189 (2008) 42–51.

[20]   D. Åsberg, M. Leśko, J. Samuelsson, K. Kaczmarski, T. Fornstedt, Method transfer from high-pressure liquid chromatography to ultra-high-pressure liquid chromatography. I. A thermodynamic perspective, J. Chromatogr. A. 1362 (2014) 206–217.

[21]   A. Felinger, Molecular dynamic theories in chromatography, J. Chromatogr. A. 1184 (2008) 20–41.

[22] J.C. Giddings, Dynamics of chromatography: principles and theory, Marcel Dekker, New York, 1965.

[23] J. Billen, K. Broeckhoven, A. Liekens, K. Choikhet, G. Rozing, G. Desmet, Influence of pressure and temperature on the physico-chemical properties of mobile phase mixtures commonly used in high-performance liquid chromatography, J. Chromatogr. A. 1210 (2008) 30–44.

[24] M. Martin, G. Guiochon, Effects of high pressure in liquid chromatography, J. Chromatogr. A. 1090 (2005) 16–38.

[25] V.L. McGuffin, S. H. Chen, Molar enthalpy and molar volume of methylene and benzene homologues in reversed-phase liquid chromatography, J. Chromatogr. A. 762 (1997) 35–46.

[26] P. Atkins, J. De Paula, Physical Chemistry, 9th ed., Oxford University Press, Oxford, 2010.

[1] P.W. Carr, D.R. Stoll, X. Wang, Perspectives on Recent Advances in the Speed of High-Performance Liquid Chromatography, Anal. Chem. 83 (2011) 1890–1900.

[2] S. Fekete, I. Kohler, S. Rudaz, D. Guillarme, Importance of instrumentation for fast liquid chromatography in pharmaceutical analysis, J. Pharm. Biomed. Anal. 87 (2014) 105–119.

[3] S. Fekete, E. Oláh, J. Fekete, Fast liquid chromatography: The domination of core–shell and very fine particles, J. Chromatogr. A. 1228 (2012) 57–71.

[4] M. Szalka, J. Kostka, E. Rokaszewski, K. Kaczmarski, Analysis of related substances in bisoprolol fumarate on sub-2-μm adsorbents, Acta Chromatogr. 24 (2012) 163–183.

[5] S. Fekete, J.-L. Veuthey, D.V. McCalley, D. Guillarme, The effect of pressure and mobile phase velocity on the retention properties of small analytes and large biomolecules in ultra-high pressure liquid chromatography, J. Chromatogr. A. 1270 (2012) 127–138.

[6] H. Poppe, J.C. Kraak, Influence of thermal conditions on the efficiency of high-performance liquid chromatographic columns, J. Chromatogr. 282 (1983) 399–412.

[7] F. Gritti, G. Guiochon, Complete Temperature Profiles in Ultra-High-Pressure Liquid Chromatography Columns, Anal. Chem. 80 (2008) 5009–5020.

[8] F. Gritti, M. Martin, G. Guiochon, Influence of Viscous Friction Heating on the Efficiency of Columns Operated under Very High Pressures, Anal. Chem. 81 (2009) 3365–3384.

[9] K. Kaczmarski, J. Kostka, W. Zapała, G. Guiochon, Modeling of thermal processes in high pressure liquid chromatography: I. Low pressure onset of thermal heterogeneity, J. Chromatogr. A. 1216 (2009) 6560–6574.

[10] K. Kaczmarski, F. Gritti, J. Kostka, G. Guiochon, Modeling of thermal processes in high pressure liquid chromatography: II. Thermal heterogeneity at very high pressures, J. Chromatogr. A. 1216 (2009) 6575–6586.

[11] V.L. McGuffin, C.E. Evans, S. H. Chen, Direct examination of separation processes in liquid chromatography: Effect of temperature and pressure on solute retention, J. Microcol. Sep. 5 (1993) 3–10.

[12] V.L. McGuffin, S.-H. Chen, Theoretical and Experimental Studies of the Effect of Pressure on Solute Retention in Liquid Chromatography, Anal. Chem. 69 (1997) 930–943.

[13] V.L. McGuffin, S.-H. Chen, Molar enthalpy and molar volume of methylene and benzene homologues in reversed-phase liquid chromatography, J. Chromatogr. A. 762 (1997) 35–46.

[14] P. Szabelski, A. Cavazzini, K. Kaczmarski, X. Liu, J. Van Horn, G. Guiochon, Experimental studies of pressure/temperature dependence of protein adsorption equilibrium in reversed-phase high-performance liquid chromatography, J. Chromatogr. A. 950 (2002) 41–53.

29

[15]   M. Martin, G. Guiochon, Effects of high pressure in liquid chromatography, J. Chromatogr. A. 1090 (2005) 16–38.

[16]   M.M. Fallas, U.D. Neue, M.R. Hadley, D.V. McCalley, Investigation of the effect of pressure on retention of small molecules using reversed-phase ultra-high-pressure liquid chromatography, J. Chromatogr. A. 1209 (2008) 195–205.

[17]   M.M. Fallas, U.D. Neue, M.R. Hadley, D.V. McCalley, Further investigations of the effect of pressure on retention in ultra-high-pressure liquid chromatography, J. Chromatogr. A. 1217 (2010) 276–284.

[18]   J.C. Giddings, H. Eyring, A molecular dynamic theory of chromatography, J. Phys. Chem. 59 (1955) 416–421.

[19]   J.C. Giddings, Dynamics of chromatography: principles and theory, Marcel Dekker, New York, 1965.

[20]   J.C. Giddings, Kinetic Origin of Tailing in Chromatography., Anal. Chem. 35 (1963) 1999–2002.

[21]   F. Dondi, M. Remelli, The characteristic function method in the stochastic theory of chromatography, J. Phys. Chem. 90 (1986) 1885–1891.

[22]   A. Cavazzini, M. Remelli, F. Dondi, Stochastic Theory of Two-Site Adsorption Chromatography by the Characteristic Function Method, J. Microcol. Sep. 9 (1997) 295–302.

[23]   A. Cavazzini, M. Remelli, F. Dondi, A. Felinger, Stochastic Theory of Multiple-Site Linear Adsorption Chromatography, Anal. Chem. 71 (1999) 3453–3462.

[24]   A. Felinger, Molecular movement in an HPLC column: A stochastic analysis, LC-GC North America. 22 (2004) 642–647.

[25]   M. Enmark, J. Samuelsson, T. Undin, T. Fornstedt, Characterization of an unusual adsorption behavior of racemic methyl-mandelate on a tris-(3,5-dimethylphenyl) carbamoyl cellulose chiral stationary phase, J. Chromatogr. A. 1218 (2011) 6688–6696.

[26]   F. Dondi, A. Cavazzini, M. Remelli, A. Felinger, M. Martin, Stochastic theory of size exclusion chromatography by the characteristic function approach, J. Chromatogr. A. 943 (2002) 185–207.

[27]   K. Horváth, M. Olajos, A. Felinger, P. Hajós, Retention controlling and peak shape simulation in anion chromatography using multiple equilibrium model and stochastic theory, J. Chromatogr. A. 1189 (2008) 42–51.

[28]   D. Åsberg, M. Leśko, J. Samuelsson, K. Kaczmarski, T. Fornstedt, Method transfer from high-pressure liquid chromatography to ultra-high-pressure liquid chromatography. I. A thermodynamic perspective, J. Chromatogr. A. 1362 (2014) 206–217.

[29]   A. Felinger, Molecular dynamic theories in chromatography, J. Chromatogr. A. 1184 (2008) 20–41.

[30]   J. Billen, K. Broeckhoven, A. Liekens, K. Choikhet, G. Rozing, G. Desmet, Influence of pressure and temperature on the physico-chemical properties of mobile phase mixtures commonly used in high-performance liquid chromatography, J. Chromatogr. A. 1210 (2008) 30–44.

[31]   G. Guiochon, D.G. Shirazi, A. Felinger, A.M. Katti, Fundamentals of preparative and nonlinear chromatography, 2nd ed., Academic Press, Boston, MA, 2006.

[32]   L.A. Cole, J.G. Dorsey, Temperature dependence of retention in reversed-phase liquid chromatography. 1. Stationary-phase considerations, Anal. Chem. 64 (1992) 1317–1323.

[33]   L.A. Cole, J.G. Dorsey, K.A. Dill, Temperature dependence of retention in reversed-phase liquid chromatography. 2. Mobile-phase considerations, Anal. Chem. 64 (1992) 1324–1327.

[34]   P. Atkins, J. De Paula, Physical Chemistry, 9th ed., Oxford University Press, Oxford, 2010.

824

825 **Figure captions**

826 **Fig. 1:** Overlaid chromatograms for HPLC (black) and UHPLC (gray). All extra-column volumes

827 have been corrected for and the retention volumes have been normalized with the HPLC and

828 UHPLC column volumes, respectively. The column temperature was set to 40°C and the

829 pressure drop over the column in HPLC was 100 bar and in UHPLC 800 bar, respectively.

830 ~~**Fig. 2:** Calculated temperature profile in UHPLC for the mobile phase 25/75, v/v~~

831 ~~acetonitrile/phosphate buffer at flow rate 1.2 mL/min. The dotted line represents the inner~~

832 ~~column wall. 782 bar over column~~**Fig. ~~6~~2:** Experimental retention factors for BTEAC (circles),

833 C7 (squares), SNS (diamonds), and OM (triangles) for different pressures. Mobile phase

834 compositions were 7, 15 and 25% acetonitrile for BTEAC, SNS and C7/OM, respectively and

835 flow rate was 0.13 mL/min.~~, with the model fit to Eq.8 (lines).~~

836 **Fig. 3:** Calculated temperature profile in UHPLC for the mobile phase 25/75, v/v

837 acetonitrile/phosphate buffer at flow rate 1.2 mL/min. The dotted line represents the inner

838 column wall. At 50 mm the center of the column is warmest (55 C) and the column wall is at

839 ca 52 C; radius 0 is the center of the column.~~.~~ The pressure over the column is 782 bar. ~~782~~

840 ~~bar over column.~~

841

842

843 **Fig. ~~4~~3:** Experimental retention factors for BTEAC (circles), C7 (squares), SNS (diamonds), and

844 OM (triangles) for different temperatures. Mobile phase compositions were 7, 15 and 25%

845 acetonitrile for BTEAC, SNS and C7/OM, respectively and flow rate was 0.13 mL/min.

858 **Fig. 65:** Stochastic modelling of BTEAC which is described by a 2-site model at different

859 temperatures. N is the column efficiency determined from Eq. 4 and the skew is determined

860 with Eq. 3b.

863 **Fig. 7:** Stochastic modelling of BTEAC which is described by a 2-site model at different

864 pressures. N is the column efficiency determined from Eq. 4 and the skew is determined

865 with Eq. 3b.

32

870 "observed" represents actual experimental result where both pressure and temperature

871 effects are present.

Figure 1
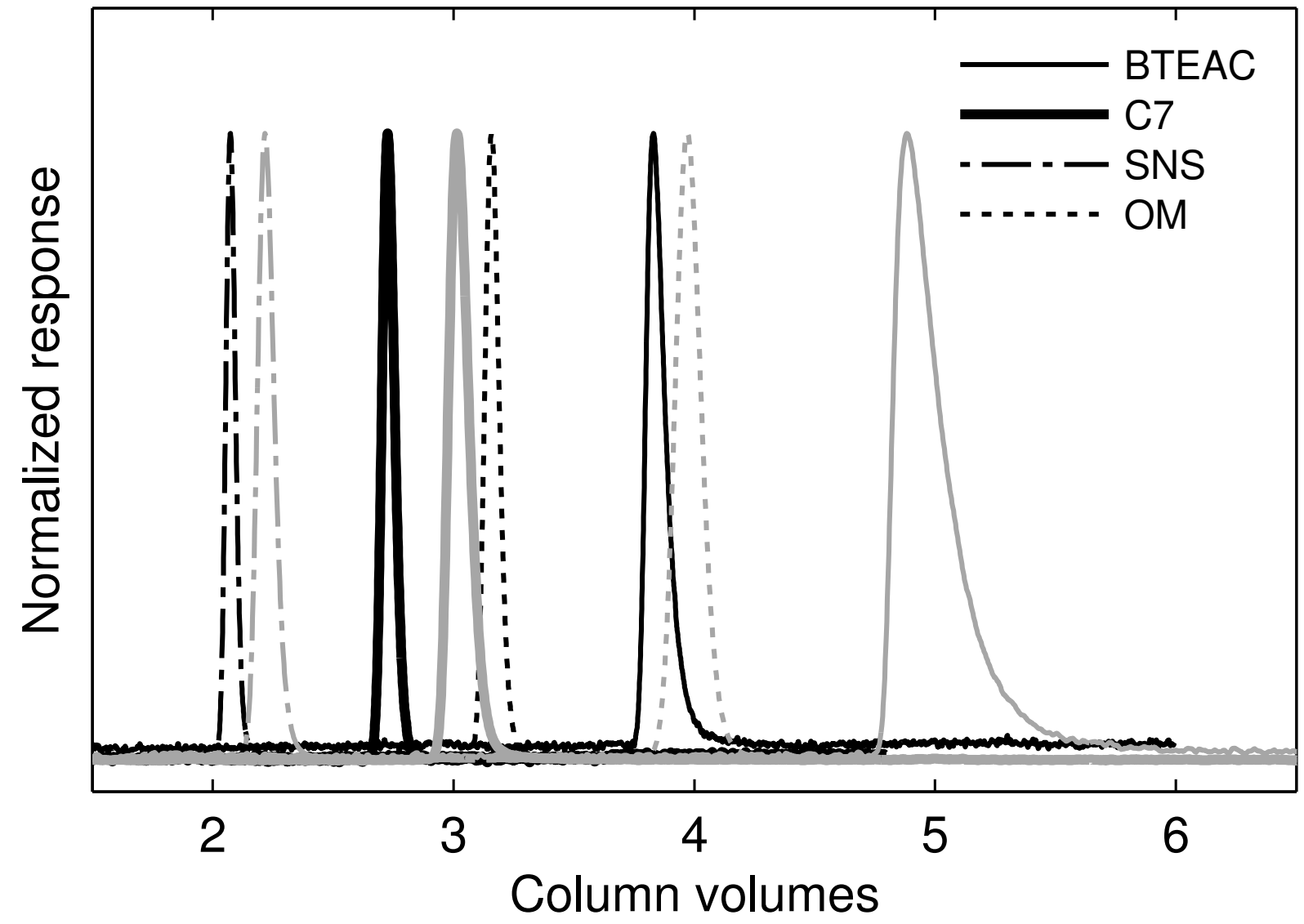
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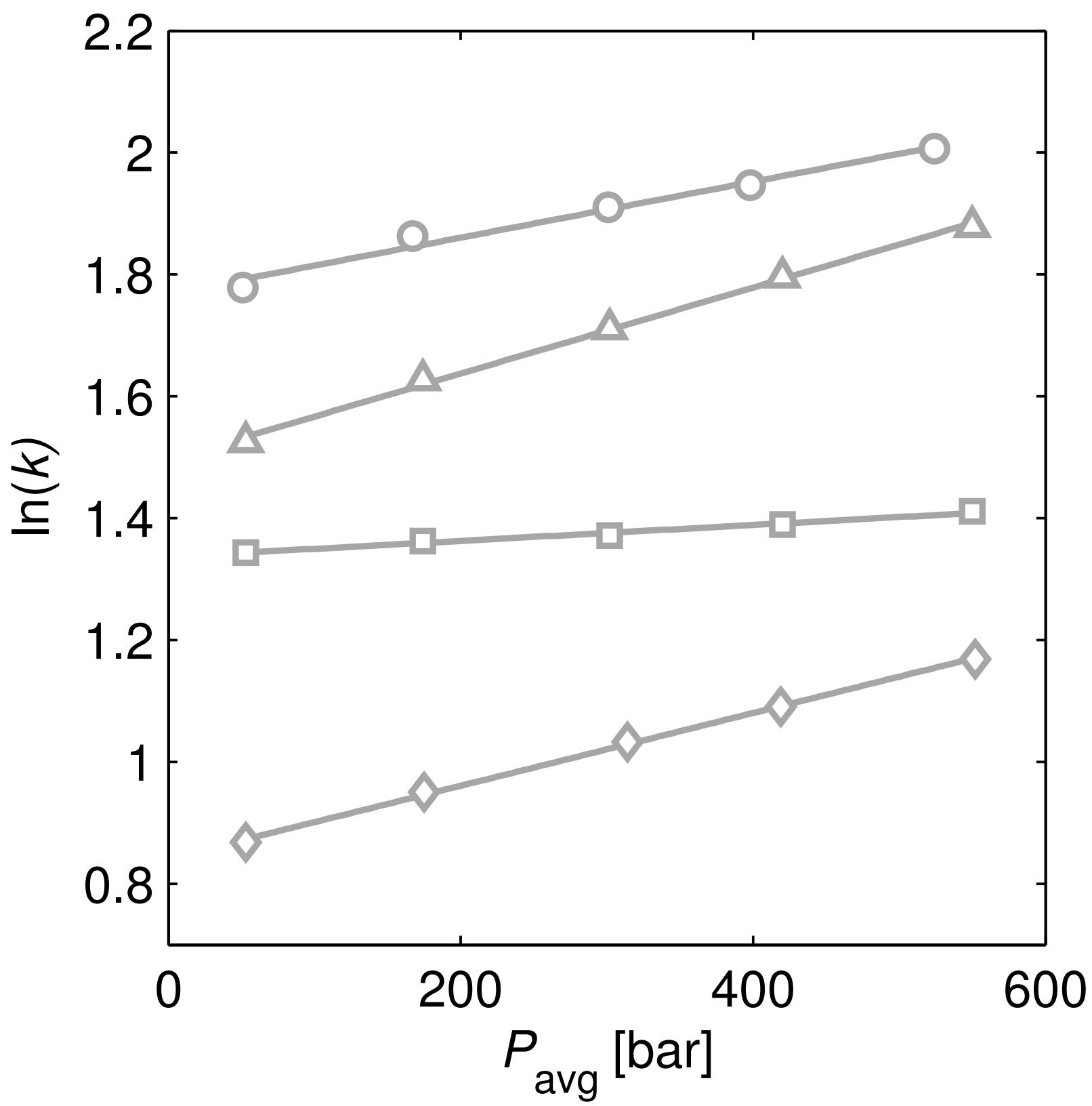
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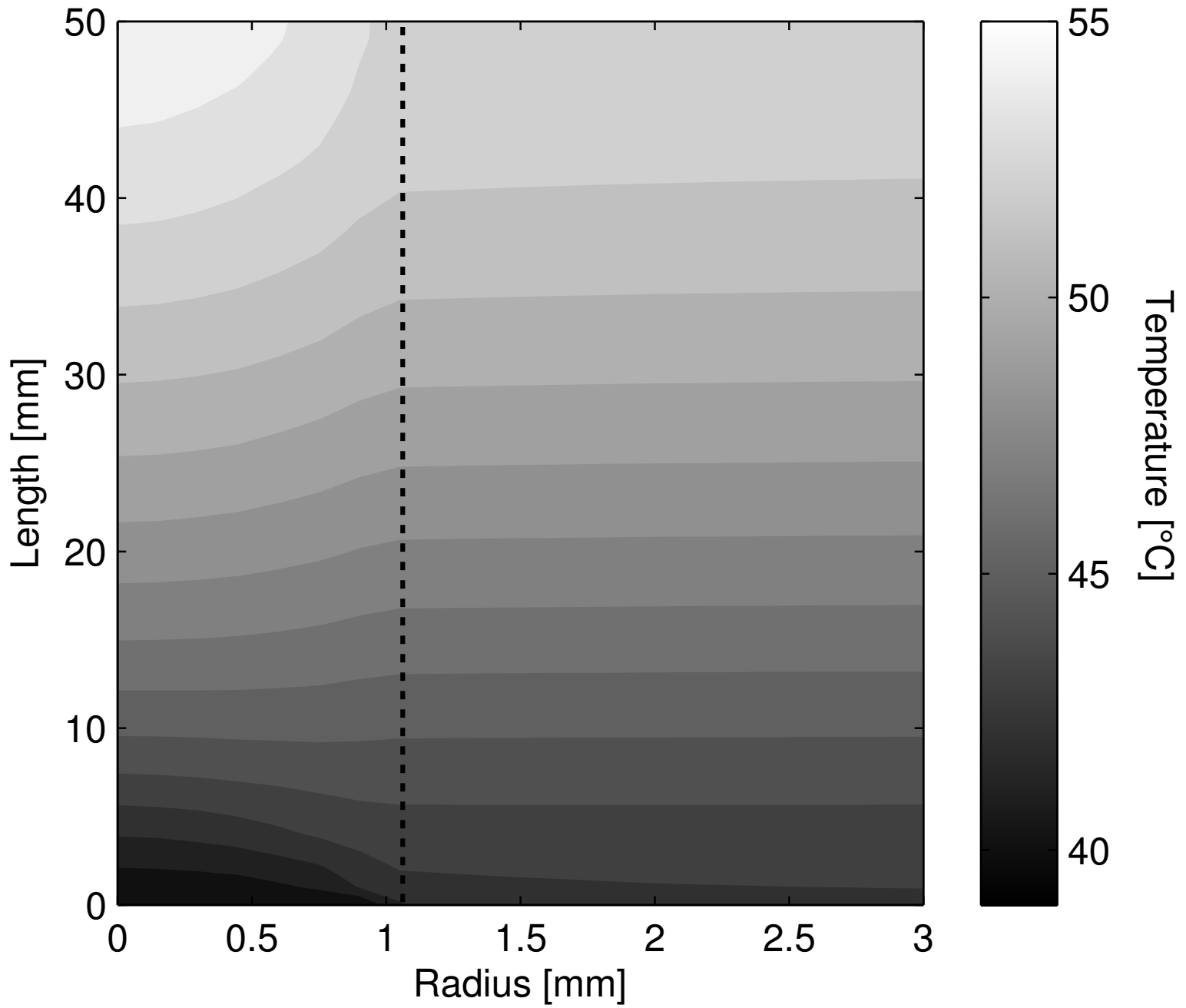
Figure 3 COLOR
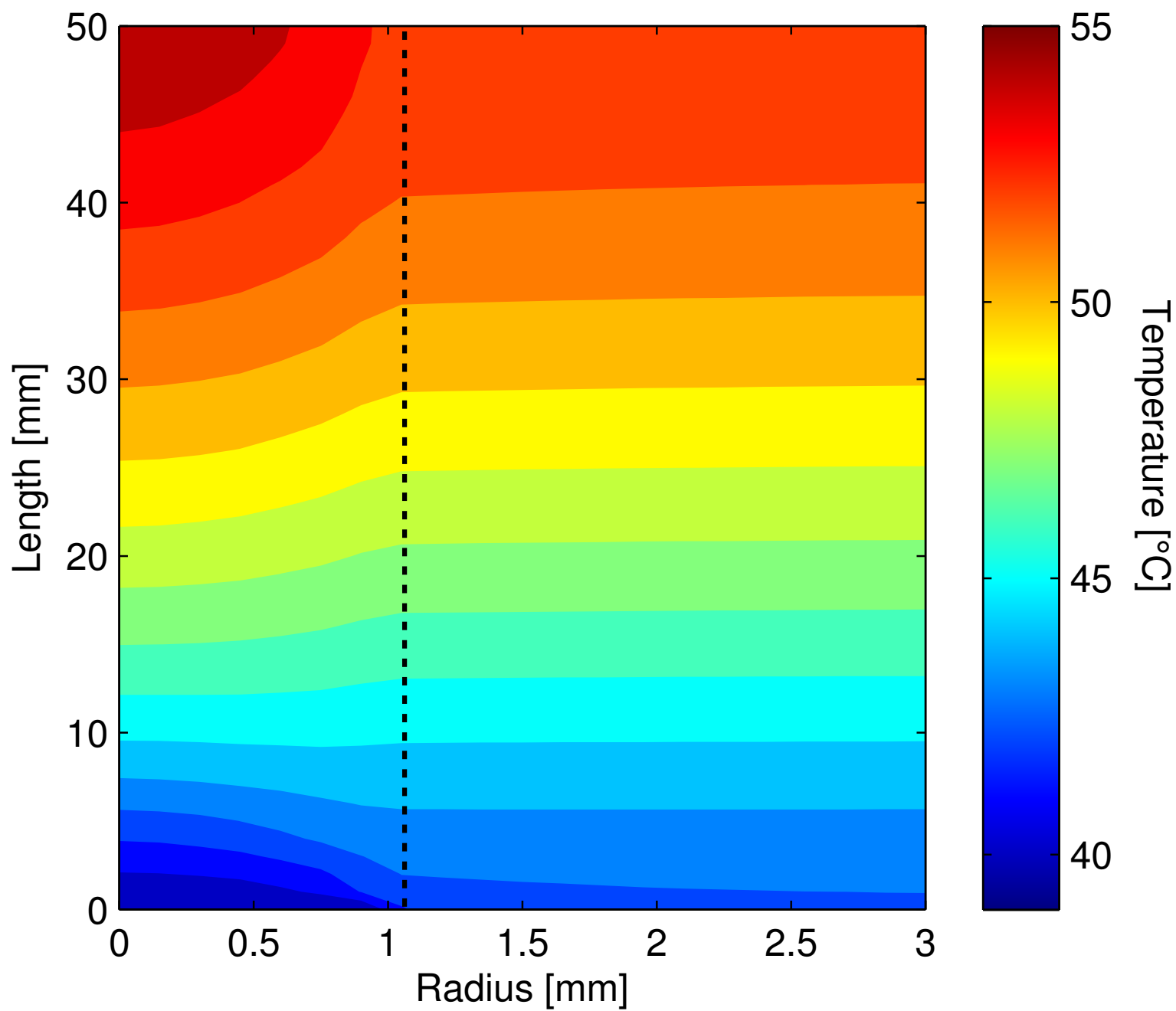
Figure 4
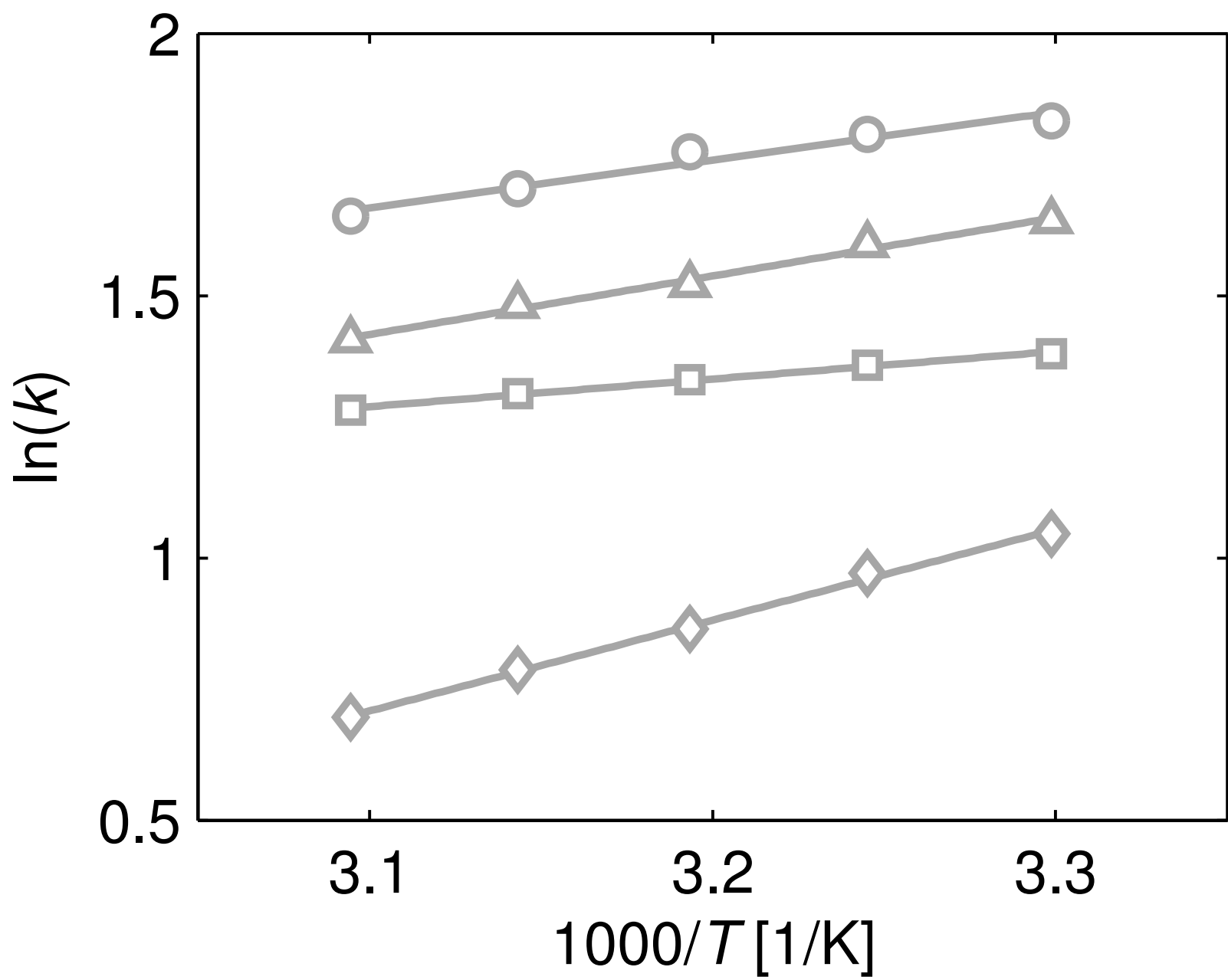
Figure 5
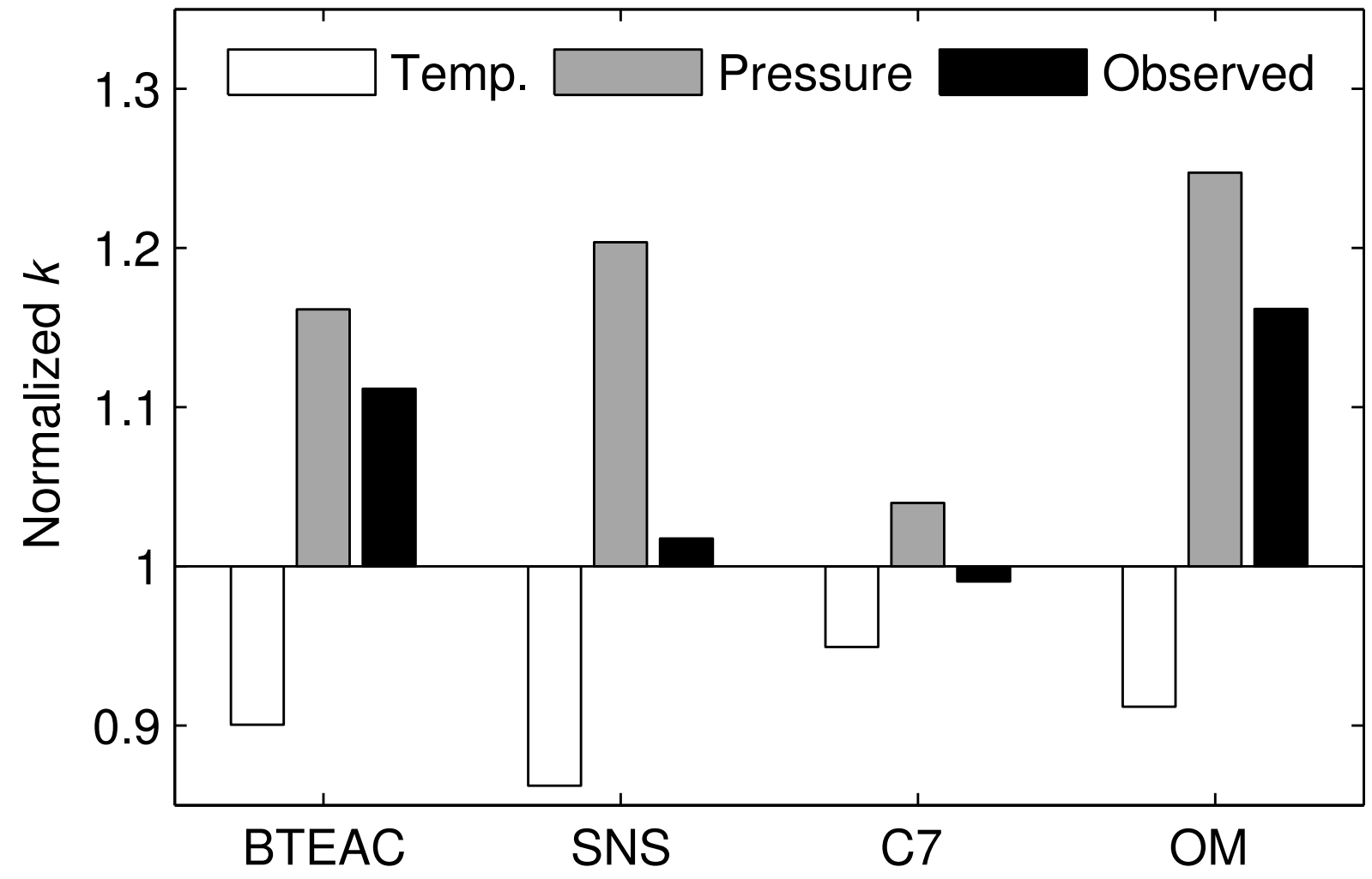
Figure 6
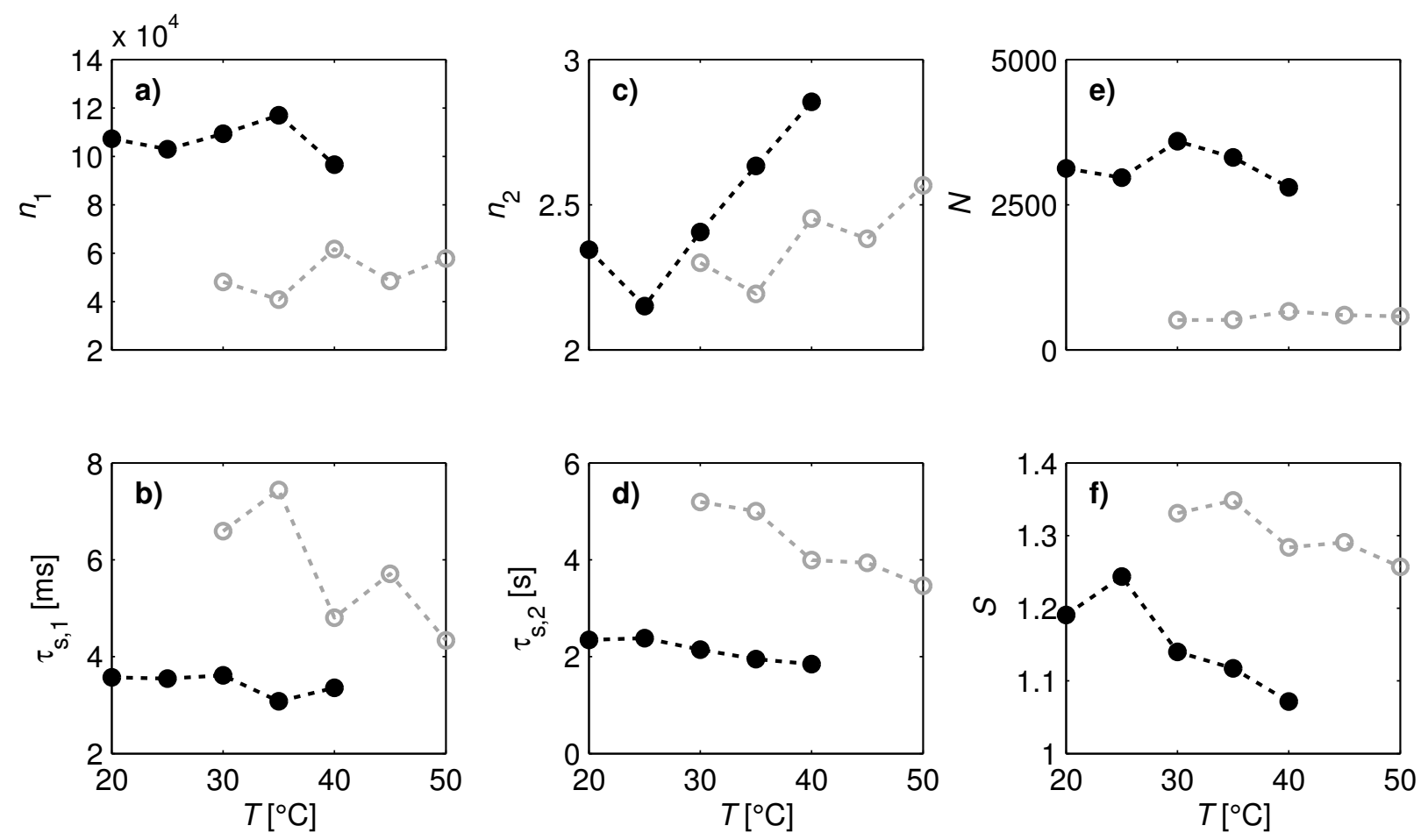
Figure 7
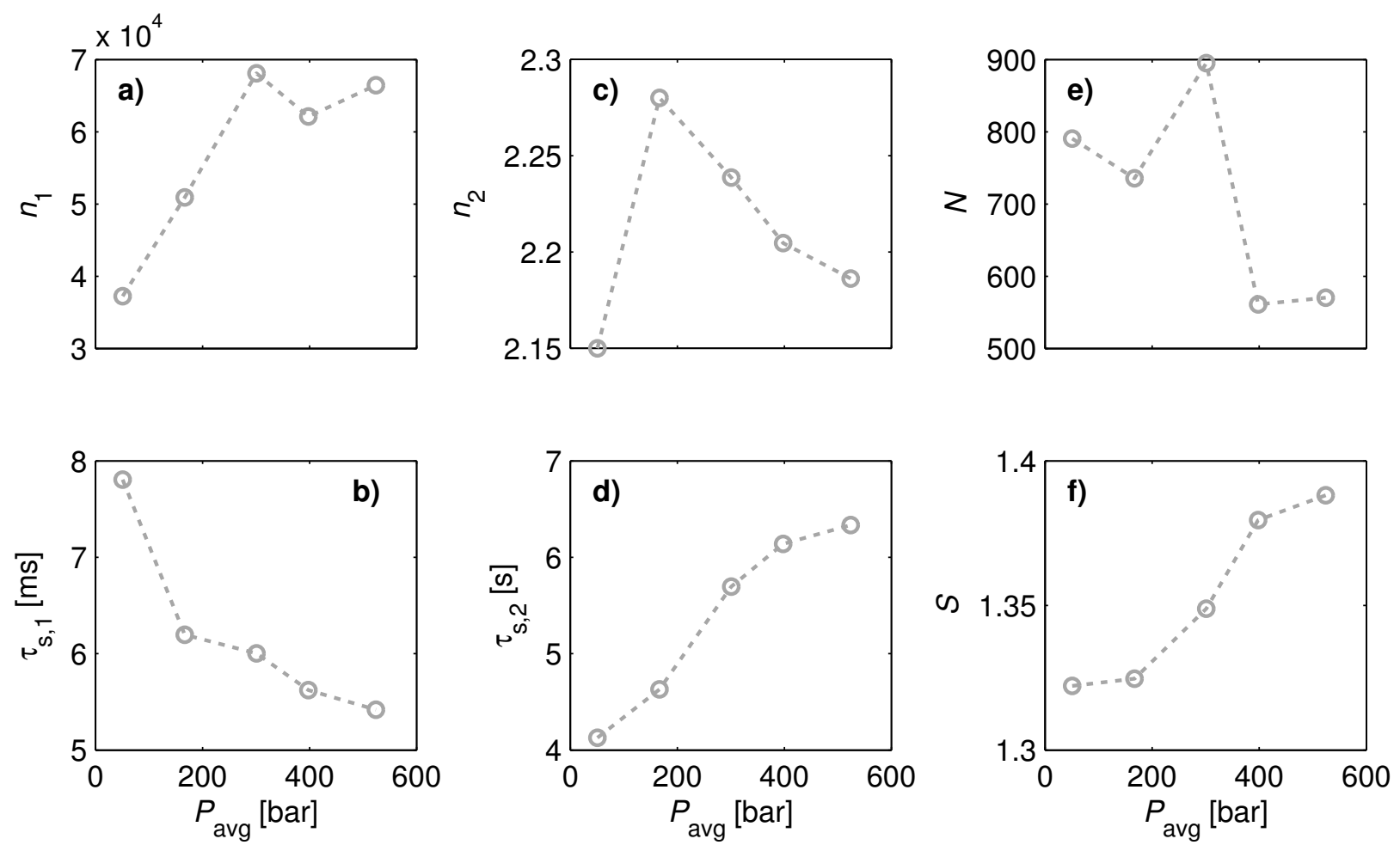
Table 1

Table 1:

Physicochemical properties from the manufacturer of the columns

Property	XBridge BEH-C$_{18}$	AQUITY BEH-C$_{18}$
Average particle size [µm]	3.5	1.7
Pore volume [cm^3/g]	0.71	0.70
Surface area [m^2/g]	184	179
Average pore diameter [Å]	138	141
Total carbon content [%]	17.88	17.40
Surface concentration [µmol/m^2]	3.36	3.07