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Investigation of plateau methods for adsorption isotherm determination in supercritical fluid chromatography☆

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ABSTRACT

The Perturbation Peak (PP) method and Frontal analysis (FA) are considered as the most accurate methods for adsorption isotherms determination in liquid chromatography. In this study we investigate and explain why this is not the case in Supercritical Fluid Chromatography (SFC), where the PP method does not work at all, using a modern analytical system. The main reason was found to be that the solute to be studied must be dissolved in the MeOH reservoir before it is mixed with CO_2. Since the solute occupies a certain partial volume in the reservoir, the larger the solute content the larger this fractional volume will be, and the final MeOH fraction in the mobile phase will then be smaller compared to the bulk mobile phase without solute in the modifier. If the retention of small injections on the concentration plateaus, i.e., "analytical-size" perturbation peaks, is sensitive to small variations of MeOH in the eluent, this will seriously decrease the accuracy of the PP method. This effect was verified and compensated for and we also demonstrated that the same problem will occur in frontal analysis, another concentration plateau method.

1. Introduction

There is currently a strong trend towards the use of preparative Supercritical Fluid Chromatography (Prep-SFC) for purification and many leading pharmaceutical industries have replaced their preparative Liquid Chromatography (Prep-LC) instruments with Prep-SFC ones, especially for purification of gram amounts in the discovery stage of drug development. The main reason for this is that the production rate can be several-fold higher in prep-SFC compared to Prep-LC [1]. In addition, the main solvent in Prep-SFC (CO_2) is already in the carbon cycle and therefore much less environmentally harmful than the organic solvents frequently used in Prep-LC. This trend is now also visible in the analytical chromatography area; one sign is that leading instrument manufacturers have recently launched new generations of analytical SFC instruments [2,3].

One inherent disadvantage of SFC is that the method is more complex than LC, mainly due to the compressibility of the supercritical mobile phase fluid that causes local variations in density, temperature and viscosity in the column. Currently the lack of fundamental knowledge hampers both understanding of the underlying mechanisms as well as reliable computer-assisted optimizations of large scale SFC processes [4]. Computer-assisted optimization can ultimately be used to design more robust separation processes with higher production rates that are cheaper, safer and more environmentally friendly. Reliable computer-assisted optimization of Prep-SFC requires both proper modeling of the separation process and accurate determination of the adsorption isotherms of the component(s) in the used phase system. With accurate adsorption isotherms, over broad solute concentration ranges, it is also possible to obtain deeper mechanistic understanding of the adsorption process; i.e., to determine the degree of heterogeneity and energy of interaction as well as the monolayer capacity of the adsorption sites. For high quality adsorption data it is possible to calculate the adsorption energy distribution (AED) and thereby, prior to the model fitting procedure, determine the particular types of interactions, e.g. dipole–dipole, van der Waals, etc., that are present in the phase system. This was recently demonstrated for liquid-based biosensor systems [5].

In a recent study we investigated the usefulness of a modern analytical SFC instrument for rapid and reliable determination of adsorption data [6]. This is important since process units in the pharmaceutical industry uses new commercial, analytical-scale SFC instruments to scout separation systems prior to scale-up. More specifically, we investigated the possibility to transfer the

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following adsorption isotherm determination methods from LC to SFC: Elution by Characteristic points (ECP), the Retention Time Method (RTM), the Inverse Method (IM) and the Perturbation Peak (PP) method. RTM, ECP and IM are based on elution profiles, whereas the PP method and Frontal Analysis (FA) are so called concentration plateau methods based on experiments where the chromatographic column is equilibrated with a constant stream of the component to be studied. For LC it has been verified and validated that concentration plateau methods are more accurate and reliable than methods based on elution profiles [7–10]. In our SFC study, the adsorption isotherm data generated by the different methods were analyzed and validated by comparing computer simulated elution profiles, using the determined isotherm data, with experimental ones [6]. Here we found that the methods based on elution profiles, i.e., ECP, IM and RTM, were able to accurately predict overloaded experimental elution profiles while the PP method, based on generating data from concentration plateaus, was not able to do so in these SFC experiments [6]. The adsorption isotherm obtained from the PP method did only coincide with the ones obtained by ECP, RTM or IM for the initial linear part of the adsorption isotherm, while at increasing component concentrations, the PP method successively deviated more and more from the other methods.

There are a small number of articles describing the determination of adsorption isotherms for SFC [11–16]. Lübbert et al. [16] used the PP method, however, the authors did not verify the determined adsorption model's ability to predict overloaded elution which makes it difficult to judge the reliability of the method for SFC. Nevertheless, the authors presented a sound approach to control pressure and temperature and in our SFC study [6] we confirmed another recent publication [17] regarding the importance of using external sensors for temperature, mass flow and pressure. In our study these sensors were used to ensure near isopycnic and isothermal conditions, which are needed to transfer adsorption isotherm determination methods from LC to SFC. Under these conditions we also showed that the ideal model, that for example are used to derive the ECP method [18,19] and RTM, can accurately describe the system [6] because modern analytical SFC systems usually have very high column efficiencies.

The aim of this study is to investigate and explain why the PP method results in an adsorption isotherm that differs from the one determined using ECP. We will also investigate if the same problem occurs in SFC for the FA method that is considered to be the most accurate method in LC [7,20–22]. Finally, AED-calculations is conducted, this has only been done once as far as we know [23].

2. Theory

As in our previous study [6], it is assumed that the column is operated under close to isopycnic and isothermal conditions and this assumption is verified experimentally by external measurements of mass flow, pressure and temperature. A short description of how to calculate density from an Equation of State is presented in Section 2.1 and the bi-Langmuir adsorption isotherm model is presented in Section 2.2. The PP, ECP and FA adsorption isotherm determination methods are presented in Sections 2.3–2.5. The theory for gradient elution is presented in Section 2.6 and Section 2.7 describes the processing of the adsorption isotherm data.

2.1. Mobile phase density and effective volumetric flow

To calculate the density of the mobile phase fluid at a certain point in the chromatographic system the Kunz and Wagner [24] Equation of State, as implemented by the National Institute of Standards and Technologies in REFPROP v 9.1 [25] was used. The inputs are the mass fractions of CO_2 and MeOH (w_{CO_2} and w_{MeOH}) and the temperature and pressure at a specific point on the column. For practical purposes, the pressure and temperature was measured at the inlet and outlet of the column (P_{inlet}, P_{outlet}, T_{inlet}, T_{outlet}). The mass fractions of CO_2 and MeOH were calculated from the total mass flow, $\dot{m}_{total}$, measured by a mass flow sensor, and the MeOH mass flow, $\dot{m}_{MeOH}$, according to,

$$w_{CO_2} = \frac{\dot{m}_{total} - \dot{m}_{MeOH}}{\dot{m}_{total}}, \qquad w_{MeOH} = 1 - w_{CO_2} \tag{1}$$

The explicit statement of near isopycnic conditions is that the density in the axial direction is nearly constant. For practical purposes, this is verified by comparing the density at the column inlet and outlet,

$$\rho(P_{inlet}, T_{inlet}) \approx \rho(P_{outlet}, T_{outlet}) \approx \rho(P_{average}, T_{average}). \tag{2}$$

The effective volumetric flow rate $F_{V,average}$ is calculated from the total mass flow and the average density, $\rho_{average}$,

$$F_{V,average} = \frac{\dot{m}_{total}}{\rho_{average}}. \tag{3}$$

In some experiments antipyrine will be introduced in the MeOH fractions. We will ignore the effect of antipyrine in the estimated mass fraction, Eq. (1), and average density, Eq. (3), since the maximum possible mass fraction of antipyrine is about 1% in the experiments. We therefore only expect small errors in these calculations. In addition, to account properly for this a suitable Equation of State for the mixture CO_2/MeOH/antipyrine will be needed and it is not available today.

2.2. The adsorption isotherm model

The adsorption isotherm relates the concentration in the mobile phase, C, and in the stationary phase, q, and in this study the bi-Langmuir model, the sum of two single-site Langmuir terms, are used,

$$q = q_{s,1}\frac{K_1 C}{1 + K_1 C} + q_{s,2}\frac{K_2 C}{1 + K_2 C}, \tag{4}$$

where $q_{s,1}$, $q_{s,2}$ is the monolayer saturation capacity and K_1, K_2 the association equilibrium constant for site 1 and 2. Here we define the first adsorption site as the low energy site and the second one as the high energy site, i.e., $K_1 < K_2$.

2.3. The Perturbation Peak (PP) method

In the PP method the adsorption isotherm is determined by injecting a small excess or deficiency of the studied component into a column that is already equilibrated with an eluent containing a constant stream of identical molecules, i.e. a concentration plateau. The perturbation peak will be a single peak in the chromatogram and its retention time is related to the slope of the adsorption isotherm according to,

$$\frac{dq}{dC_0} = \frac{V_R(C_0) - V_M}{V_a}, \tag{5}$$

where $V_R(C_0)$ is the retention volume of the perturbation peak at an established concentration plateau with concentration C_0, V_a is the stationary phase volume, V_M is the column hold up volume and dq/dC_0 is the slope of the adsorption isotherm. By repeating this experiment at several different concentration plateau levels, the whole adsorption isotherm can be determined. Eq. (5) is derived from the ideal model of chromatography and assumes constant volumetric flow and constant amount of modifier in the eluent. However, because only the first moment of the perturbation peak is used, low column efficiency will only reduce the height of the perturbation peak.

2.4. The elution by characteristic points (ECP)

In the ECP method, the adsorption isotherm data is usually obtained by integrating the diffuse part of an overloaded elution profile [20,26,27]. However, this method requires that the point where the concentration is zero is known and since this concentration range usually is very noisy this introduces uncertainties in the estimated adsorption isotherm [26]. By instead determining the raw slope of the adsorption isotherm this source of error can be eliminated [26],

$$\frac{dq}{dC} = \frac{V_R(C) - V_M - V_{inj}}{V_a}, \quad (6)$$

here $V_R(C)$ is the elution volume corresponding to the mobile phase concentration C and V_{inj} is the injection volume. The ECP method is derived using the ideal model and therefore requires moderately to highly efficient separation systems [18,19]. However, the efficiency alone is not a good measurement because the shape of an overloaded elution zone is dependent both on the column load and the efficiency [20,28–30]. A better measurement of this should be the Shirazi number [28,30], showing how good the ideal model describes the experimental data.

2.5. Frontal analysis (FA)

FA is generally regarded as the most accurate method to determine adsorption isotherms in LC [20,31,32]. It is usually carried out by injecting a large zone of solute so that a concentration plateau is established. The retention volume of the front of the elution zone is associated with the adsorbed amount on the column surface,

$$q(C) = \frac{(V_R(C) - V_{sys} - V_M) \cdot C}{V_a} \quad (7)$$

where $V_R(C)$ is the retention volume of the elution front for concentration C and V_{sys} is the system dead volume without the column. By repeating this experiment for different injection concentrations the whole adsorption isotherm can be determined.

2.6. Gradient theory

We base our interpretation of the varying conditions on gradient theory for LC, especially in Fig. 2. In analytical LC the separation is usually performed using gradient mode, i.e., having a varying volume fraction of a modifier during the elution. The retention factor of the eluted components are often described using the Linear Solvent Strength (LSS) theory [33,34] which provides the following relationship between the retention factor (k) and the volume fraction of organic modifier in the mobile phase, φ,

$$k(\phi) = k_0 \exp(-S\phi), \quad (8)$$

where k_0 is the retention factor without any modifier in the eluent and S is a constant that describes the modifier's effect on the separation system's retention factor.

For nonlinear adsorption isotherms it is usually assumed that the adsorption isotherm model itself is not affected by the fraction of organic modifier in the mobile phase, only the adsorption isotherm parameters change [35–41]. In this study we have also assumed that the saturation capacity is not affected by the modifier. If we expand the equilibrium constants in the bi-Langmuir model Eq. (4) using Eq. (8) we obtain,

$$q(C, \phi) = q_{s,1} \frac{K_{1,0} \exp(-S_1\phi)}{1 + K_{1,0} \exp(-S_1\phi)} + q_{s,2} \frac{K_{2,0} \exp(-S_2\phi)}{1 + K_{2,0} \exp(-S_2\phi)}. \quad (9)$$

2.7. Analysis of adsorption data

A three step approach for processing adsorption data from LC, prior to the model fit with statistical evaluation using an F-test [31], was recently developed [31,42–44]. In Step 1 the type of adsorption isotherm is determined by plotting the slope of the adsorption data to see if it contains any inflection points. In Step 2, a Scatchard plot is used to detect the characteristics of the adsorption isotherm, e.g. a linear plot is only true for the Langmuir model and a concave plot indicates a Tóth or a bi-Langmuir model. In Step 3, an Adsorption Energy Distribution (AED) [45–47] is calculated to estimate the number of adsorption sites.

The adsorption data is fitted to a potential adsorption model that fulfills the requirements/observations from Step 1 to 3 above. The fitted adsorption model is validated by comparing predicted overloaded elution profiles with experimental ones; if they differ a new model must be tried.

3. Experimental and procedures

3.1. Materials

Antipyrine (1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazol-3-one) pharmacopoeia grade (Sigma-Aldrich, Steinheim, Germany), HPLC grade MeOH (Fischer Scientific, Loughborough, UK) and CO_2 (>99.995%, AGA Gas AB, Sweden) were used. The SFC system was a Waters UPC^2 system (Waters Corporation, Milford, MA, USA). The column used was a Kromasil silica 100 × 4.6 mm column, packed with 5 μm nominal particle size, 100 Å pore size (AkzoNobel Eka, Bohus, Sweden). The column temperature was measured with two PT-100 4-wire resistance temperature detectors with an accuracy of ±0.2 °C (Pentronic AB, Gunnebo, Sweden). The inlet and outlet pressures were measured, in the same way as in [6], using two absolute pressure transmitters (model EJX530A, Yokogawa Electric Corporation, Tokyo, Japan) with an accuracy of ±1 bar. The signals were logged using a PT-104 data logger from Pico Technology (Cambridgeshire, United Kingdom). The mass flow was measured using a Bronkhorst mini CORI-FLOW model M12 (Bronkhorst High-Tech B.V., Ruurlo, Netherlands) with an accuracy of ±0.2% of the mass flow. The MeOH mass flow was measured by connecting the mass flow meter between the solvent pump and the vent valve, before the mixer, while the total mass flow was measured directly after the mixer.

3.2. Procedures

All experiments were conducted at a set flow rate of 1 mL/min, at a set back pressure of 150 bar and at a column temperature of 35 °C. The set MeOH composition was varied between 8 and 12 (v/v)%$_{set}$ (notice that here we will use the notation %$_{set}$ for the set composition and %$_{act.}$ for actual or measured composition). The settings were verified by additional measurements of mass flow, pressure and temperature to ensure all adsorption isotherm determination methods use the true conditions, see Section 2.1. The dead volume of the system capillaries and connections from the injector to the detector was taken from [6]. The actual volume fraction MeOH in the samples can be calculated using the following relation,

$$(v/v)\%_{act} = \frac{m_{tot} - m_{antipyrine}}{\rho_{MeOH} \cdot V_{tot}} \times 100, \quad (10)$$

where $m_{antipyrine}$ is the amount of antipyrine, m_{tot} is the total mass of the prepared solution with volume V_{tot} and ρ_{MeOH} is the density of MeOH. Ten solutions were prepared in MeOH in the concentration range between 10 and 115 g/L. The exact concentrations of the solutions where chosen so the actual MeOH fraction in the system could be adjusted precisely by the SFC instrument. The smallest

Table 1
Experimentally obtained values of the MeOH volume fraction in samples with different concentrations of antipyrine at 22 °C. Details on the calculations used are presented in Section 3.2.

Antipyrine (g/L)	0	13.52	25.59	37.43	49.05	60.46	71.67	82.67	93.47
MeOH (volume fraction)	1	0.990	0.981	0.971	0.963	0.954	0.945	0.937	0.928

change in fluid composition using the UPC^2 system is 0.1 (v/v)%$_{set}$. For example: if a 10 (v/v)%$_{act.}$ MeOH content is sought on a perturbation plateau and the modifier solution equilibrating the column contains 95 (v/v)%$_{act.}$ MeOH the system needs to compensate this by pumping about 10.5 (v/v)%$_{set}$ MeOH. The solutions fulfilling these requirements are listed in Table 1. All overloaded elution profiles used a 250 g/L solution of antipyrine in MeOH. Partial loop injections of 10, 20, 30 and 40 μL were made using a 50 μL fixed loop. Both small perturbation injections and larger partial loop injections were compared with some full loop injections with excellent agreement.

3.3. Calculations

The adsorption energy distribution was calculated using the expectation maximization method [46] with 300 grid points and 500 000 iterations. Chromatographic simulations were done using the Rouchon algorithm with 2 500 theoretical plates (N) [20,48]. All calculations and plots were conducted using Python 3.3.2, Numpy 1.7.1, Scipy 0.13.0 and Matplotlib 1.3.1.

4. Results and discussion

Previously we have noticed that adsorption isotherms determined using the PP method deviates considerably from the ones determined with other methods such as the ECP method, the inverse method and the retention time method [6]. Our hypothesis is that this deviation is due to the fact that the amount of MeOH modifier in the column decreases with increasing solute concentration in the established concentration plateaus. The reason for this is that the solute will displace some modifier and this will result in a reduction of MeOH fraction in the eluent, although the instrumental setting of the CO_2/MeOH ratio is constant. The difference between set and true CO_2/MeOH ratio will increase with increasing solute concentration in the established concentration plateau.

Below we verified the above effect by measuring the adsorption isotherm of the studied component (antipyrine) at different CO_2/MeOH ratios and then used two different approaches to rectify this effect. The first approach used was to ensure that the MeOH fraction was constant at the different plateau concentrations used in the PP method. The second approach was to conduct normal PP experiments and compare the retention time of the perturbation peak at different plateau concentrations with overloaded injections with corresponding MeOH fraction in the eluent.

4.1. System verification

The performance of the chromatographic system was verified by external measurement of pressure, temperature and mass flow. The flow rate of the SFC-system was set to 1 mL/min at a column temperature of 35 ± 0.5 °C and the Back Pressure Regulator (BPR) was set to 150 ± 1 bar. Pressure, temperature and mass flow were measured at 9 different CO_2/MeOH compositions from 88.7/11.3 to 91.2/8.8 (v/v)%$_{set}$. At the set condition of 90/10 (v/v)%$_{set}$ CO_2/MeOH, the system was estimated to deliver 90.3/9.7 (v/v)%$_{act.}$ CO_2/MeOH with a density of 0.86 g/mL at a flow rate of 1.1 mL/min. At the set condition of 91.2/8.8 (v/v)%$_{set}$ CO_2/MeOH (the lowest relevant MeOH composition) the system was estimated to deliver 91.6/8.4 (v/v)%$_{act.}$ CO_2/MeOH with a density of 0.86 g/mL at a flow rate of 1.1 mL/min. For more details on all MeOH fractions, see Table 2.

For all methanol fractions, the axial temperature gradient was measured to be 0.5 °C. It cannot be assumed that radial temperature gradients across the column do not exist, and if so, these would need to be measured using a different approach. However, if there are small gradients, these will be present for all experiments, i.e. not only for the PP but also for the ECP measurements. Since ECP and PP deviates strongly in this study we can assume we can ignore these effects.

For all set conditions evaluated, the system was estimated to deliver a slightly higher volume fraction of CO_2 and the relation between the set and estimated volume fractions was linear and could therefore easily be interpolated. The deviations from set and estimated volume fraction are in general small and it is reasonable to assume that the system actually delivers very close to the set conditions. For the CO_2/MeOH eluent ratios used in this study, the relative error between the measured density and the density calculated by the REFPROP software was less than 1.75% according to the study [49]. This error might increase with the increasing mass fraction of the component in the eluent; however the maximum mass fraction of antipyrine is only about 1% in the experiments.

4.2. Adsorption of antipyrine for different methanol content

It is generally recognized that the retention factor decreases with increasing amount of modifier in the eluent. In Fig. 1(a), the natural logarithm of the retention factor for antipyrine, at different fractions of MeOH in the eluent, is plotted. As we can see, the LSS-theory, Eq. (8), describes the retention time well with an S-value of 15 (note that we use the natural logarithm and not the base ten logarithm that is also commonly used). This is rather high compared to the S-values of about 8 that is generally observed in reversed phase LC for molecules with molecular weight 188 g/mol like antipyrine [50]. The higher sensitivity of the retention times in SFC with respect to the MeOH fraction has also been reported in a study by Berger et al. [51], here a large retention shift of 4-hydroxybenzoic acid eluted on a diol phase was observed, even for small increases in MeOH content of the eluent. The authors went even further and isolated the effect of change in pressure, density and composition on the retention and their conclusion was that composition, i.e., the MeOH content of the eluent, was by far the most important parameter. In the current study it was estimated that there was no measurable difference between the average density at the lowest and the default 90/10 (v/v)%$_{set}$ composition. There is however a pressure gradient of about 10 bar, see Table 2, and this gradient is identical regardless of the set eluent composition. The only change that is then measurable is the eluent composition and this should therefore further be investigated.

In order to quantify how the adsorption isotherm of antipyrine is affected by the amount of MeOH in the eluent, the adsorption isotherm was determined using the ECP method [26] for different MeOH content. Prior to the adsorption isotherm model fit the raw adsorption isotherm data was analyzed using Adsorption Energy Distributions (AED) calculations and Scatchard plots to reduce the number of potential adsorption models. In Fig. 1(b) the AED from the raw slope of the adsorption isotherm data is plotted, as can be seen the AED is at least bimodal with a low energy site that diverge off scale. This is probably because the low energy site is not

Table 2
Performance verification of the Waters UPC² system. Temperature (T), pressure (P), effective volumetric flow ($F_{v,average}$) and density (ρ) at the column inlet and outlet together with mass flow of CO_2 ($\dot{m}_{CO_2}$) and of MeOH ($\dot{m}_{MeOH}$). Details of procedures and calculations are presented in Section 2.1 and 3.

Set (v/v)%	Column Position	$\dot{m}_{CO_2}$ (g/min)	$\dot{m}_{MeOH}$ (g/min)	T (°C)	P (bar)	ρ (g/mL)	Estimated (v/v)%	$F_{v,average}$ (mL/min)
88.7/11.3	Inlet	0.84	0.09	34.4	169	0.87	89.0/11.0	1.1
	Outlet			33.9	156	0.87		
	Average			34.2	163	0.87		
90/10	Inlet	0.85	0.08	34.4	169	0.86	90.3/9.7	1.1
	Outlet			33.9	156	0.86		
	Average			34.2	163	0.86		
91.2/8.8	Inlet	0.86	0.07	34.4	168	0.86	91.6/8.4	1.1
	Outlet			33.9	156	0.86		
	Average			34.2	162	0.86		

saturated; however, the lower capacity/higher energy site is totally resolved. The adsorption energy of the resolved site decreases with increasing amount of MeOH in the eluent (except for 10%$_{set}$ and 12%$_{set}$ MeOH, probably due to experimental noise). Because the AED was at least bimodal and the Scatchard plot (not shown) was convex the adsorption data can be described with models such as the bi-Langmuir or bi-Tóth adsorption model. In Fig. 1(c), the bi-Langmuir model fit to the adsorption data is presented. The initial slope of the adsorption isotherm is clearly decreasing with increasing amount of MeOH in the eluent as expected from the reduction of retention factor with increasing amount of MeOH in the eluent; see Fig. 1(a).

In Fig. 2, the determined bi-Langmuir adsorption isotherm parameters from the model fit in Fig. 1(c) are plotted. We can observe that the monolayer saturation capacity is more or less unaffected by the MeOH concentration, see Fig. 2(a) for the low energy site and Fig. 2(c) for the high energy site where the lines are the average monolayer layer saturation capacity. The equilibrium constant is decreasing with increasing amount of MeOH in the eluent; see Fig. 2(b) for low energy site and Fig. 2(d) for the high energy site. The observed S-values, see Eq. (9), are 13.9 and 17.5 for the low and high energy site, respectively. This clearly shows that the equilibrium constants are responsible for reduction of the retention time with increasing amount of MeOH in the eluent and not the changes in the saturation capacity. In this context it should be mentioned that the accuracy of the low energy site, both for the saturation capacity and the equilibrium constant, is probably low. The reason for this is that the maximum concentration of antipyrine used in this study is not high enough to saturate the low energy site, as clearly indicated from the AED-calculations, see Fig. 1(b). However, it is hard to reach higher antipyrine concentration because this would require larger injections that increases the risk of errors due to the introduction of larger MeOH sample plugs [52,53].

4.3. Standard perturbation peak and frontal analysis experiments

The basic procedure for the PP method used here is identical to the one described in our previous study and the reader is referred to this for more details [6]. Each plateau was established by mixing CO_2 and the modifier stream with a known concentration of dissolved antipyrine for 3 hours before injecting 3 μL of the modifier solution diluted 10 times. Solutions of exactly 13.5, 25.6, 37.4, 49.0, 60.5, 71.7, 82.7 and 93.5 g/L antipyrine were used in the PP experiments and a total of 4 injections were done on each plateau to measure the average retention time of the perturbation peaks. Increased asymmetry of the perturbation peaks could be observed if the concentration of the perturbation pulse was significantly different from the approximate plateau concentration,

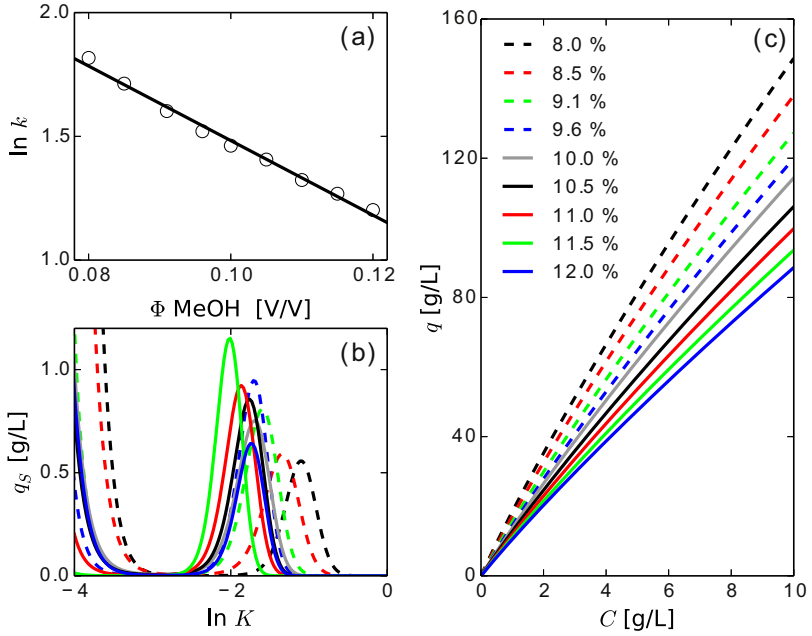

Fig. 1. In (a) the natural logarithm of the retention factor for antipyrine (k) is plotted for 8, 8.5, 9.1, 9.6, 10, 10.5, 11, 11.5 and 12 (v/v)%$_{set}$ MeOH in the eluent (circles) and the line is the best fit of the data to Eq. (8). In (b) the adsorption energy distributions calculated for adsorption data determined using the ECP method for the same MeOH compositions as in figure (a). In (c) the bi-Langmuir model fit to the ECP adsorption data at the same eluent compositions as for figure (a).

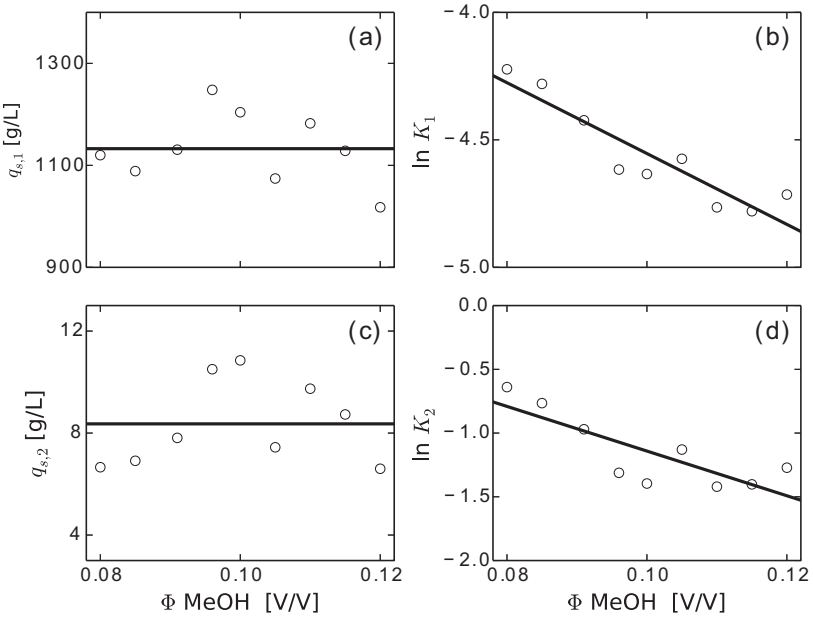

Fig. 2. The determined adsorption isotherms parameters using the ECP method for volume fractions 0.08–0.12 MeOH is presented. In (a) and (c) the saturation capacities of the two Langmuir sites (circles) and their averages (lines). In (b) and (d) the association equilibrium constants of the same Langmuir sites (circles) and the best fit to Eq. (9) (lines).

for example by injecting pure MeOH. The system was set to pump 90/10 (v/v)%$_{set}$ CO_2/MeOH, for which the real volume fraction MeOH was determined to be 9.7%$_{act.}$ and this volume fraction was used to calculate the plateau concentration. For example, using a modifier stream concentration of antipyrine of 13.5 g/L the plateau concentration would be 1.30 g/L (13.5 × 0.097) The average retention time of the obtained perturbation peaks are plotted in Fig. 3(a), labeled "PP uncorrected", together with the elution profile obtained when injecting 40 μL of 250 g/L antipyrine for the same set conditions. It is apparent that the perturbation points do not lie on the diffuse rear of the elution profile and that the distance from the elution profile and the perturbation points increases with increasing plateau concentration. These observations were apparent already in the previous study [6].

FA experiments were conducted by first equilibrating the column with pure eluent followed by changing the modifier to MeOH

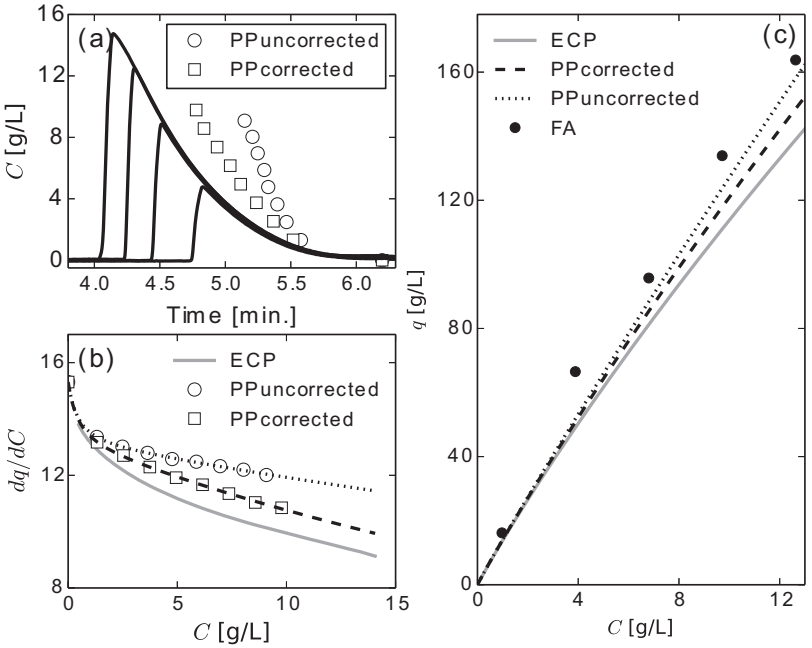

Fig. 3. In (a) experimental elution profiles for 10, 20, 30 and 40 μL injections of 250 g/L antipyrine using an instrumental setting of 10 (v/v)%$_{set}$ MeOH. The symbols are the perturbation peak retention times obtained from 3 μL perturbation injections on established 0, 13.5, 25.6, 37.4, 49.0, 60.5, 71.7, 82.7 and 93.5 g/L antipyrine plateaus. The circles using the "standard" approach with set modifier fraction of 10 (v/v)%$_{set}$ MeOH and the squares using the "compensated" approach with set modifier fractions of 10, 10.1, . . ., 10.8 (v/v)%$_{set}$ MeOH. In (b) ECP adsorption isotherm slopes (solid line), symbols are experimental data from perturbation peaks at an instrumental setting of 10%$_{set}$ MeOH (circles) or using compensated (see above) MeOH fractions (squares) with fitted bi-Langmuir model (dashed and dotted line). In (c) the adsorption isotherms obtained using the ECP (solid grey), PP uncorrected (dotted), PP corrected (dashed) and FA (circles) methods.

containing 10, 40, 70, 100 and 130 g/L antipyrine. The fronts of the breakthrough curve were then used to calculate the raw adsorption isotherm data points, see Eq. (7), that is plotted in Fig. 3(c) together with the corresponding adsorption isotherms estimated using the ECP and PP methods. The ECP data can accurately be used to predict elution profiles and is therefore regarded more or less a "reference". As can be seen the estimated adsorbed amount, q, by the PP and FA methods are higher compared to the ECP method and it can therefore be concluded that the FA and PP methods will, at least for this particular system, overestimate the adsorbed amount of solute component.

In the standard PP and FA methods the partial volumes of mobile phase and solute changes when varying the plateau concentration of the solute. In the current experimental set up the solute is delivered via the modifier stream of MeOH and then mixed in a passive mixer in the SFC system. In Section 3.2 it was described how the partial volume of a solution of antipyrine and MeOH was determined. The MeOH volume fraction was determined for different concentrations of antipyrine from 10 g/L to 150 g/L and the fraction varied linearly from 99%, for 10 g/L antipyrine, to 88%, for 150 g/L antipyrine. This means that while the system delivers a constant volumetric flow of modifier, the fraction of MeOH in this stream decreases with increasing concentration of antipyrine. In Section 4.2 it was shown that the retention of antipyrine is sensitive to the MeOH content in the eluent. The variation of the MeOH volume fraction is therefore a reasonable explanation to why the distance between the perturbation points and the diffuse rear of the elution profile in Fig. 3(a) increases with increasing plateau concentration and only the first PP points overlap with the elution profile's rear. We assume that the FA method will also suffer from this effect and a decrease in MeOH content will result in longer breakthrough times that will lead to the observed overestimation in the adsorbed amount, see Eq. (7).

4.4. Rectifying methanol variations in the perturbation peak experiments

Here an approach for compensating the decrease in modifier content in the eluent in PP experiments is presented. For the elution profile in Fig. 3(a) the system is set to deliver 10 (v/v)%$_{set}$ MeOH but the calculated and measured amount is estimated to be 9.7 (v/v)%$_{act.}$ However, only when there is no component in the eluent the volume fraction of MeOH will be 9.7 (v/v)%$_{act.}$; on a 1 g/L plateau the volume fraction of MeOH is 9.6 (v/v)%$_{act.}$ (9.7×0.99) and on the 15.0 g/L plateau it is only 8.5 (v/v)%$_{act.}$ (9.7×0.88), i.e., the difference increases with increasing plateau concentration. Knowing this, it should be possible to compensate the lowered MeOH fraction by setting the system to deliver a larger fraction of MeOH to the eluent. Obviously, the plateau concentration will then be different for the compensated plateau because the volume fraction of the modifier stream varies.

Due to limitations of the SFC system, the smallest change in MeOH volume fraction is 0.1%$_{set}$, i.e., it can be set at 10, 10.1%$_{set}$, etc. This means that it will not be possible to exactly compensate for arbitrarily chosen plateau concentrations. Therefore, only solutions with a MeOH volume fraction that could be exactly compensated by steps of 0.1 where prepared. Because the volume fraction MeOH varies linearly with the concentration of antipyrine, this can be done by simple interpolation. For example, for an instrumental setting of 10.1 (v/v)%$_{set}$ MeOH, the system is estimated to deliver 9.8 (v/v)%$_{act.}$ and a solution of antipyrine with a MeOH volume fraction of 0.99 would compensate this to 9.7 (v/v)%$_{act.}$ A solution of exactly 13.5 g/L antipyrine has a volume fraction of 0.99 and was thus prepared as the first corrected perturbation point. The plateau concentration for an instrumental setting of 10.1 (v/v)%$_{set}$ MeOH would be 1.32 g/L (13.5×0.098). Setting the system to 10.8 (v/v)%$_{set}$ MeOH would require a solution of exactly 93.5 g/L antipyrine to compensate to a MeOH content of 9.7 (v/v)%$_{act.}$ on the plateau. The actual perturbation peak analysis was performed in the same way as described in Section 4.3, except now the system was set at 10.1, 10.2, . . ., 10.8 (v/v)%$_{set}$ MeOH (9.8–10.5 (v/v)%$_{act.}$ MeOH measured) when equilibrating the plateau using solutions of 13.5, 25.6, 37.4, 49.0, 60.5, 71.7, 82.7 and 93.5 g/L antipyrine, respectively.

When comparing the average retention time of the corrected perturbation peaks with the uncorrected perturbation peaks, it is apparent that the difference increases with increasing compensation and that the compensated perturbation peaks elute increasingly faster with increasing compensation, see Fig. 3(a). This is not surprising since the retention of antipyrine decreases with increasing MeOH fraction in the mobile phase, see Fig. 1(a). Comparing the corrected perturbation peaks retention time with the diffuse rear of the elution profile, the absolute difference only increases slightly with the plateau concentration. Compared to the uncorrected perturbation peaks retention time, the corrected peaks are much closer to the diffuse rear; in theory the points should exactly match the same concentration on the diffuse rear. The results from the corrected perturbation peak experiments strengthen the hypothesis that the displacement of MeOH in the mobile phase due to the solute is a major reason for the observed difference between adsorption data determined using PP and ECP method.

4.5. Overloaded injections and perturbation peaks using the same methanol fraction

Each retention time obtained by standard PP experiments corresponds to the slope of the adsorption isotherm for a unique mobile phase (CO_2/MeOH) composition. This is why the points in Fig. 3(a) cannot be compared with the retention time of the same concentration on the overloaded elution profile. However, it should be possible to compare the PP retention times with elution profiles obtained for the same unique mobile phase fluid composition. For the perturbation peaks obtained on plateaus when pumping modifier solutions of 25.59, 49.0 and 114.5 g/L this would correspond to make overloaded injections with the system set to 9.8, 9.6 and 9.1 (v/v)%$_{set}$ MeOH. These elution profiles are plotted in Fig. 4(a–d) where the elution profile obtained at a set condition of 10 (v/v)%$_{set}$ is plotted as a reference (dashed grey). From these figures it is clear that the retention of antipyrine increases significantly with decreasing MeOH content in the mobile phase.

The perturbation points plotted in Fig. 4(a–d) are the same as the "uncorrected" ones in Fig. 3(a) and, as explained above, each perturbation point can only be compared to an elution profile with the same mobile phase (MeOH/CO_2) composition. The perturbation point with no component in the eluent is marked with a star in Fig. 4(a) and should be compared with the elution profile obtained at a set MeOH content of 10.0 (v/v)%$_{set}$. The perturbation points obtained on plateaus established when using modifier solutions of 25.59, 49.0 and 114.5 g/L antipyrine are marked with stars in Fig. 4(b–d) and should be compared with the overloaded elution profiles obtained with 9.8, 9.6 and 9.1 (v/v)%$_{set}$ MeOH, respectively. Except for the perturbation point with no component in the eluent, it is obvious that the distance between the perturbation point and the corresponding elution profile always is smaller than the distance between the perturbation point and a non-corresponding elution profile. In theory the perturbation points should lie exactly on the corresponding elution profile. However, the results here also strengthen the hypothesis that it is the displacement of MeOH which makes the traditional PP method not applicable in SFC.

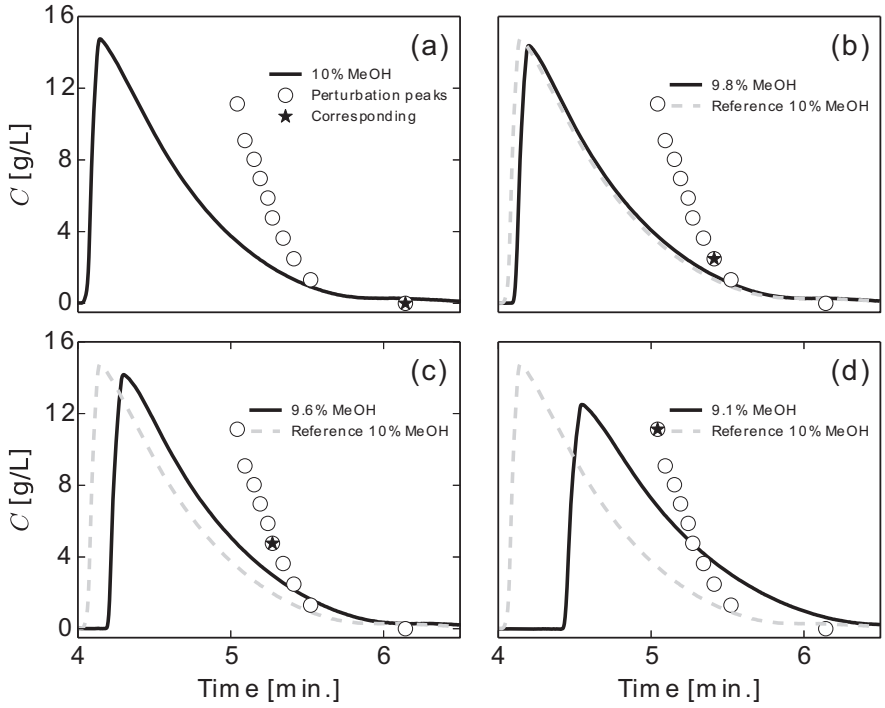

Fig. 4. The retention time of the perturbation peaks at an instrumental setting of 10%$_{set}$ MeOH (circles) with experimental elution profiles for a 40 μL injection of 250 g/L antipyrine at 10%$_{set}$ MeOH (gray lines) and at different MeOH composition (black lines): in (a) 10, in (b) 9.8, in (c) 9.6 and in (d) 9.1 (v/v)%$_{set}$ MeOH in the eluent where the star symbol is the corresponding perturbation peak retention time for the used fraction of MeOH.

4.6. Predicting elution profile using different approaches

The adsorption isotherms obtained using the ECP method, the uncorrected PP method and the corrected PP method were used to simulate a 40 μL injection of 250 g/L antipyrine with an instrumental setting of 10 (v/v)%$_{set}$ MeOH. The simulations were performed using the Equilibrium-Dispersive model, with 3000 theoretical plates, as described in the previous study [6]. In Fig. 5(a) the experimental elution profile (solid black) nearly perfectly coincides with the elution profile simulated using the ECP adsorption isotherm (solid grey). The simulation using the uncorrected PP method adsorption isotherm gives very poor prediction. The

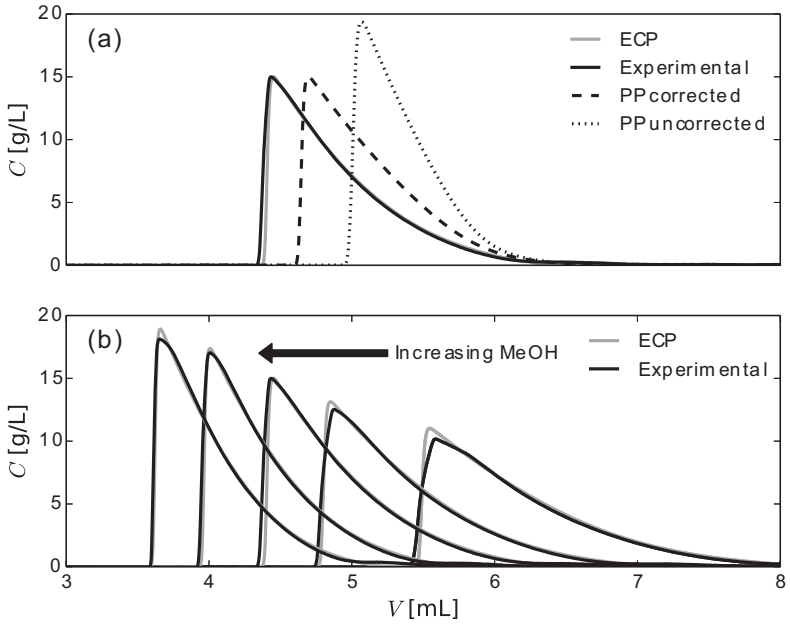

Fig. 5. A 40 μL injection of 250 g/L antipyrine experimental elution profile (black solid line) together with elution profiles calculated using adsorption isotherms determined by different methods. In (a) elution profiles calculated using adsorption isotherms determined by ECP (gray solid line), by corrected PP (dashed black line) and by uncorrected PP (dotted black line). In (b) experimental elution profiles for different MeOH fractions (8, 9.1, 10, 11 and 12%$_{set}$) together with the corresponding calculated elution profiles using adsorption isotherms determined by ECP (solid gray line).

simulation using the adsorption isotherm from the corrected PP method is significantly better, though still gives a poor prediction, i.e., even with correction the PP method is not a good choice for adsorption isotherm determination. In Fig. 5(b) experimental elution profiles obtained when using instrumental settings of 8, 9.1, 10, 11 and 12%$_{set}$ MeOH are presented together with simulations using the corresponding determined ECP adsorption isotherm. It is clear that the adsorption isotherm obtained using the ECP method, in contrast to the PP method, can accurately predict experimental elution profiles.

One reason for the deviation between the adsorption isotherms determined using corrected PP and ECP could be the existence of an injection plug [52]. The adsorption of antipyrine in the injection solvent plug is practically zero. This means that the solute will travel at a different velocity in the plug compared to the mixed phase. To minimize this effect we keep the injections small. Any effect will however be incorporated in the ECP adsorption data and not in the PP data. The effect of the solvent plug could theoretically be mitigated by injecting the solute in a solvent with identical elution strength as the eluent, which is the preferred way in LC. However, it is difficult, or almost impossible, to accomplish this in practice for SFC.

5. Conclusions

Recently LC methods for adsorption isotherm determination were transferred to SFC under close to isopycnic conditions and it could be concluded that methods based on overloaded elution profiles worked well also for SFC. The PP method is not based on elution profiles, instead small perturbations on constant concentration plateaus of the studied component are made and from the retention times of the eluted perturbation peaks the adsorption isotherm can be derived. However, the PP method will in practice result in incorrect adsorption isotherms in SFC and the higher the plateau concentration the larger the error.

Here we investigated from a practical perspective why the PP method gives incorrect results for SFC and we verified that the reason for this was the principal difference in experimental setup between SFC and LC. More specifically, the component to be studied must be delivered in the organic modifier, here MeOH, via the modifier pump and not from the same pump that contains the main part of the mobile phase (CO_2). This means that the component displaces only the MeOH in the organic modifier which causes deviations from the set MeOH/CO_2 ratio in the final mobile phase and this deviation increases with increasing component concentration. As the MeOH content affects the adsorption isotherm, these deviations will mean that *different* adsorption isotherms at each component plateau in the PP experiments are measured, not the same one. This is not a failure of the PP method itself, or its theory, but is instead a major complication when measuring adsorption isotherms in SFC using the PP method, i.e., it is in practice not possible to use the PP method for SFC instruments.

We could verify that variations of MeOH in the eluent was the main reason why the PP method gives incorrect adsorption isotherms for SFC using two different approaches. In the first approach the MeOH fraction was adjusted so it was kept constant on all component concentration plateaus. In the second approach we compared the retention times of the perturbation peaks with ordinary overloaded elution profiles recorded using the same MeOH content as for the perturbation peaks.

The reason why the procedure to maintain a constant MeOH fraction for all concentration plateaus did not give perfect overlap with the diffuse rear or the elution profiles remains to be investigated. A possibility is the effect of an injection plug. The magnitude of this effect would increase with increasing injection volume and this might explain why the deviation increases with higher concentration. A comprehensive study of the plug effect requires extensive work and is beyond the scope of this article, but we are currently performing experiments to investigate this.

The FA method will probably also give incorrect results in SFC due to the same reasons as for the PP method. Concentration plateau methods can therefore not be recommended in SFC and other methods should be used to properly determine adsorption data. Finding suitable methods will be of importance for example when predicting scale-up from an analytical SFC instrument to an industrial preparative one or for understanding molecular interactions in SFC systems.

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