



# Adsorption equilibria of proline in hydrophilic interaction chromatography

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## ABSTRACT

The adsorption behavior of proline under hydrophilic interaction chromatography conditions was investigated from six aqueous solutions of acetonitrile. Proline adsorption isotherms were recorded at each mobile phase composition by frontal analysis and inverse method. The BET model was found to be the best choice to describe the nonlinear behavior of proline adsorption under hydrophilic interaction chromatography conditions. The adsorption isotherm parameters were derived from two independent parameter estimation methods. The parameters derived from regression analysis of the frontal analysis data and from overloaded elution bands were found to be in good agreement with the excess isotherm of water. The mobile phase composition at which the maximum excess adsorption of water was observed corresponded to the maximum saturation capacity measured for proline.

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## 1. Introduction

Hydrophilic interaction chromatography (HILIC) [1] is the mode for the separation of polar compounds generally from aqueous solution of acetonitrile, with high acetonitrile concentration. With the decrease of the acetonitrile concentration, a range of mobile phase compositions with low sample retention can be observed. When the acetonitrile concentration is below a threshold, the retention of the samples may increase with the decrease of the concentration of acetonitrile (per aqueous liquid chromatography, PALC) [2]. The width of the composition boundary between HILIC and PALC modes depends on the adsorbent and the sample properties.

Significant enrichment of water has to be considered using aqueous solutions as mobile phase on polar adsorbents [3]. The retention of solutes may be due to the combination of adsorption and liquid–liquid partition mechanism, which – together with possible additional effects, such as ion-exchange – control the retention [4]. The water-rich layer above the surface governs the mixed retention of polar compounds under HILIC conditions. The higher the organic modifier concentration the stronger is the repulsion of the polar analytes from the aqueous-organic bulk mobile phase and the retention volumes increase, what results the ‘HILIC-half’ of the U-shape plot of the retention factor as a function of the organic modifier content [5].

The most accurate method to describe the physical–chemical properties of the adsorbent surface is the frontal analysis (FA)

[6]. An alternative method with lower chemical requirement for isotherm determination is the modeling of overloaded elution bands using the inverse method (IM) [7]. Nonlinear chromatography data can give a detailed view of the surface processes in comparison with the data derived from analytical injections. The nonlinear behavior of polar analytes can help optimize the preparative scale separations.

The goal of our study was to determine the effect of the preferential water adsorption on silica surface on the nonlinear behavior of proline as test compound under HILIC conditions from aqueous solution of acetonitrile as mobile phase.

## 2. Theory

### 2.1. Measurement of solvent adsorption

In hydrophilic interaction chromatography, an adsorbed layer of water is formed on the surface of the polar stationary phase. The excess amount of water present at the surface can be characterized by measuring its excess isotherm. The determination of the excess isotherms starts with measuring the thermodynamic void volume of the column by the minor disturbance method. The equilibrium between the binary mobile phase and the adsorbed phase is perturbed and the elution volume of the perturbation is recorded at every mobile phase composition from pure water to pure acetonitrile.

For the determination of the thermodynamic void volume of the system, the NA (nothing-is-adsorbed) convention was used [15] to determine the Gibbs dividing plane. The  $v_{NA}$  (the concentration is expressed in volume fraction) and  $n_{NA}$  (the concentration is in

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molar fraction) conventions were used and the results are compared in our study. The thermodynamic void volume determined by the NA conventions should be equal to the void volume in the chromatographic system if determined with an ideal mixture of noncompressible liquids.

When the composition of the mobile phase is expressed by volume fraction (vNA convention), the thermodynamic void volume of the column is derived as:

$$V_{0/vNA} = \int_0^1 V_R(\phi) d\phi \quad (1)$$

where  $\phi$  is the volume fraction of water in the mobile phase, and  $V_R$  is the retention volume of the disturbance peak. In the case of the nNA convention the equation is:

$$V_{0/nNA} = \int_0^1 V_R(x) dx \quad (2)$$

where  $x$  is the molar fraction of water in the mobile phase.

The excess adsorption isotherm of water can be calculated from the data above as [8]:

$$\Gamma_{vNA}(\phi) = \frac{1}{V_{a/vNA}} \int_0^\phi [V_R(\phi') - V_{0/vNA}] d\phi' \quad (3)$$

$$\Gamma_{nNA}(x) = \frac{1}{V_{a/nNA}} \int_0^x [V_R(x') - V_{0/nNA}] dx' \quad (4)$$

where  $V_{a/vNA}$  and  $V_{a/nNA}$  are the volume of the adsorbent in the case of the two respective conventions, and they are given by:

$$V_{a/vNA} = V_G - V_{0/vNA} \quad (5)$$

$$V_{a/nNA} = V_G - V_{0/nNA} \quad (6)$$

where  $V_G$  is the total geometrical volume of the column.

## 2.2. Frontal analysis

Frontal analysis measurements were carried out using the step-wise technique [9]. One channel of the multichannel solvent delivery system was used to deliver the sample solution and the other to pump the pure mobile phase. Adsorption isotherms were measured at six acetonitrile–water compositions. Acetonitrile percentage was varied in the range 70–85% (v/v). At each solvent composition, the maximum concentration of proline was fixed at 4 g/dm<sup>3</sup>. Breakthrough curves were recorded at 215 nm.

The integral mass balance of breakthrough curves between the equilibrium mobile phase concentrations  $C_i$  and  $C_{i+1}$  (in g/dm<sup>3</sup> of the solution) shows that the equilibrium concentration of the analyte on the stationary phase is given by [10]:

$$q_{i+1} = q_i + \frac{(C_{i+1} - C_i)(V_{F,i+1} - V_0 - V_{ex})}{V_a} \quad (7)$$

where  $q_i$  and  $q_{i+1}$  are the adsorbed concentrations of the analyte (in g/dm<sup>3</sup> of the adsorbent), when the stationary phase is in equilibrium with the solute mobile phase concentrations  $C_i$  and  $C_{i+1}$ , respectively, at the  $i$ th and  $(i+1)$ th step.  $V_{F,i+1}$  is the retention volume of the breakthrough curve at the  $(i+1)$ th step and  $V_{ex}$  the extra-column volume. The retention volume of each front was derived using the equal area method [9].

## 2.3. Modeling overloaded elution bands

In addition to FA, adsorption isotherms were also measured by the IM. The equilibrium dispersive model was used to model the experimental data. This model assumes that all contributions due to the non-equilibrium can be lumped into an apparent axial

dispersion term. It represents a reasonable approximation of the real system when the mass transfer in the chromatographic column is controlled only by molecular diffusion across the mobile phase flowing around the packing particles and if the exchange of feed components between the stationary and mobile phase is very fast. The mass-balance equation was solved using the Rouchon backward–forward algorithm [9]. The injection profile (boundary condition of mass-balance equation) was modeled as the convolution between an exponential modified Gaussian function and a rectangular wave [11]. Measured and calculated band profiles were compared by evaluating the following objective function:

$$\min \sum_i (C_i^{\text{calc}} - C_i^{\text{meas}})^2 \quad (8)$$

where  $C_i^{\text{calc}}$  and  $C_i^{\text{meas}}$  are the calculated and measured concentrations at point  $i$ . At the end of each iterative loop, the isotherm parameters are changed to minimize the objective function using a super-modified simplex algorithm [12]. UV detector calibration was carried out using the plateau absorbances recorded in FA for the different equilibrium mobile phase concentrations of proline. A polynomial equation was used to fit absorbance-concentration data.

## 3. Experimental

### 3.1. Instrumentation and chemicals

The measurements were performed using an Agilent (Palo Alto, CA, USA) liquid chromatograph. This instrument includes a 1100 binary-solvent delivery system, a manual injector with a 20- $\mu$ L sample loop, 1100 series variable wavelength UV detector, column thermostat and a data acquisition station.

Acetonitrile, methanol and proline were purchased from Sigma–Aldrich. Water was purified using Milli-Q system (Millipore, El Paso, TX, USA).

The column employed for the measurement of the water excess isotherm, was a 250 mm  $\times$  4.6 mm Waters Atlantis porous silica with 5  $\mu$ m average particle diameter. The adsorption isotherms of proline were determined on a 150 mm  $\times$  4.6 mm Waters Atlantis column packed with the same silica gel. All measurements were carried out with a constant 1.0 cm<sup>3</sup>/min flow rate, at 278 K.

## 4. Results and discussion

### 4.1. Measurement of hold-up and extra-column volumes

In addition to the thermodynamic determination (Eqs. (1) and (2)),  $V_0$  was also determined by the weight difference method (wd) [13,14]. The column was successively filled with water and organic modifier and  $V_{0/wd}$  calculated as:

$$V_{0/wd} = \frac{m_{H_2O} - m_{org}}{\rho_{H_2O} - \rho_{org}} \quad (9)$$

where  $m_{H_2O}$  and  $m_{org}$  are the masses of the column filled with water and organic modifier, respectively, and  $\rho_{H_2O}$ , and  $\rho_{org}$  the corresponding densities. The measurements were carried out with acetonitrile as organic modifier. The resulting values are listed in Table 1. The volume of the adsorbent was calculated as the difference of the geometric column volume and the void volume. The weight difference method gives another opportunity to estimate the thermodynamic void volume of the columns. This method assumes the total volume of the liquid phase in the column as the mobile phase. At this point the void volumes determined by Eqs. (1) and (2) become comparable with the results obtained from the independent weight difference method.

**Table 1**

Main characteristics of the two Waters Atlantis silica columns determined by the minor disturbance and the weight difference method.

Column dimension [mm]	$V_C$ [cm <sup>3</sup> ]	$V_0$ [cm <sup>3</sup> ]			$V_0$ [cm <sup>3</sup> ]		
		vNA	nNA	wd	vNA	nNA	wd
250 × 4.6	4.155	3.554	3.657	3.582	0.601	0.500	0.573
150 × 4.6	2.493			2.100			0.393

In HILIC mode, a fraction of the liquid phase in the column becomes part of the stationary phase (adsorbed water layers), the rest of the liquid phase will remain part of the mobile phase. The amount of the adsorbed water is not static, it varies with the mobile phase composition. The NA convention is a suitable point of reference to investigate the adsorption of water and analyte on the adsorbent surface.

For FA measurements, system extra-column volume includes the volume from the mixer to the detector. It was determined by replacing the column with a zero-volume connector and by injecting low-concentration finite pulses of proline. The extra-column volume of the system was  $V_{ex} = 0.71 \pm 0.01$  cm<sup>3</sup>.

#### 4.2. Excess isotherm of water

The excess isotherm of water on porous silica surface was measured from water–acetonitrile mixtures, with increasing water concentration. 20  $\mu$ L of water was injected three times into the column equilibrated with the mobile phase containing 0, 1, 3, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 97, 99, and 100% (v/v) of water, respectively. The elution volumes of the disturbance peaks were recorded at 190 and 254 nm. The disturbance caused by the injection of water moves along the column in two bands, one is related to the vacancy of acetonitrile, and the other to the excess of water. At 254 nm water does not absorb the UV light, but a disturbance appears at this wavelength caused by the injection of water and that helps to identify the water disturbance peak recorded at 190 nm.

At the first step, the thermodynamic void volumes were calculated according to Eqs. (1) and (2). The thermodynamic void volumes and other column properties are listed in Table 1. The void volume derived using the vNA convention is lower than the one calculated by the nNA convention. The value derived with the weight difference method lies between those two former ones.

After the estimation of the void volumes, the excess isotherm of water was calculated according to Eqs. (3) and (4) in the case of vNA and nNA conventions, respectively.

Fig. 1 compares the excess isotherms of water calculated using the two different conventions. For the sake of a simpler comparison, the excess amounts are plotted against volume fractions regardless whether they were calculated on the basis of volume or molar fractions. There is a significant difference in the excess amplitudes between the two representations. The minor deviation between the void volumes derived from different conventions is one of the reasons for the two different excess isotherms.

Out of the two approaches, the calculation of the excess amount by the nNA convention is more exact [16,17]. The weakness of the vNA convention is that it calculates the excess adsorbed amount from the concentration of the homogeneous bulk phase multiplied by the total volume of eluent in both the bulk phase and the heterogeneous, adsorbed phase.

The calculation of the excess isotherm using molar fractions rather than volumetric concentration does not affect the location of the extrema, but causes a significant decrease in the amplitude of both the positive and the negative parts of the isotherm. In a HILIC system, the apparently large negative excess of the water isotherm was observed and attributed to the preferential adsorption of acetonitrile on the siloxane bridges [18]. Small molecules such as

acetonitrile cannot be completely excluded from the adsorbed phase, but the large preferential adsorption of an apolar molecule is questionable on a naked silica surface. The excess isotherm derived from the molar fraction composition definitely gives a more sound physical representation of the phenomenon.

The maximum excess amount of water can be observed at around 20% (v/v) of water. That point represents the conditions where water adsorption on the silica is the most favorable. The adsorption of water on the silica modifies the retention properties of polar compounds such as proline in HILIC mode. The correlation between the excess amount of water on the surface and the retention properties of proline will be discussed.

#### 4.3. Frontal analysis of proline

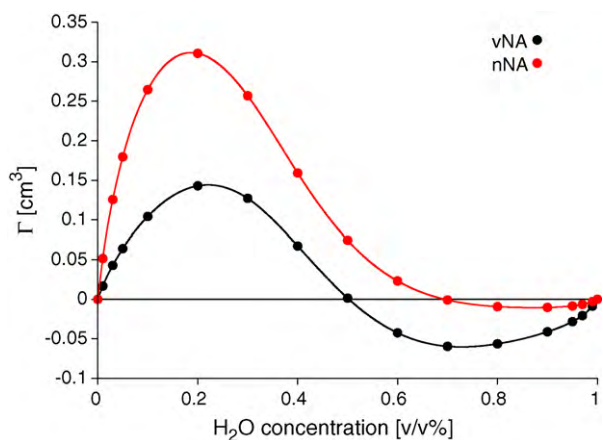
The consecutive breakthrough curves of proline were recorded from six solvent compositions. The acetonitrile concentration was chosen according to the rapid increase of the retention factor of proline. The volume fraction of acetonitrile was 70, 75, 78, 80, 82 and 85% (v/v) in the solvent. The breakthrough curves were recorded at 215 nm, and the discrete isotherm data points were calculated according to Eq. (7).

The shape of the isotherm plot corresponds to the type III van der Waals adsorption isotherm [19] uniformly in every mobile phase composition. To model this behavior, the BET equation was chosen [20]. The equation for liquid–solid adsorption can be written as:

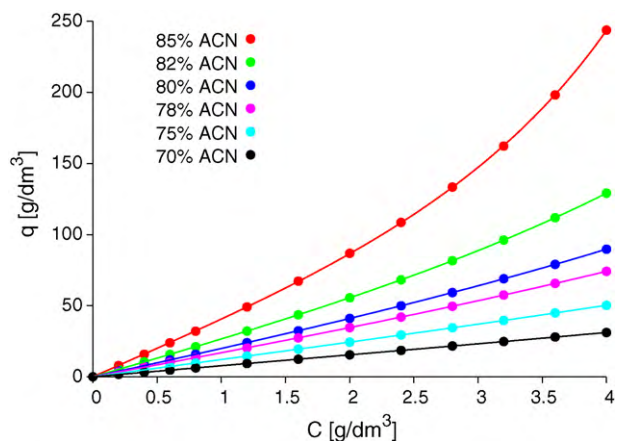
$$q = \frac{q_s b_s C}{(1 - b_L C)(1 - b_L C + b_s C)} \quad (10)$$

where  $q_s$  is the column saturation capacity,  $b_s$  is the equilibrium constant of the interaction between solute molecules and the surface, and  $b_L$  is the equilibrium constant of the interaction between the molecules in the adsorbed layers.

Nonlinear regression analysis was performed and the data were fitted to the BET isotherm equation to model the adsorption behavior of proline. The isotherm points and the best fitted isotherm



**Fig. 1.** Excess isotherm of water on porous silica column, calculated using the vNA and nNA conventions. Note the significant difference between the negative parts of the isotherms.



**Fig. 2.** Adsorption isotherm data points and the best fitted BET isotherm models of proline with the change of the mobile phase composition.

models are summarized in Fig. 2, the best fitted isotherm parameters are summarized in Table 2.

The most conspicuous effect of the increase of the concentration of the acetonitrile is the stronger multilayer behavior of proline. The decrease of the solubility of proline in the mobile phase results the stronger interaction between the adsorbed molecules. Fig. 3 shows the initial linear region of the adsorption isotherms of proline. These regions of the isotherms govern the retention properties of proline under analytical conditions. The Henry constant  $a$  (or initial slope) of the isotherm is in direct relation with the retention factor  $k'$ :

$$k' = Fa = Fq_s b_s \quad (11)$$

where  $F$  is the phase ratio of the column. The enrichment of proline in the lower regions is nearly tenfold at the isotherm with the lowest slope and more than thirtyfold at the highest running isotherm.

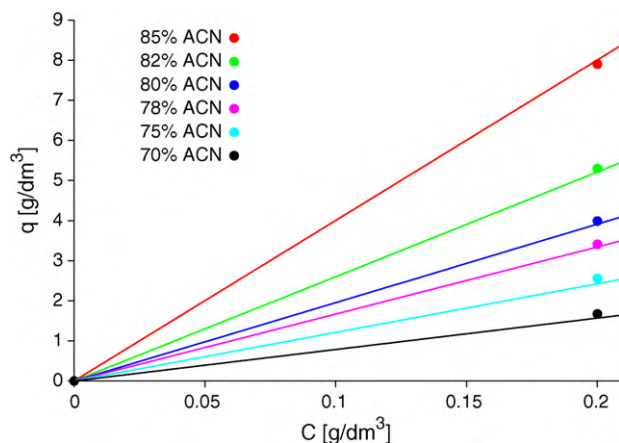
#### 4.4. Modeling of overloaded elution bands of proline

Series of overloaded elution bands are shown in Fig. 4. The decrease of the acetonitrile concentration reduces the fronting

**Table 2**

The best fitted BET parameters of proline derived from regression analysis and inverse method with three different injection concentrations of 4, 2 and 0.8 g/dm<sup>3</sup>. The injection volume was uniformly 1 cm<sup>3</sup>.

ACN% (v/v)	FA	IM <sub>4g/dm<sup>3</sup></sub>	IM <sub>2g/dm<sup>3</sup></sub>	IM <sub>0.8g/dm<sup>3</sup></sub>
$q_s$ [g/dm <sup>3</sup> ]				
85	128.6	127.1	119.4	109.2
82	142.8	137.0	134.9	142.4
80	151.5	155.7	155.9	152.5
78	138.6	139.4	148.6	129.6
75	107.6	103.3	117.1	99.00
70	66.69	65.14	64.14	66.50
$b_s$ [dm <sup>3</sup> /g]				
85	0.3119	0.3217	0.3391	0.3691
82	0.1819	0.1907	0.1927	0.1830
80	0.1288	0.1260	0.1230	0.1274
78	0.1203	0.1191	0.1095	0.1267
75	0.1128	0.1098	0.0963	0.1147
70	0.1180	0.1130	0.1125	0.1087
$b_L$ [dm <sup>3</sup> /g]				
85	0.1501	0.1419	0.1513	0.1627
82	0.0989	0.0964	0.0968	0.0859
80	0.0725	0.0645	0.0657	0.0587
78	0.0655	0.0592	0.0573	0.0568
75	0.0544	0.0518	0.0478	0.0500
70	0.0509	0.0479	0.0515	0.0441



**Fig. 3.** The initial linear region of the adsorption isotherms of proline.

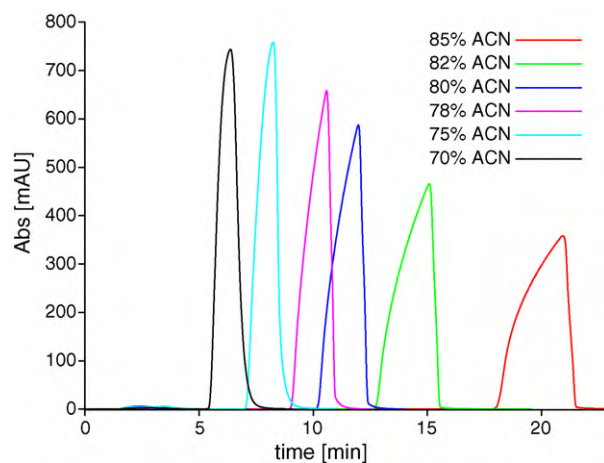
behavior of the elution band, and tends toward Gaussian-like peak. The reduced fronting part of the peaks indicates the lower column overloading, the weaker adsorption of proline.

The overloaded elution bands were generated using the pumps to inject large sample volumes. The injection volumes of the proline samples were uniformly 1 cm<sup>3</sup>. 4.0, 2.0 and 0.8 g/dm<sup>3</sup> proline solutions were injected to cover broader concentration range. The overloaded elution bands were modeled using the inverse method. The initial parameters were derived from the regression analysis of the frontal analysis data. The measured and the calculated band profiles are in good agreement at higher concentrations and at higher retentions. The accuracy of the parameter estimation is the highest at those conditions. The BET parameters derived from the IM are summarized in Table 2.

#### 4.5. Change of the isotherm parameters with the mobile phase composition

Fig. 6 shows the BET parameters, and the retention factors derived from the inverse method and regression analysis as a function of the mobile phase composition. The retention factor data were calculated according to Eq. (11).

The plot of the column saturation capacities  $q_s$  shows maximum at 20% (v/v) of water. This is in an excellent agreement with the excess isotherm data where the maximum excess of adsorbed water was found at the same mobile phase composition. The for-



**Fig. 4.** Series of overloaded elution bands of proline. The concentration of proline was 4 g/dm<sup>3</sup>, the injected volume was 1 cm<sup>3</sup>. UV detection at 215 nm.

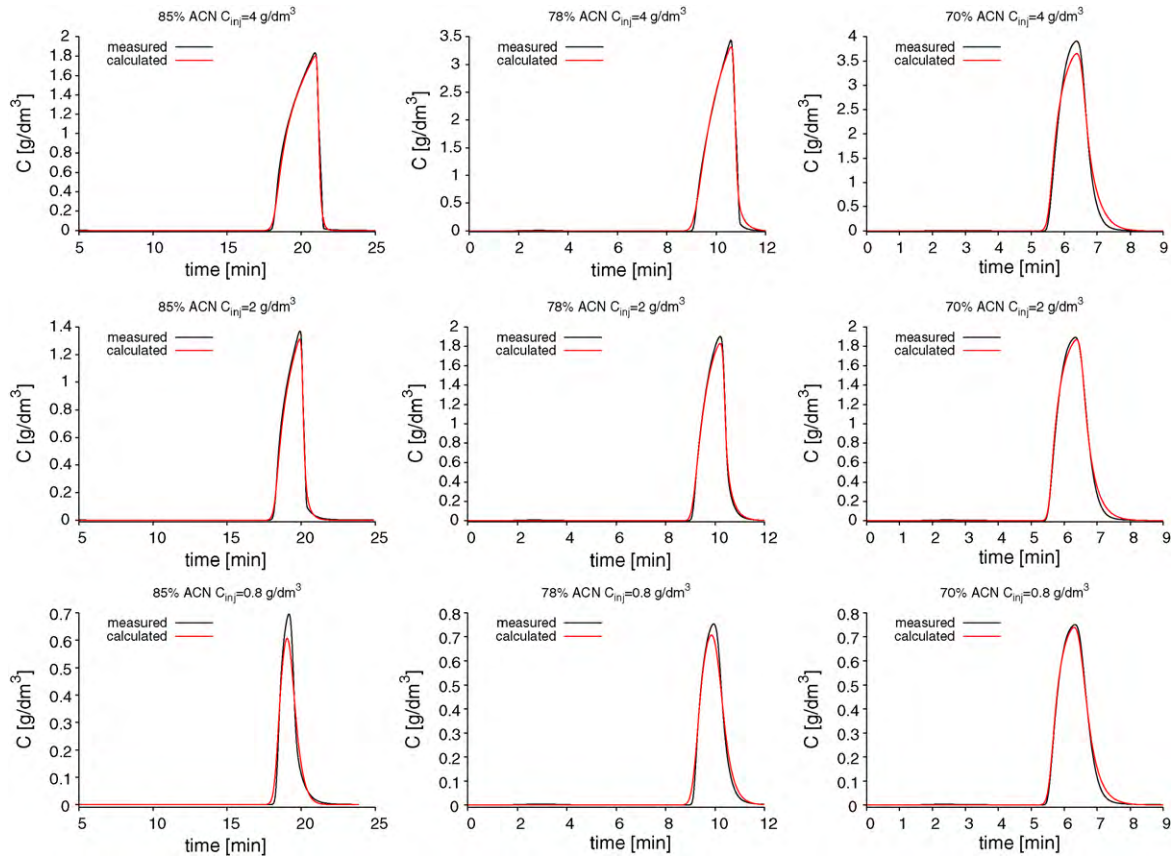


Fig. 5. Comparison between the calculated and measured overloaded elution bands of proline.

mation of the enriched water layer above the surface makes the partition of proline favorable. Moving away from that mobile phase composition triggers the decrease of the surface excess amount of water, and accordingly the saturation capacity of proline decreases.

For preparative scale applications that mobile phase composition is the best choice.

The equilibrium constants  $b_s$  and  $b_L$  behave in a similar manner and show a rapid decrease at higher acetonitrile content

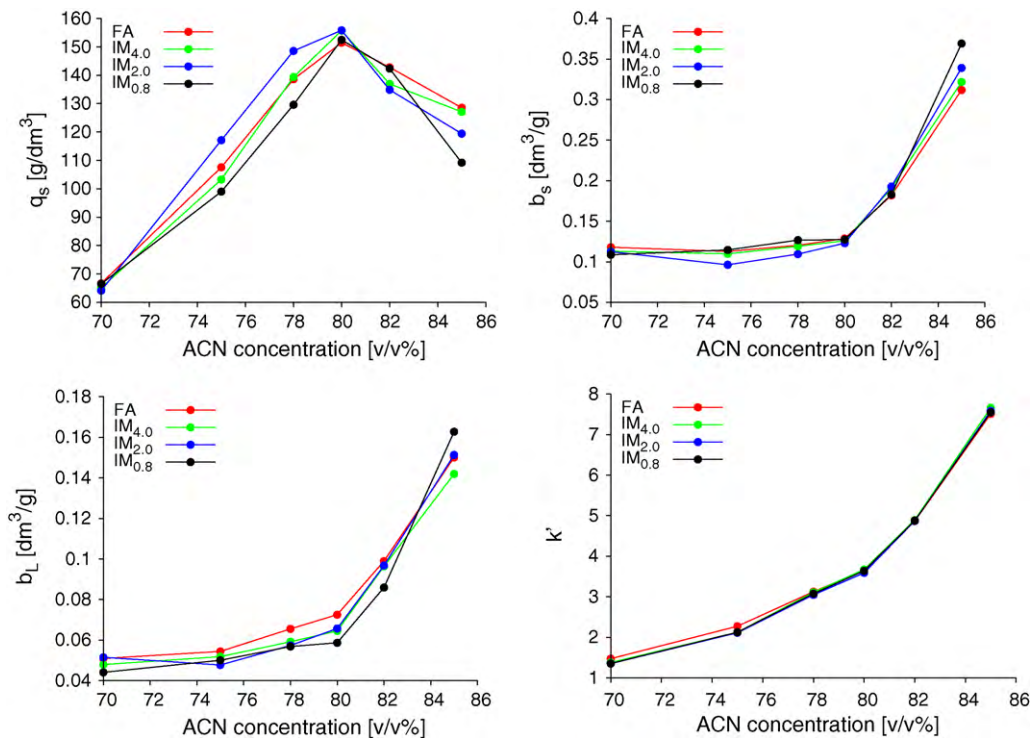


Fig. 6. The column saturation capacity  $q_s$ , the equilibrium constants  $b_s$ ,  $b_L$  and the retention factor  $k'$  of proline as the function as the mobile phase composition.

and moderate decrease at lower acetonitrile concentrations. It seems that the retention of proline under linear conditions is governed by mostly the change of the equilibrium constant of the partition-adsorption ( $b_s$ ). The shape of the plot of the retention factor  $k'$  follows the change of the equilibrium constants. This phenomenon results in the U-shaped plot of retention factor observed in HILIC.

## 5. Conclusions

The nonlinear behavior of proline adsorption from six different aqueous solutions of acetonitrile was measured. The extended liquid–solid BET isotherm accounts best for the multilayer adsorption of proline. The multilayer adsorption becomes stronger with the increase of the acetonitrile concentration. The fronting of the overloaded bands becomes more pronounced from less polar mobile phases.

The column saturation capacity changes parallel with the excess adsorption of water. The partition of proline between the bulk mobile phase and the adsorbed water-rich layer is the most favorable in 20% (v/v) water–80% (v/v) acetonitrile mobile phase composition. The retention of proline under analytical conditions is governed by the equilibrium constant of the partition-adsorption  $b_s$ .

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