# Dependence of Elution Curve and Adsorption Isotherms of Insulin on Composition of Mobile Phase of Frontal Analysis in Reversed Phase Liquid Chromatography

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**With frontal analysis (FA), the dependence of adsorption isotherms of insulin on the composition of mobile phase in reversed phase liquid chromatography (RPLC) has been investigated. This is also a good example to employ the stoichiometric displacement theory (SDT) for investigating solute adsorption in physical chemistry. Six kinds of mobile phase in RPLC were employed to study the effects on the elution curves and adsorption isotherms of insulin. The key points of this paper are: (1) The stability of insulin due to delay time after preparing, the organic solvent concentration, the kind and the concentration of ion-pairing agent in mobile phase were found to affect both elution curve and adsorption isotherm very seriously. (2) To obtain a valid and comparable result, the composition of the mobile phase employed in FA must be as same as possible to that in usual RPLC of either analytical scale or preparative purpose. (3) Langmuir Equation and the SDT were employed to imitate these obtained adsorption isotherms. The expression for solute adsorption from solution of the SDT was found to have a better elucidation to the insulin adsorption from mobile phase in RPLC.**

**Keywords** reverse phase liquid chromatography (RPLC), solid-liquid system, stoichiometric displacement theory (SDT), adsorption mechanism, adsorption isotherm, frontal analysis (FA), insulin

## Introduction

In the reported papers,[1-4] the retention mechanism of solute in reversed phase liquid chromatography (RPLC) was theoretically and experimentally proved to be stoichiometric displacement and follows the stoichiometric displacement model for retention (SDM-R) of solute. Additionally, three of four puzzles[5] for retention mechanism of solute which had not been solved for a long time in RPLC were solved quantitatively.[1-4] The last one of them, "Can generalizations be made that apply to most samples and reversed-phase systems?", will be answered in this study.

The SDM-R was proposed for the retention model of proteins and small solutes in RPLC by Geng and Regnier.[6-8] Geng and Bian[9-11] further proposed a unified SDM-R being valid for many kinds liquid chromatography (LC) except size exclusion chromatography. A stoichiometric displacement model for adsorption (SDM-A) of solute in liquid-solid system was presented by Geng and Shi.[12] Based on the SDM-A, the empirical equations of Freundlich[11,13] and the extended Langmuir[14,15] in liquid-solid system were theoretically derived and experimentally proved. Thus, the stoichiometric displacement model (SDM) has actually become a unified model for both retention mechanism in LC and adsorption mechanism in physical chemistry.

If we only concern with RPLC, the SDM-R was examined experimentally by Kaibara[16], compared with the empirical equation presented by Valko *et al.*[17] and the solubility parameter model decribed by Schoenmakers *et al.*[18] It was concluded that the stoichiometric displacement parameter $Z$ value was the more reasonable approach. The SDM-R was listed as one of four very popular retention models in RPLC in a special volume of *Journal of Chromatography* in 1993.[17] Moreover, the SDM-R is known as the only model to explain the retention mechanism of biopolymers.[19,20] All of those applications have actually answered the last puzzle mentioned above already before.

The SDM-R has now been extended by Geng *et al.* to protein folding with simultaneous purification and the changes in molecular conformation of proteins in molecular biology.[21,22] No matter either from the theoretical standpoint of view, or from the applications in broad regions, it has, so far, not been found that any retention mechanism in LC and adsorption mechanism in physical chemistry can be comparable to the SDM-R and SDM-A. The SDM from a model presented in 1984 actually has now been developed into a systematic theory, stoichiometric displacement theory (SDT) in natural science applying in separation science, chemistry, biology, and pharmacy. Several reviews of the recent developments and applications of the SDT for protein folding by LC[11,23] and in the whole area[11,24] were published.

Because, as described above, a lot of examples in usual

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RPLC have been solved by the SDT, in this study the answer to this last puzzle will be done by means of an example to solve some problems of the solute adsorption from solution in physical chemistry. Frontal analysis (FA) of insulin in RPLC employed in the reported papers[1-4] would be still employed in this study.

RPLC due to its simple, rapid and high resolution, is referred as one of the most useful LC. During the process of purification and separation of proteins with RPLC, protein molecules usually lose their three-dimensional structure. Its application for purification purpose in large-scale is, therefore, limited. However, denaturation of many proteins in RPLC system appears to have a completely or partially reversible character. In other words, with removing denaturing conditions, such as desorbing from a stationary phase, completely removing organic solvent, and ion-pairing agent from mobile phase, some of the denatured proteins can be renatured either completely or partially. Insulin produced from *E coli* and purified with RPLC in large-scale is one of these successful examples.[25]

The adsorption isotherm of a solute can be employed to predict its chromatographic behavior in large-scale LC. Thus, the investigation in this field has became a vital project in nonlinear chromatography theory. Though the expected accuracy depends on many factors, the adsorption isotherm and equation to imitate it are referred to be the most important effecting factors. Poppe published a review paper about the adsorption isotherms measured with RPLC.[26] Adsorption isotherms of a solute may be obtained with either static or chromatographic methods. Many papers deal with adsorption isotherms of solutes by LC.[27-31]

When an adsorption isotherm of a solute is employed for predicting the chromatographic behavior of a solute in large-scale LC, two important things should be considered. First, the chromatographic conditions employed in FA should be as same as possible with that in preparative chromatography.[32] The chromatographic conditions here include the kind of stationary phase, and the type and/or the concentration of both mobile phase and ion-pairing agent. Second, it is necessary to find a quantitative equation having strongly theoretical foundation to fit the obtained adsorption isotherm of the solute well. Based on the equation and the adsorption isotherm and combining together with a suitable kinetic equation, the peak shape and retention of the solute in preparative chromatography should be predicted closer to the experimental one. Unfortunately, many papers involving this subject not only ignore the two important points mentioned above but also deal with merely small solutes.

Isolation and purification of therapeutic proteins produced in biotechnology have been an important but a very difficult project. The changeable rules in terms of retention and peak shape of a therapeutic protein with the increases in sample loading have not been fully understood. They would affect the optimized purificational technology for the aim protein.

Insulin is one of the polypeptides with molecular weight about $5.7 \times 10^3$ Dalton, but it has some characters of usually typical proteins. Insulin is also a special therapeutic protein. Thus, insulin was selected as a typical biopolymer to investigate its elution curve and adsorption isotherm in RPLC system.

The Langmuir equation is widely employed for solute adsorption from solution. The Langmuir equation in its linear form may be shown as:

$$c_m/c_s = 1/a + (b/a)c_m \quad (1)$$

where $c_s$ and $c_m$ represent the concentration of solute on the absorbent and in bulk solution, respectively. From the expanded Langmuir equation,[14,15] the physical meaning of the parameter $a$ represents the product of the overall thermodynamic equilibrium constant of solute displacing solvent and the total number of active sites on the adsorbent surface. The parameter $b$ is the overall thermodynamic equilibrium constant for one solute interacting with the adsorbent at a certain concentration of the solvent. When an adsorption system is given, both $1/a$ and $b/a$ are constants. Thus, Eq. (1) is a linear equation. With a plot of $c_m/c_s$ versus $c_m$, the slope, $b/a$, and intercept, $1/a$, can be obtained.

Considering each interaction among solute, solvent and absorbent, and treating those with thermodynamic equilibria, one expression of the SDT, the stoichiometric displacement for adsorption (SDM-A) of solute, specially derived from liquid-solid system was presented.[11,12,15] The SDM-A was derived from the standpoint of pure physical chemistry and experimentally proved to be better than Langmuir equation for the adsorption from solution[11] and also comparable to that from gas-liquid system.[33] It was also proved by calorimetric method recently.[34,35] The SDM-A in physical chemistry has two expressions.[11,12] First, the quantitative relationship between the logarithm of the equilibrium concentration of solute (mmol/$m^2$) on the absorbent, $\log c_s$ and the logarithm of the equilibrium concentration (mol/L) of solute in a bulk solution, $\log c_m$, as shown in Eq. (2):

$$\log c_s = \beta + nr/Z \log c_m \quad (2)$$

where $n$ represents the moles covering the ligands on the stationary phase by one mole solute. The term $r$ is the numbers of the adsorbed layer of the solvent. The $nr$ and $q$ denote the moles of displacer or solvent released at the surface of an absorbent and the surface of solute molecules, respectively, as one mole of the solute is adsorbed by the absorbent and $Z$ is the sum of the $nr$ and $q$ as shown in Eq. (3):

$$Z = nr + q \quad (3)$$

Second, the logarithm of the partition coefficient of the solute in the two phases, $\log P$ relates to $\log c_m$, as shown in Eq. (4):

$$\log P = \beta - q/Z \log c_m \quad (4)$$

The term $\beta$ in Eqs. (2) and (4) contains a group of constants.

$$\beta = \log K + n \log K' \quad (5)$$

where, $K$ and $K'$ are the thermodynamic equilibrium constants for one mole of solute displacing solvent and its reversed process, *i.e.*, one mole of solvent displacing solute in the same process, respectively. SDM-A is based on five chemical equilibria in a liquid-solid adsorption system[11,12] and can lastly unify the five chemical equilibrium constants to a general one. Thus, how many equilibrium constants are involved in a liquid-solid adsorption system is really not important. A conclusion is obtained that as long as the formation of a multi-layer on an adsorbent is due to chemical equilibrium and the molecular conformation of a protein does not significantly change, the SDM-A should be valid for various kinds of liquid-solid adsorption systems with both mono- and multiple adsorption layers.[11] This point was proved with pure physical chemistry a few years ago.[13-15,33-35]

## Experimental

*Equipment and chemicals*

A Hewlett Packard 1090 liquid chromatograph with a diode-array detector and a Hewlett Packard color Pro plotter was used. SynChrompak HPLC column RP-P, C-18 (100 × 4.6 mm, I. D.; particle size, 5.6 $\mu$m; pore diameter, 30 nm; specific surface area, 53 $m^2/g$; ligand density, 4 $\mu mol/m^2$; packings weight of the column, 1.0 g) was purchased from SynChrom Inc. (West Lafayette, IN, USA). The column temperature was controlled at (25 ± 0.50) ℃ with a water bath.

Insulin (bovine pancreas, HPLC) was bought from Sigma Co. Methanol, 2-propanol and acetonitrile were obtained from EM Science (Gibbstone, NJ, USA). Absolute alcohol was bought from McCormick Distilling Co., Inc. (Perkin, IC, USA). Trifluoroacetic acid (HPLC/spectro grade) was obtained from Pierce (Rockfold, IL, USA). Hydrochloric acid (Ultrex, Ultrapure Reagent) was obtained from T. Baker. Acetic acid (glacial, Fisher Chemical) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pure water is double-deionzed water.

Except the mobile phase was 47.0% methanol in water, ($V_{methanol}/V_{water}$) with 0.030% hydrochloric acid (HCl), ($V_{MP}/V_{HCl}$) and 32.0% ethanol in water ($V_{ethanol}/V_{water}$) with 0.15% TFA ($V_{MP}/V_{TFA}$), four other organic solvents-water ($V_{solvent}/V_{water}$) with 0.1% TFA ($V_{mobile\ phase}/V_{TFA}$) as: (1) 45.0% methanol; (2) 32.0% ethanol; (3) 18.0% 2-propanol; and (4) 27.50% acetonitrile.

Two solutions were used as the strong solution in company with the six kinds of mobile phase to do gradient elution for the column cleaning. (1), 50% acetic acid; and (2), 90% methanol/water + 0.03% ($V_{MP}/V_{HCl}$) HCl. The insulin solution of 1.0 mg/mL was separately dissolved into the six kinds of mobile phases.

*Procedure*

The procedure of FA in RPLC in this study was followed the equipment scheme employed in the previous paper[1] and insulin was dissolved in each of the six kinds of mobile phase.

The elution curves of insulin in the six kinds of mobile phase were obtained according to the same procedure as the reported paper.[1,2] The concentration of organic solvent in the mobile phase employed was selected according to the capacity factor of insulin to be in range of 2 to 10. The other experimental procedures were selected as same as those in the previous papers.[1]

All data were recorded at wavelength 254 nm with reference wavelength 550 nm.

## Result and discussion

*Factors of effecting insulin adsorption*

The effect of organic solvent concentration on the adsorption isotherm of solute was reported by one of the author.[32] A conclusion is that the adsorbed amount of solute from solution depends on the concentration of organic solvent in mobile phase. Therefore, when the isotherm of the solute is needed to predict either the chromatographic behavior of the solute or the real adsorption, the selection of the concentration of the organic solvent in bulk solution should be as same as possible to that in the real application. Despite the effect of the kind and concentration of organic solvent in mobile phase on solute adsorption, the contributions of ion-pairing agents are still important in RPLC.

*Stability of insulin with time delay*

Insulin in various kinds of solutions can be unstable and its self-association in these solutions is very complicated.[36] Thus, the investigation of insulin stability in RPLC conditions becomes firstly important. In the reported paper,[2] it was reported that insulin can interact with methanol and water in the presence of HCl to have different rates. Because the delay time after insulin dissolving was only three and five hours, the measured methanol increments were different, but their two elution curves in the two circumstances were found to the same. This fact indicates that the self-association of insulin does not appear in that circumstance. However, in a reported paper,[4] we found that when the mobile phase consisted of ethanol, water and 0.1% TFA, compared to methanol and 2-propanol, the obtained $\beta$ value of insulin imitated by SDM-A was abnormal. This was attributed to the self-association of insulin, the changes in its molecular conformation and polymerization. The question is that are the elution curves of insulin still the same, if the employed mobile phase is still ethanol, water, and 0.1% TFA and the delay time is signifi-

cantly different?

To prove this point further, Fig. 1 shows the effect of insulin self-association with time delay after dissolving in the mobile phase consisting of 32% ethanol, water and 0.10% TFA. Curves 1 and 2 separately indicate that the insulin adsorption was accomplished from the same concentration, 0.10 mg/mL insulin, but time delayed for 1 and 20 hours after insulin dissolving; respectively, while curves 3 and 4 show that from 0.20 mg/mL insulin solution but time delayed for 6 and 25 hours.

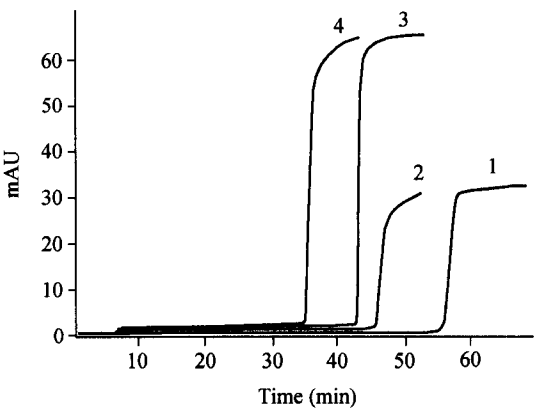


**Fig. 1** Comparisons of the stability of insulin solution. The chromatographic conditions are: SynChrompak HPLC column RP-P (100 × 4 mm), 32% (*V/V*) aqueous ethanol + 0.10% (*V/V*) TFA solution; flow rate, 0.40 mL/min; detection wavelength, 254.4 nm; with reference wave length 550 nm; column temperature, (25 ± 0.50) ℃. Curves 1 and 2 are 0.10 mg/mL insulin solution standing for 1 and 20 h after dissolving, respectively, and pumped at 7.50 min after program, while curves 3 and 4 are 0.20 mg/mL insulin 6 and 25 h after preparing and with the same starting time for pumping insulin solution. Detection was at 254 nm with reference wavelength 550 nm.

From Fig. 1, on the one hand, the break-through time (BTT) of insulin and the corresponding plateau height depend on insulin concentration in the mobile phase employed. The higher the concentration of insulin in mobile phase, the shorter the BTT is. This is a normal circumstance. On the other hand, when insulin concentration is the same, the heights and the BTT of the plateau of insulin were found to be affected by the delay time after insulin dissolving. The longer the delay time after insulin dissolving, the higher the plateau height and the longer the BTT are, and *vice versa*. This phenomenon can be attributed to the changes in the molecular conformation, self-association, denaturation or/and polymerization of insulin in the mobile phase.[36] The decrease in their BTT of insulin indicates the decrease in insulin retention in usual RPLC. The changes in molecular conformation or denaturation of insulin may also cause the decreases in the protein retention in usual RPLC. The changes in plateau height and the BTT would affect on the measured adsorption amount of insulin calculated on the stationary phase of RPLC, as does its adsorption isotherm. However, our experimental data showed that these changes may be ignored with time delay, as long as the delay time is over 24 h after insulin dissolving.

From the reported conclusion,[1,2] it would be expected that with the changes in the plateau height and the BTT of insulin with various time delay, a plateau of ethanol increment should appear before the insulin plateau on the elution curve of insulin. To prove this point, with enlarging that section before insulin plateau with zoom-technique, a plateau was found really to exist (not shown here). Although the plateau height of the ethanol increment were found to depend on insulin concentration, their BTT are almost the same.

*Selection of type and concentration of ion-pairing agents*

It is necessary for protein separation with RPLC to put an ion-pairing agent into the mobile phase employed. TFA is one of the most popular used in RPLC. Three functions of TFA are important for biopolymer separation in RPLC.[37] First, interactions with proteins to make TFA concentration decreases in the sample solution of biopolymers. Second, TFA can be adsorbed by the stationary phase of RPLC and accumulated on it to modify its character. Third, TFA can join in the stoichiometric displacement process between biopolymers and organic solvent as if it was a solute. The existence of TFA was also reported to affect protein retention in FA of RPLC.[2] A reasonable postulation is that TFA should have an effect on the adsorption isotherm of proteins. Fig. 2 shows the comparison of this effect of TFA concentration between 0.10% (curve 1) and 0.15% (curve 2), as ethanol concentration in the mobile phase is 32%. From Fig. 2, the insulin adsorption is stronger and more with TFA in 0.15% than in 0.10%. This result coincides with the reported that the retention of protein increases with the TFA concentration.[37]

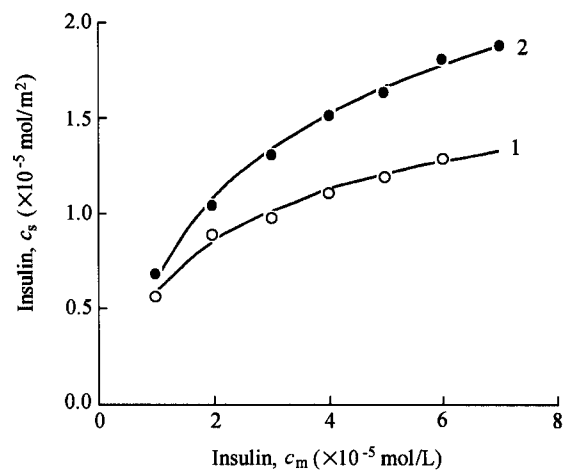


**Fig. 2** Effects of TFA concentration on the adsorption isotherms of insulin. Insulin solution with standing for 24 h after preparing in 32% ethanol with different concentration of TFA was pumped at 7.50 min after program starting. The chromatographic conditions are: flow rate, 0.40 mL/min; column temperature (25 ± 0.50) ℃. Curves 1 and 2 are the adsorption isotherms of insulin obtained from 32.0% ethanol with 0.10% and 0.15% TFA, respectively.

Different kinds of ion-pairing agent should also have an effect on the adsorption isotherm of insulin. Hydrochloric acid (HCl) has been often used to separate peptides[38,39] and, sometimes, to separate proteins in RPLC,[1] though it is harmful for stainless steel. As pointed out above, a comparable retention of protein should be the criterion for selecting the type and concentrations of ion-pairing agent and organic solvent. Thus, it is impossible to simultaneously satisfy the two conditions. In other words, if the same kind of organic solvent accompanying the different ion-pairing agent is selected to obtain a comparable retention, the concentration in the two circumstances must be different. The functions of TFA and HCl for protein separation may be quite significantly different. Based on experimental results from usual RPLC, insulin retention from 45% methanol with 0.10% TFA solution can be comparable to that from 47% methanol with 0.03% HCl solution. Even though we kept the retention of insulin in the two circumstances as same as possible, as shown in Fig. 3, insulin adsorption was greater from the latter than the former. This fact can be explained by the conclusion in the previous paper,[2] that compared to the polarity of TFA with HCl, the former has strong non-polarity and stay in the region IV close to mobile phase reported by Miller *et al.*[40] and Sentell,[41] while the later has strong polarity and stay in the region II nearby the silica surface.

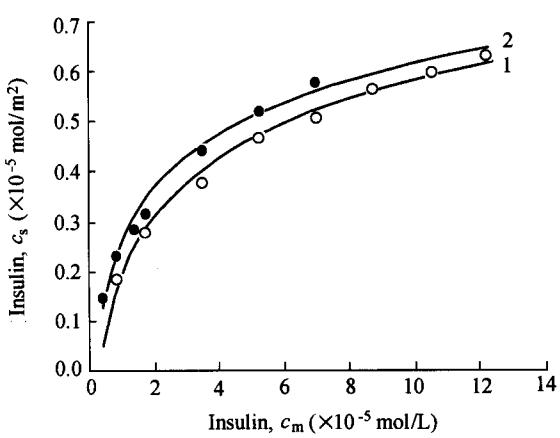


**Fig. 3** Effects of the kinds of ion-pairing agent on the adsorption isotherms of insulin. The chromatographic conditions are: 47% (*V/V*) aqueous methanol + 0.03% hydrochloric acid solution (curve 2) and 45% (*V/V*) aqueous methanol + 0.10% TFA solution (curve 1). The other conditions are the same as that shown in Fig. 2.

It is dynamically easier to displace TFA than HCl, because TFA is closer to mobile phase than HCl. However, it is thermodynamically easier to displace HCl than TFA, because the non-polarity of TFA adsorbed by the stationary phase of RPLC is much stronger than HCl. For usual RPLC, because solute has not enough time to penetrate into the interior of the bonded phase layer (BPL), dynamics is important, while for FA in RPLC, solute has enough time to adsorb and desorb, so thermodynamics is important. A conclusion is thus obtained that the presence of TFA can make insulin be adsorbed less than that in the presence of HCl.

By the way, the foregoing experimental result also elucidates that if we want to obtain a reproducible result of adsorption isotherm, both of the kinds and concentrations of TFA and/or organic solvent in mobile phase employed must keep invariable during a whole set of FA. This is one of the reasons why degassing is never used during the insulin adsorption process in FA of RPLC.[1-4]

*Adsorption isotherms of insulin from various kinds of mobile phase*

To investigate the effect of kind of mobile phase on adsorption isotherms o insulin, six kinds of mobile phase used in RPLC were selected. Elution curves, adsorption isotherms, equations to elucidate the adsorption isotherms, and parameters in these equations were compared and evaluated in details.

*Imitation of adsorption isotherms of insulin in six kinds of mobile phase by two equations*

The usual method employed in FA to measure the adsorbed amount of insulin was used in this paper. In other words, each adsorbed amount of insulin could be obtained by the product of each BTT of insulin from the six kinds of mobile phase times their corresponding insulin concentration in the mobile phase employed.

The adsorption isotherms of insulin were determined from the six kinds of mobile phase consisting of: (1), 45% aqueous methanol with 0.10% TFA; (2), 18% 2-propanol with 0.10% TFA; (3), 32% ethanol with 0.10% TFA; (4), 22.5% acetonitrile solutions plus 0.10% TFA; (5), 32% aqueous ethanol plus 0.15% TFA and (6), 47% methanol plus 0.030% hydrochloric acid. Except aqueous methanol with 0.030% HCl, the others have a broader range of insulin concentration.

The obtained adsorption isotherms were imitated by the Langmuir Eq. (1) and the SDM-A Eq. (2). On the one hand, from their regression coefficients $R^2$ listed in Table 1, all of the $R^2$ obtained from the SDM-A are greater than 0.99, but one of them for Langmuir equation are less than 0.99. Thus, based on the goodness of $R^2$ value, we may conclude that the SDM-A is better than the Langmuir equation. On the other hand, because the SDM-A uses log-log plot, only evaluation by using $R^2$ may be insufficient. The parameters obtained from the two equations should be used further to explain the characteristics of the insulin adsorption from different mobile phases.

*Parameters of two equations*

Both Langmuir and SDM-A equations have two parameters, $1/a$ and $b/a$ as well as $nr/Z$ and $\beta$, respectively. All of these parameters obtained from the six kinds of mobile phase were also listed in Table 1. The physical meaning of

**Table 1** Comparisons among Langmuir, and SDM-A

| Mobile phase | Equation | | | | | |
|---|---|---|---|---|---|---|
| | Langmuir | | | SDM-A | | |
| | $R^2$ | $b/a$ | $1/a$ | $R^2$ | $nr/Z$ | $\beta$ |
| Methanol (45% + 0.1% TFA) | 0.9939 | 128220 | 4.22 | 0.9958 | 0.472 | - 3.33 |
| Methanol (47.0% + 0.03% HCl) | 0.9929 | 138764 | 2.70 | 0.9988 | 0.482 | - 3.23 |
| Ethanol + 0.1 TFA | 0.9920 | 60422 | 1.99 | 0.9981 | 0.448 | - 3.10 |
| Ethanol + 0.15 TFA | 0.9972 | 36850 | 2.02 | 0.9924 | 0.523 | - 2.66 |
| 2-Propanol + 0.1% TFA | 0.9890 | 79350 | 3.12 | 0.9991 | 0.419 | - 2.82 |
| Acetoniyrile + 0.1% TFA | 0.9903 | 45546 | 2.45 | 0.9969 | 0.516 | -2.82 |

the two constants, $1/a$ and $b/a$ in Eq. (1) in liquid-solid system has also been taken from gas-solid system. As pointed above, they have different meanings from the extended Langmuir equation described above. The SDM-A was specially derived from liquid-solid system and also has two constants $\beta$ and $nr/Z$ or $q/Z$, shown in Eqs. (2) and (4), respectively. Thus, $\beta$, $nr/Z$ and $q/Z$ have exact physical meanings and we firstly should compare to the reasonability for the magnitudes and their physical meanings of the obtained two constants between Langmuir and the SDM-A.

Because both $nr$ and $q$ are the fractions of $Z$, as shown in Table 1, all $nr/Z$ values are less than unity. It coincides to that expected in theoretical part. Three solvents, methanol, ethanol and 2-propanol employed may be referred as a pseudo-homologue in RPLC.[4] When 0.10% TFA is used as an ion-pairing agent and insulin solution is stable enough, the adsorbed characteristics of insulin from the three mobile phases should relate to the carbon number of the pseudo-homologue of alcohols, $N_c$. This point was proved in the reported paper already.[4] However, the parameters, neither $1/a$ and $b/a$ shown in Table 1, nor $a$ and $b$ themselves in Langmuir equation have these linear relations.

From Table 1, the result from the SDM-A really obeys to the homologue rule, while Langmuir equation does not, indicating the SDM-A to be better than the Langmuir equation in this study.

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