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Simple method for determining the amount of ion-exchange groups on chromatographic supports

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Abstract

The objective of this study was to develop a fast, simple, non-destructive, non-toxic and low-priced method for determining the amount of ionic groups on resins, since the conventional titration method fails to give proper results on methacrylate monoliths. After the column had been pre-saturated with a high concentration buffer solution, a low concentration buffer solution of the same pH value was pumped through the column. Measuring pH and absorbance, the profiles with a shape of typical break-through curve were obtained. It was shown that the time of the pH transient, which appeared under such conditions, could be used as a measure of the total ionic capacity of ion-exchange monolithic columns. The effect of the column length, linear velocity and varying concentrations of buffer solutions on the time of the pH transient was examined. The method was shown to be suitable for determining the amount of ionic groups on both anion and cation monolithic columns. In addition, it could also be applied to particle bed columns. The time of the pH transient and the protein dynamic binding capacity were also compared and it was concluded that for a given monolith the protein capacity can be derived from the data obtained by the new method.

Keywords: Ion exchangers; Total ionic capacity; pH transient; Monolithic columns; Convective Interaction Media (CIM); Resin characterization

1. Introduction

Ion-exchange chromatography is one of the most frequently used techniques for the purification and separation of samples consisting of charged biomolecules [1,2]. The technique is based upon the reversible binding of a charged species to oppositely charged groups attached to an insoluble matrix. The weak van der Waals forces and non-polar interactions make a minor contribution in an ion-exchange separation [1]. The retention and separation of the charged molecules on the ion-exchanger depend on sample molecules, mobile phase composition and type of ion exchanger [1,2] as well as on the properties of the matrix. Separation efficiency is related to the matrix structure, which to a large extent determines surface accessibility and consequently binding capacity, as well as to the amount of the surface ligand, which influences capacity, selectivity and recovery [3]. Therefore, besides the structure of the matrix precise control of the ligand density is crucial to preparing chromatographic resins with reproducible properties.

A quantitative measure for characterizing an ion-exchange support's ability to take up exchangeable counter-ions is capacity. There are several methods for determining the ionic capacity. The total ionic capacity is defined by the number of charged substituent groups per gram of dry ion exchanger or per milliliter of swollen gel [1] and it is a constant based upon the material's character and is independent of the experimental conditions [4]. It is usually measured by potentiometric titration with a strong acid or base solution [1]. However, the time to achieve equilibrium (the stationary state the potentiometric titration is based upon) is very long [4,5] and the procedure involves a large number of preparation steps [4] making it relatively complicated.

Due to the above-mentioned limitations, a few other methods have been developed to characterize chromatographic supports. The ionic capacity can be calculated from elemental analysis data [6]. To determine ionic capacity also in-

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column frontal analysis has been used. A method commonly used for determining the amount of anion-exchange groups is performed by pumping a mobile phase containing $NaNO_3$ through the resin and observing when saturation occurs by measuring absorbance at 254 nm [6]. For certain resins, those mainly intended to separate large molecules, resin capacity can also be determined, e.g. by bovine serum albumin (BSA) dynamic capacity which more closely approximates the capacity under operating conditions [1]. However, this value does not necessarily reflect the density of the ionic groups as it is influenced by surface accessibility, too.

While in principal any of the suggested methods can be applied to any conventional resins, applying them to monolithic stationary phases seems to be more problematic. In the case of particulate resins, a small representative sample can be taken from the entire batch and analyzed according to the selected method. Monoliths, on the other hand, consist of a single block of material and each block is considered a single batch. This is why characterization data are only valid for the tested monolith and the results cannot be extrapolated to other monoliths, so elemental analysis is not a proper method to determine the capacity of the monolithic columns. The in-column frontal analysis using $NaNO_3$ solution is very fast and elegant and gives similar results to elemental analysis and potentiometric titration but it is limited to anion-exchange resins [6,7]. On the other hand, the application of the methods using proteins is problematic since they contaminate the matrix making them unusable for the separation or purification of target compounds. This is the case especially for medical or pharmaceutical applications where chromatographic media must be contaminant free, so such matrix would be unacceptable or would need an intensive validated cleaning. Because of this, a different method for determining the amount of ion-exchange groups would be beneficial.

A method that is quite impractical for particulate resins, but might be well suited for monoliths, could be to determine the amount of introduced groups during the activation process by measuring the mass change of a dry monolith before and after the reaction. Knowing a reaction stoichiometry, the amount of the introduced groups can be accurately determined. While this can be easily applied on small monoliths [3], larger ones are much more sensitive towards drying, which might cause cracks in the structure thus practically destroying the monolith.

For the separation of macromolecules, mobile phase gradients (ionic strength or pH) are routinely used for elution from an ion exchanger [1]. If a non-adsorbed buffering species is used for that purpose, changes in ionic strength or pH propagate throughout the column with the velocity of a non-adsorbed solute. On the other hand, ionic strength and pH gradients can be retained by a column if an adsorbed buffering species is present or if an adsorbent with a buffering capacity is used [8]. The time at which the pH front exits the column is proportional to the slope of the adsorption isotherm of the buffering species [9,10]. Since the slope of the adsorption isotherm implicitly involves the total amount of the ionic groups present on the matrix, we speculated that a method could be developed to determine the ionic group density.

Recent experiments showed that such a pH front can be achieved by a stepwise change of a high concentration buffer solution to a low one having the same pH value [11].

During the course of our study, similar observations were reported by Pérez and Frey [12] and a local-equilibrium theory was used to explain this phenomenon and to predict pH profiles.

The objective of this study was to develop a fast, simple, non-destructive, non-toxic and low-priced method for determining the capacity of ion exchangers. A classic titration method was tested and a new method based upon the phenomenon of an internally produced pH transient is introduced.

2. Theoretical background

From a steady state material balance relation for buffering species inside the column, using the boundary itself as the frame of reference, the velocity of a stepwise change in composition can be developed [4]. Assuming sufficiently fast mass transfer, resulting in equilibrium conditions at each point throughout the column, and negligible effects of axial dispersion, the time at which the pH front exits the column is given by equation [8,9,13]:

$$t(\mathrm{pH}) = \frac{L}{v_{\mathrm{fluid}}}\left[1 + \frac{(1-\alpha)\varepsilon}{\alpha} + \frac{(1-\alpha)(1-\varepsilon)}{\alpha}\frac{\mathrm{d}q_{\mathrm{A}}}{\mathrm{d}C_{\mathrm{A}}}\right] \quad (1)$$

where L represents the column length, v_{fluid} is the linear velocity of unadsorbed solute, α and ε are column and particle porosities, respectively, and $\mathrm{d}q_{\mathrm{A}}/\mathrm{d}C_{\mathrm{A}}$ is the slope of the adsorption isotherm for the buffering species A that depends on the adsorbing compound and the matrix. The methacrylate monoliths are continuous homogeneous phases, i.e. made from one piece, with particle porosity (ε) 0, so the second term in Eq. (1) becomes 0 and the equation simplifies into:

$$t(\mathrm{pH}) = \frac{L}{v_{\mathrm{fluid}}}\left[1 + \frac{(1-\alpha)}{\alpha}\frac{\mathrm{d}q_{\mathrm{A}}}{\mathrm{d}C_{\mathrm{A}}}\right] \quad (2)$$

As can be seen from Eq. (2), all terms, L, v_{fluid} and α, are independent of the interaction between the solute and the support, except $\mathrm{d}q_{\mathrm{A}}/\mathrm{d}C_{\mathrm{A}}$, which represents the slope of adsorption isotherm. If the gradient is strongly retained by the column packing, the term inside the square brackets is large compared to unity and t(pH) becomes directly proportional to $\mathrm{d}q_{\mathrm{A}}/\mathrm{d}C_{\mathrm{A}}$ [10]:

$$t(\mathrm{pH}) = \frac{L}{v_{\mathrm{fluid}}}\left[\frac{(1-\alpha)}{\alpha}\frac{\mathrm{d}q_{\mathrm{A}}}{\mathrm{d}C_{\mathrm{A}}}\right] \quad (3)$$

Adsorption isotherm describes the equilibrium of the adsorption process at a defined temperature and can also be expressed by the fractional coverage θ (i.e. the fraction of the number of occupied sites) as a function of equilibrium con-

centration (activity) of solute in the solution $f(C_A)$:

$$\theta_A = \frac{q_A}{q_t} = f(C_A) \quad (4)$$

where q_A is adsorbed phase concentration of buffer species A and q_t represents the total ionized functional group concentration in the stationary phase. Differentiation of Eq. (4) gives:

$$\frac{dq_A}{dC_A} = q_t f'(C_A) \quad (5)$$

and the combination of the Eqs. (3) and (5) results in:

$$t(\text{pH}) = \frac{L}{v_{\text{fluid}}}\left[\frac{(1-\alpha)}{\alpha} q_t f'(C_A)\right] \quad (6)$$

For a particular solute/ion exchanger system at a defined temperature the fractional coverage θ_A and the slope of the isotherm at a chosen point is defined by the solute concentration C_A (sometimes the ratio of equilibrium concentration of solute in the solution near the surface and the concentration of solute in the bulk solution C_A^*/C_A is used). If all variables (the type of interaction solute/ion exchanger, temperature, solute concentration) are fixed, than the ratio q_A/q_t is constant and the concentration of adsorbed species A, q_A, is proportional to the total concentration of ion-exchange sites, q_t:

$$t(\text{pH}) = \frac{L}{v_{\text{fluid}}} k q_t \quad (7)$$

where k is the constant, defined by column porosity (void volume) and solute/ion exchanger system (for various solute/ion exchanger systems the adsorption isotherms and therefore $f'(C_A)$ are different).

According to Eq. (7) total ionic capacity of ion-exchange resins could be estimated by measuring the time at which the retained pH front exits the column.

3. Experimental

3.1. Instrumentation

For potentiometric measurements MA 5736 pH meter (Metrel, Horjul, Slovenia) with InLab 406 combination pH electrode (Mettler Toledo, Urdorf, Switzerland) and one Knauer Type 64 analytical pump (Knauer, Berlin, Germany) were used. Capacity measurements were obtained by two HPLC systems; the first was built with two Knauer Type 64 analytical pumps and the second with two Knauer K-1800 preparative pumps. The detectors used were Knauer UV–Vis absorbance detectors model K-2500 with a 10 mm optical path (analytical system) and 2 mm optical path (preparative system), operated at 210 nm for ionic capacity measurements and 280 nm for protein capacity measurements. The pH of the eluent was measured using a low-volume on-line sampling cell and a Model 450 CD combination pH electrode (Sensorex, Stanton, CA, USA) attached to a Model CG 843 pH meter (Schott, Stafford, UK). Both the pH meter and the UV detector were connected via a Knauer interface box and Eurochrom 2000 software to a personal computer for real time data acquisition. To determine the mass of dry monolithic columns, the monolithic columns were dried in an SP 8 dryer (Kambič, Semič, Slovenija) and weighed by Chyo JL-180301503 analytical balance (Chyo Balance Corporation, Japan).

3.2. Materials

Measurements were carried out on Convective Interaction Media (CIM) diethylaminoethyl (DEAE), CIM quaternary amine (QA), CIM sulfonyl (SO3) and CIM carboxymethyl (CM) ion-exchange monoliths of different volumes (0.34, 8, and 80 ml) packed into a CIM polyether ether ketone (PEEK) disk housing, CIM 8 and 80 ml tube housings all from BIA Separations (Ljubljana, Slovenia). Additionally, measurements were made on two particle based ion exchangers: HiTrap Q HP (quaternary amine, bed volume 1 ml) from Amersham Biosciences (Uppsala, Sweden) and Econo-Pac High Q Cartridge (quaternary amine, bed volume 1 ml) from Bio-Rad Labs. (Hercules, CA, USA).

All solutions were prepared using water purified by a Watek IWA-80 roi (Ledeč nad Sázavon, Czech Republic) water purification system and analytical grade reagents. Sodium dihydrogenphosphate dihydrate, disodium hydrogenphosphate monohydrate, sodium hydrogencarbonate, ammonium sulfate, sodium hydroxide, potassium hydroxide, sodium chloride, potassium chloride, hydrochloric acid (37%) and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany). Bovine serum albumin was purchased from Sigma (St. Louis, MO, USA). Nitrogen was obtained by Messer (Ruše, Slovenia).

Buffer solutions were made by adding a known mass of each buffering species to a known volume of deionized water. Phosphate and hydrogencarbonate buffer solutions were than titrated with sodium hydroxide (so no other contaminant ions were introduced into the solution) and Tris–HCl buffer solution was titrated by HCl to the appropriate pH. All solutions were passed through a 0.45 μm pore size filter composed of sartolon polyamide (Sartorius, Göttingen, Germany) and degassed in an ultrasound chamber.

3.3. Methods

3.3.1. Potentiometric measurements

The first potentiometric measurements were performed using cation exchangers in H^+ form, which is obtained during activation of the monolithic support using a strong acid solution. A known amount of base solution, which was purged by nitrogen before use, was continuously circulated through the CIM cation-exchange disk monolithic column for various periods of time. During circulation, H^+ ions were exchanged by cations and the pH of the circulated solution decreased. The solution was then titrated by an acid solu-

tion. The difference between the amounts of used acid solution for the blank experiment and the experiment with the ion-exchange column represented the amount of exchanged cations. For CIM anion-exchange disk (in OH^- form) monolithic columns the circulation was performed by an acid solution which was titrated by a base solution afterwards and the amount of exchanged anions was calculated.

In the second part of the potentiometric measurements, a defined amount of salt solution, purged by nitrogen, of known pH was pumped through the ion exchanger in H^+ (cation exchanger) or OH^- (anion exchanger) form. After pumping, the solution was titrated by a base (for cation exchanger) or acid (for anion exchanger) solution. The amount of exchanged ions was calculated from the difference in used base (acid) for the blank experiment and the experiment with an ion exchanger.

3.3.2. Mass conversion measurements

For the determination of the total ionic capacity of CIM ion-exchange monolithic columns, mass conversion measurements were performed. The difference in mass of dry monolithic support before and after the chemical modification was calculated and normalized on the initial monolith mass. Dividing the normalized mass difference with the known stoichiometry of the reaction the amount of introduced groups per gram of monolith was obtained. This value was considered as a total ionic capacity.

3.3.3. pH transient time measurements

The pH transient time measurements were performed by using two buffer solutions that varied in concentration, but had the same pH. The column was first equilibrated with a high concentration solution until the pre-saturation absorbance at 210 nm and pH were reached. The mobile phase flowing through the column was then instantly switched to the low concentration solution of the same solute having the same pH in order to form the inadvertent pH transient [12]. The experiment ended when the absorbance and the pH of the effluent reached the absorbance and the pH of the solution at the column inlet. The time between switching the mobile phases and reaching 50% absorbance of the "breakthrough" was determined as described in Fig. 1.

3.3.4. Protein dynamic capacity measurements

The protein dynamic capacity of the CIM DEAE monolithic columns was determined using frontal analysis experiments. The column was first equilibrated with a standard buffer solution (20 mM Tris–HCl, pH 7.4) and then switched to a protein solution (1 mg/ml BSA disks or 3 mg/ml tubes in 20 mM Tris–HCl buffer solution, pH 7.4) at a flow rate of 3 ml/min (disks), 16 ml/min (8 ml tubes) or 160 ml/min (80 ml tubes). The absorbance at 280 nm was measured and the protein capacity at 50% breakthrough was calculated.

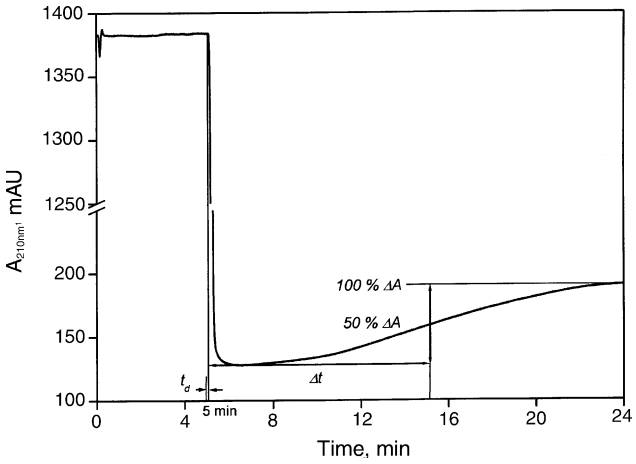

Fig. 1. Determination of the time of the pH transient (Δt or t(pH) in the text) for a CIM QA disk monolithic column. Method: 0.5 M $NaHCO_3$, pH 8.8 (5 min), 20 mM $NaHCO_3$, pH 8.8 (60 min). Flow rate: 4 ml/min. Detection: absorbance at 210 nm. Time of the transient was estimated at 50% of the absorbance. t_d is the dead time of the chromatographic system.

4. Results and discussion

4.1. Determination of ionic capacity by potentiometric titration

To determine the amount of ion-exchange groups on CIM monolith ion exchangers, a classical titration method was used. The experiment was performed by pumping a titration solution through the monolithic column in a closed circle while continuously measuring the pH value. When the equilibrium was established, a drop of acid or base solution (depending on the type of resin and the starting pH value) was added to the circulating solution. In this way, a titration curve was obtained and the amount of groups was easily calculated. However, it took a very long time to reach the equilibrium. In fact, after each drop addition, it sometimes took several hours for equilibration to occur. While this is an extremely time consuming process, instrument drift might additionally contribute to inaccuracy of the measurement. To avoid these problems, we narrowed our experiments to determining only the total amount of groups present on the monolith. This was done by circulating a solution through initially conditioned monolithic columns for several days and then retitrating afterwards. The results presented in Table 1

Table 1
Percentage of total ionic capacity[a] obtained by continuous circulation[b] of base solution[c] through CIM SO3 disk monolithic column for different period of time and retitration with acid solution[d] afterwards

Time (day)	Percentage of total ionic capacity (%)
1	28.0
4	52.2

[a] Total ionic capacity was determined by mass conversion (see Section 3.3.2).
[b] Flow rate: 2 ml/min.
[c] 20 ml of 0.03 M KOH solution was used.
[d] 0.1 M HCl solution was used.

demonstrate that even after 4 days of continuous circulation only approximately one-half of the groups, determined by mass conversion (see Section 3.3.2 for method description), were titrated. To eliminate possible diffusional limitations, the monolith was grinded up and the reaction was allowed to proceed in a batch mode for up to 7 days. In this case, almost a total titration of the groups was achieved (over 90%). Based on these results it can be concluded that practically all groups present on the resin can contribute to the titration process but the time required to perform such experiments is extremely long. However, due to the necessary monolith destruction, this method cannot be used for quality control. Furthermore, in the case of anion exchangers, exposure to strong acidic conditions for several days results in the generation of an amine smell indicating degradation of the resin.

Obviously, not all of the groups can be titrated in a short time, but there might still be a certain percentage, which could react quickly. We speculated that maybe pumping a defined amount of acid or base solution through the conditioned monolithic column causes titration of a constant percentage of the total ionic groups regardless of the amount or density. If this would be the case, the total amount of the groups could be simply calculated. A set of experiments using QA and DEAE anion-exchange monoliths was performed to verify this approach. Anion-exchange monolithic columns with different ligand densities were prepared and the ratio of the groups that were titrated versus all ionic groups was calculated. It was found that the ratio was far from being constant ranging for QA monolithic columns between 1 and 50% and for DEAE monolithic columns between 5 and 15%. Interestingly, the trends for weak and strong anion exchangers were opposite. Currently we have no explanation for this behavior.

From the results of the performed experiments, it can be concluded that using titration to determine the amount of ion-exchange groups cannot be efficiently implemented for methacrylate monoliths. Because of this, a different method has to be developed. We speculated that it can be based on the formation of the pH transient.

4.2. pH and UV absorbance measurements

To verify the applicability of the above conclusions, an experiment using CIM DEAE disk monolithic column was performed. After the column was pre-saturated with a high-concentration buffer solution (0.5 M phosphate buffer solution, pH 6.8), the low-concentration buffer solution (20 mM phosphate buffer solution, pH 6.8) of the same pH value was pumped through the column and the pH was measured to find whether the inadvertent pH transient is formed (Fig. 2a). The pH transient produced by two solutions of the same salt differing in concentration, but having the same pH, is clearly indicated by a decrease of approximately 1 pH unit. The change of the pH value might be a consequence of selective anion desorption. In the case of the buffering salt, e.g. phosphate buffer pH 6.8 we used for CIM DEAE monolithic columns, adsorbed molecules are mainly in two forms: $H_2PO_4^-$ and

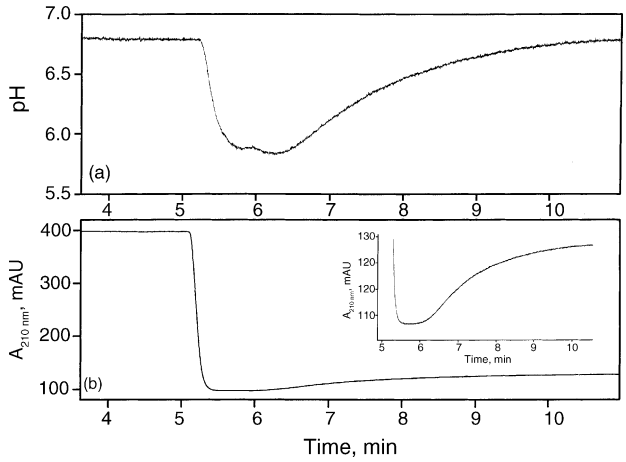

Fig. 2. pH (a) and absorbance (b) measurement of the step change from high to low concentration buffer solution on a CIM DEAE disk monolithic column. Method: 0.5 M phosphate buffer solution, pH 6.8 (5 min), 20 mM phosphate buffer solution, pH 6.8 (60 min). Flow rate: 4 ml/min. Detection: pH and absorbance at 210 nm.

HPO_4^{2-}. Since they are charged differently, their strength of interaction with the matrix is different. During the desorption process, $H_2PO_4^-$ ions are preferentially desorbed resulting in their higher concentration in the solution. Therefore, the pH value of the solution decreases.

Because a pH detector is not always available in a chromatographic system, a UV detector was used to find out whether the change of pH also reflects a change of the absorbance. Fig. 2b shows a very similar profile of the absorbance measured at 210 nm as it was observed from pH measurements. To answer the question whether the change in absorbance is really representative and is a consequence mostly of pH changes or also some concentration changes, the effect of the pH value on absorbance at 210 nm was determined by buffer solutions having the same concentration but differing in pH value. The results are shown in Fig. 3. Using a calculated calibration curve (see Fig. 3b), the pH curve was converted to an absorbance curve and compared to the real absorbance measurements (Fig. 4). The calculated absorbance curve coincides with experimental data in terms of amplitude. A slight time shift of the data can be attributed to the time de-

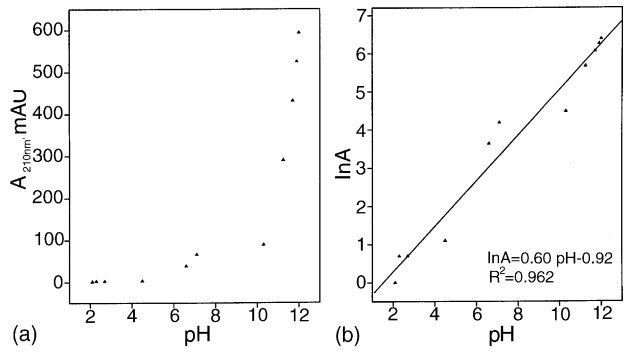

Fig. 3. Dependence of absorbance (a) and ln *A* (b) on pH for 20 mM phosphate buffer solutions. Absorbance was measured at 210 nm.

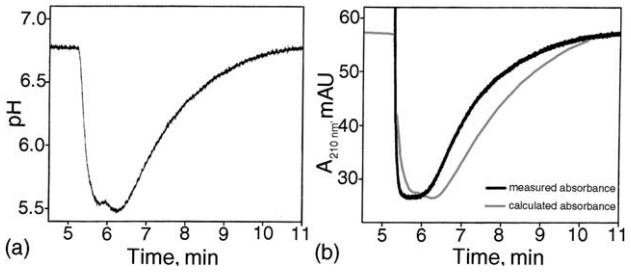

Fig. 4. Measured pH transient curve (a) and comparison of measured (solid line) and calculated (scattered line) absorbance (b) for a CIM DEAE disk monolithic column. Method: 0.5 M phosphate buffer solution, pH 6.8 (5 min), 20 mM phosphate buffer solution, pH 6.8 (60 min). Flow rate: 4 ml/min. Detection: pH and absorbance at 210 nm.

lay between the two detectors. Additional confirmation, that a change in absorbance is mainly due to the change in pH of the buffer solution, was shown by negligible changes in the conductivity of the effluent solution. Therefore, it can be concluded that in this case, the pH and absorbance measurements are basically equivalent and therefore, absorbance can be used to monitor the formation of the pH transient.

While for a single CIM disk monolithic column the pH transient resembles more like a pulse response, i.e. the time of the transient is quite short due to a low amount of ionic groups. For larger units, e.g. 80 ml CIM DEAE tube monolithic columns, an obvious "breakthrough curve" is obtained (Fig. 5), indicating a much higher amount of anion-exchange groups.

4.3. Influence of column length and linear velocity

First, to verify whether the data can be described by Eq. (7), the pH transient times for weak-base anion-exchange CIM DEAE disk monolithic columns having different bed lengths were measured. The bed length of the CIM DEAE

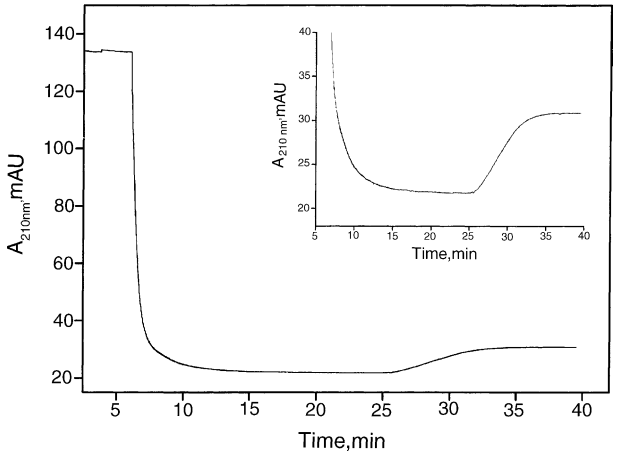

Fig. 5. Absorbance measurement of the step change from high to low concentration buffer solution on a CIM DEAE 80 ml tube monolithic column. Method: 1.0 M phosphate buffer solution, pH 6.8 (5 min), 20 mM phosphate buffer solution, pH 6.8 (60 min). Flow rate: 80 ml/min. Detection: absorbance at 210 nm.

Table 2
Dependence of the time of the pH transient on the column length for CIM DEAE disk monolithic columns[a]

Column length (mm)	Time (min)
2.0	1.3
3.0	2.1
5.0	3.7
6.0	4.0

[a] Method: 0.5 M phosphate buffer solution, pH 6.8 (5 min), 20 mM phosphate buffer solution, pH 6.8 (60 min). Flow rate: 4 ml/min. Detection: absorbance at 210 nm.

monolithic columns was varied by placing several disks in a single housing [14]. Data summarized in Table 2 shows the time of the transient using columns of different lengths. Fitting the data gives a linear proportion of the pH transient time to the column length:

$$t(\text{pH}) = 0.7L - 0.025$$

where t(pH) is the time of the pH transient (see also Eq. (7)) and L represents the column length. The correlation coefficient (R^2) is 0.982.

Secondly, various flow-rates were applied reflecting a change of the linear velocity. According to Eq. (7) the time at which the pH front exits the column should be inversely related to the linear velocity (flow rate) of the solution. Data of the time of the pH transient for a CIM DEAE disk monolithic column are summarized in Table 3. Fitting with a rational reciprocal function gives the following equation:

$$t(\text{pH})\phi_V = 9.26$$

where t(pH) is the time of the pH transient and ϕ_V is the volume flow rate. R^2 is 0.994.

From these two sets of measurements, it can be concluded that the results are in accordance with Eq. (7).

4.4. Correlation of pH transient time and maximum ion-exchange capacity

The effect of the amount of groups present on the monolith was tested. The times of pH transient for CIM DEAE disk and 8 ml tube monolithic columns with different concentrations of ion-exchange groups using phosphate buffer solutions (pH 6.8) were measured (Fig. 6). The amount of ion-exchange groups for each column was determined by mass conversion as described in Section 3.3.2. CIM DEAE 80 and 800 ml

Table 3
Time of the pH transient at different flow rates, measured for CIM DEAE disk monolithic column[a]

Flow rate (ml/min)	Time (min)
1.1	8.6
2.0	4.4
4.0	2.1

[a] Column length: 3 mm. Method: 0.5 M phosphate buffer solution, pH 6.8 (5 min), 20 mM phosphate buffer solution, pH 6.8 (60 min). Detection: absorbance at 210 nm.

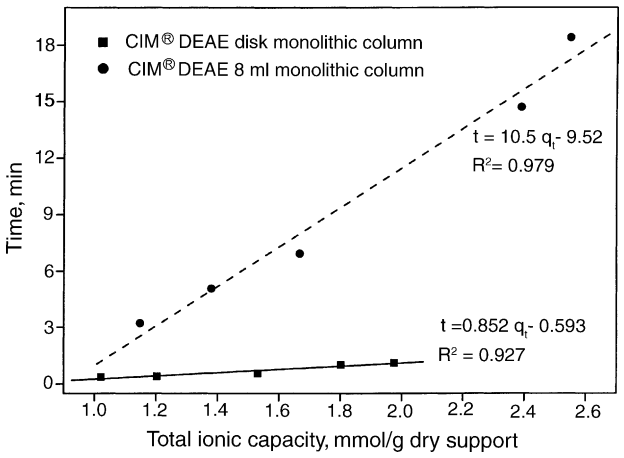

Fig. 6. Time of the pH transient as a function of total ionic capacity for CIM DEAE disk and CIM DEAE 8 ml tube monolithic columns. The total ionic capacity was determined by mass conversion measurements. The pH transient times were measured using a 0.5 M and 20 mM sodium phosphate buffer solutions, pH 6.8. Flow rate: 10 column volumes per minute (disks), 1 column volume per minute (tubes). Detection: absorbance at 210 nm.

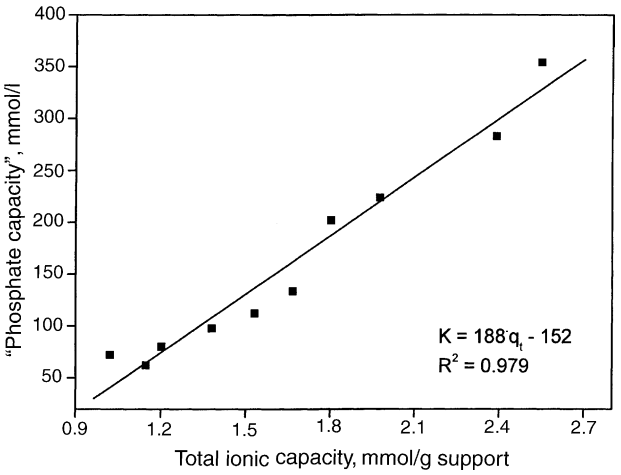

Fig. 7. Correlation between "phosphate capacity" and total ionic capacity for CIM DEAE disk and CIM DEAE 8 ml tube monolithic columns. The "phosphate capacity" was calculated by using Eq. (8). The total ionic capacity was determined by mass conversion measurements.

tube monolithic columns were not included in this part of the study since drying of larger monolithic blocks to determine total ionic capacity by mass conversion causes crack formation or even breakage of the monoliths. Tested monolithic columns demonstrate a good linear relation of the measured time of the transient and the total ionic capacity for this type of ion exchangers. This conclusion again confirms that Eq. (7) properly describes experimental results and is especially important because it indicates that the proposed method can be used for determining the ionic group density.

To determine the ionic capacity for larger monolithic columns, which are cylindrical shape operating in radial mode, proper values for column length (L) and linear velocity (v_{fluid}) should be used. Due to a different geometry of the columns such calculation might be time consuming. Since the ratio L/v_{fluid} (see Eq. (7)) actually represents residence time of the non-retained molecule in the column, it can be simply calculated as a ratio of the column volume (V_c) and the flow rate (ϕ_V). For the columns of the same chemistry the following equation should be used:

$$K = \frac{\phi_V t(\mathrm{pH}) C_2}{V_c} \quad (8)$$

where ϕ_V is the volume flow rate, t(pH) is the time of the pH transient, C_2 is the concentration of elution buffer solution and V_c is the column volume. K is the measure for "phosphate capacity" and is given in mmol/l. Calculation of "phosphate capacity" enables the direct comparison of the total ionic capacity for various CIM DEAE monolithic columns (Fig. 7). This is very convenient especially for larger, 80 and 800 ml CIM DEAE tube monolithic columns where direct measurement of the total ionic capacity by mass conversion becomes very problematic.

4.5. Effect of buffer concentration on method sensitivity

To evaluate the applicability of the developed method, the effect of various parameters that influence the method's results should be closer investigated. According to Eq. (3) in the case of a specific selected column, column length (its volume) and column porosity are constant, so only the flow rate and the slope of the adsorption isotherm affect method results. As already shown in Section 4.3, lowering the flow rate (linear velocity) results in longer transient times and, in some cases, more accurate determination of ionic capacity. However, if the data acquisition rate is high enough, the flow rate has no influence on the method's sensitivity.

On the other hand, this is not the case for the slope of the adsorption isotherm. While for a selected column the total ionic functional group concentration (q_t) is constant, the buffer solution concentration plays a significant role (Eq. (5)). Measurements of the time of the pH transient using different concentrations of the phosphate buffer solutions for a CIM DEAE disk monolithic column are presented in Table 4. It is observed that changing the concentration of the high con-

Table 4
Time of the pH transient for different concentrations of buffer solutions, measured for CIM DEAE disk monolithic column[a]

Concentration of solution 1[b] (M)	Concentration of solution 2[c] (M)	Time (min)
0.1	0.020	2.2
0.5	0.005	10.9
0.5	0.010	4.5
0.5	0.020	2.1
1.0	0.020	2.4

[a] Column length: 3 mm. Method: high phosphate buffer concentration solution, pH 6.8 (5 min); low phosphate buffer concentration solution, pH 6.8 (60 min). Flow rate: 4 ml/min. Detection: absorbance at 210 nm.
[b] 1 refers to high buffer concentration solution.
[c] 2 refers to low buffer concentration solution.

centration buffer solution only slightly affects the time of the transient. In contrast, changing the concentration of the low concentration buffer solution significantly influences the results (Table 4), while the effect on the slope of the isotherm, i.e. the value of $f'(C_A)$ from Eq. (6), is stronger. As shown in Table 4, the time is increased when the concentration is decreased which is expected as the buffer capacity of the solution having the same pH raises with the total concentration of the buffering species; therefore the resistance of the solution to changes in pH is stronger [15] and the retained pH front moves faster. So, the sensitivity of the method in the case of the selected column and constant flow rate mostly depends on the concentration of the low concentrated buffer solution.

4.6. Other CIM ion exchangers

Although the method was developed on weak anion-exchange columns, it can be easily transferred to strong anion-exchange CIM monolithic columns, such as QA. Furthermore, by selection of a proper buffering species, it can be implemented also on cation-exchange CIM monolithic columns.

The experiments on CIM QA disk monolithic columns, having strong anion-exchange groups, showed that time of the pH transient using phosphate buffer solutions was very short and the sensitivity of the method was low. Because of this, some other salt solutions were tested. The best results were obtained by using sodium hydrogencarbonate buffer solutions (pH 8.8). Fig. 8 shows a good linear correlation between the transient time and the total ionic capacity of the resin, determined by mass conversion, and confirms usefulness of the method to determine the total ionic capacity of strong-base anion exchangers.

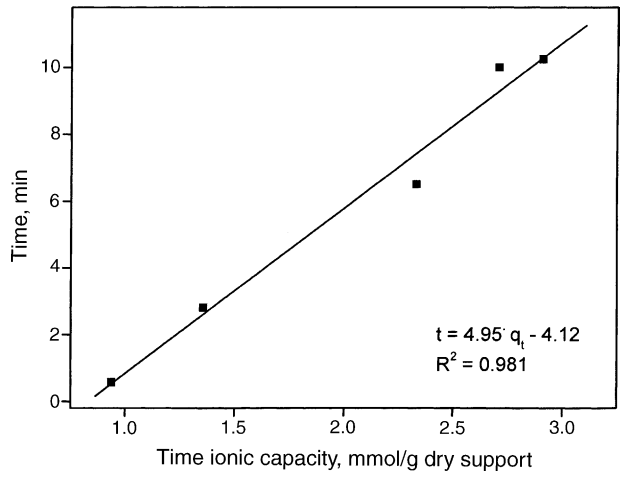

Fig. 8. Time of the pH transient as a function of total ionic capacity for CIM QA disk monolithic columns. The total ionic capacity was determined by mass conversion measurements. The transient time was measured using a 0.5 M and 20 mM sodium carbonate solutions, pH 8.8 at 4 ml/min. Detection: pH and absorbance at 210 nm.

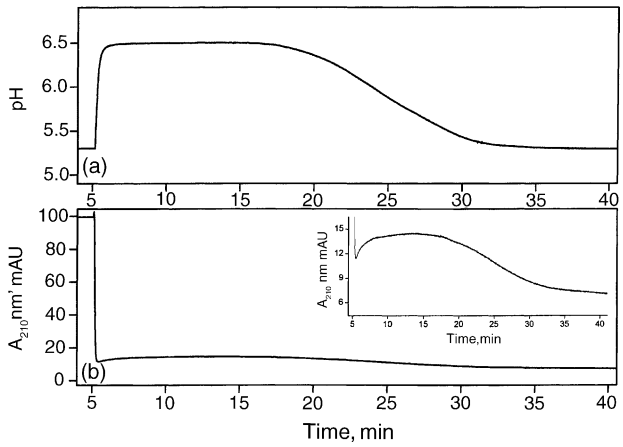

Fig. 9. Measurement of pH (a) and absorbance (b) during the step change from high to low concentration solution on a CIM SO3 disk monolithic column (L = 3.0 mm). Method: 0.5 M ammonium sulfate solution, pH 5.3 (5 min), 20 mM ammonium sulfate solution, pH 5.3 (60 min). Flow rate: 4 ml/min. Detection: pH and absorbance at 210 nm.

For measurements of the amount of ion-exchange groups on cation-exchangers, both weak- and strong-ones (CIM CM and CIM SO3 monolithic columns), tests were performed using ammonium sulfate buffer solutions (pH 5.3). By measuring pH and absorbance at 210 nm, inversed transient curves were observed in comparison to the results on anion exchangers (Fig. 9). The correlations between the transient time and the column length for CIM SO3 disk monolithic columns are presented in Table 5. Linear fitting to data gives the equation:

$$t(\mathrm{pH}) = 5.40L + 1.50$$

where t(pH) is the time of the pH transient and L represents the column length. R^2 is 0.98. The high correlation coefficient certifies that the method can also be used to estimate the amount of ion-exchange groups on strong-acid cation exchangers.

The pH transient was also obtained using weak-acid cation exchangers (CIM CM disk monolithic columns) with ammonium sulfate solutions.

4.7. Particle bed columns

As the inadvertent pH transient was first described for columns comprised of particle shaped resins [12], the measurements using strong-base anion-exchange particle bed

Table 5
Dependence of the time of the pH transient on the column length for CIM SO3 disk monolithic columns[a]

Column length (mm)	Time (min)
2.15	11.6
3.0	19.6
5.15	29.4
6	33.4

[a] Method: 0.5 M ammonium sulfate solution, pH 5.3 (5 min), 20 mM ammonium sulfate solution, pH 5.3 (60 min). Flow rate: 4 ml/min. Detection: pH and absorbance at 210 nm.

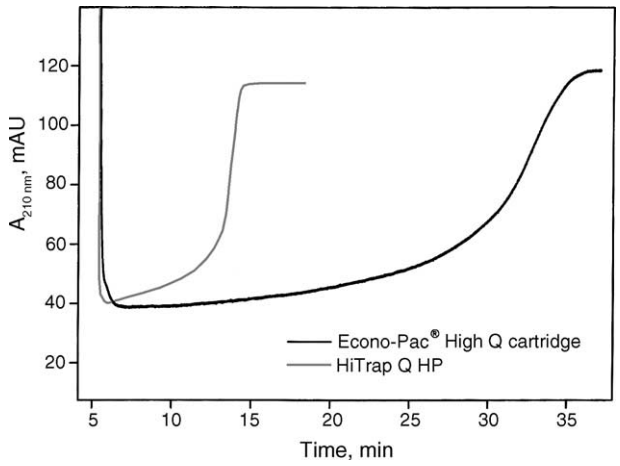

Fig. 10. Measurements of the time of the pH transient on Econo-Pac High Q cartridge (1 ml) from Bio-Rad Labs. and HiTrap Q HP (1 ml) from Amersham Biosciences. Method: 0.5 M $NaHCO_3$, pH 8.8 (5 min), 20 mM $NaHCO_3$, pH 8.8 (60 min). Flow rate: 4 ml/min. Detection: absorbance at 210 nm.

columns from Bio-Rad Labs. and Amersham Biosciences were made (Fig. 10) to prove the method could also be used for conventional columns (Table 6). The time of the pH transient measured at different flow rates for HiTrap Q HP column (Amersham Biosciences) again shows an inverse relationship, i.e. the higher the flow rate the shorter the time of the transient.

Assuming that the chemistry of the strong anion-exchange groups for columns from Table 6 is quite similar, $f'(C_A)$ from Eq. (6) can be set as a constant and calculated from data for CIM QA disk monolithic column. Consequently, the total ionic functional group concentration (i.e. total ionic capacity) for other columns can be estimated. $f'(C_A)$ for CIM QA disk from Table 6 is approximately 135 ml/mmol (q_t determined by mass conversion is 1.3 mmol/ml support, v_{fluid}, calculated from the flow rate and cross sectional area of the column, is 3.5 cm/min, L is 3 mm, α is 0.6). Total ionic capacity calculated for HiTrap Q HP column, using some approximations (for example, column porosity, α, was roughly estimated by using void volume of the column, 0.33 [16]), was 0.13 mmol/ml gel. That is very close to the actual total ionic capacity, which is between 0.14 and 0.20 mmol/ml gel given in technical specifications of the column by the manufacturer [17]. Unfortunately, the calculations could not be done for Econo-Pac High Q cartridge as there was no information about porosity or void volume of the column. However, assuming that the column porosity is similar to the HiTrap Q HP column, it can be speculated from the data in Table 6 that the total ionic capacity for Econo-Pac High Q cartridge is higher then the HiTrap Q HP column (column volume was the same for both).

Table 6
Time of the pH transient for various strong-anion exchange columns[a]

Column type	Flow rate (ml/min)	Time (min)
Econo-Pac High Q cartridge[b]	4.0	26.3
HiTrap Q HP[c]	4.0	8.3
	2.0	15.1
CIM QA disk[d]	4.0	10.0

[a] 0.5 M and 20 mM sodium hydrogencarbonate solutions pH 8.8 were used. Detection: pH and absorbance at 210 nm.
[b] Manufacturer Bio-Rad Labs. (Hercules, CA, USA).
[c] Manufacturer Amersham Biosciences (Uppsala, Sweden).
[d] Manufacturer BIA Separations (Ljubljana, Slovenia).

4.8. Comparison of time of pH transient and protein dynamic capacity

CIM ion-exchange monolithic columns are largely used for the purification and separation of macromolecules. Determining the capacity of large molecules under real application conditions is more indicative for users of such columns. This is why a correlation between dynamic binding capacity, determined by a classical method using proteins, and the "phosphate capacity", which represents the ionic capacity, was investigated. The dynamic binding capacity for bovine serum albumin and the "phosphate capacity" of CIM DEAE disk and 8 ml tube monolithic columns were determined. Fig. 11 shows that the BSA capacity raises with "phosphate capacity" until a plateau is reached and the capacity for the BSA becomes independent from the charged ligand density on the resin's surface, i.e. the ionic capacity of the resin. This is a normal consequence of the surface limitation since the large molecule cannot access all possible binding sites, which are accessible to small ions. From this data, it can be concluded that for a given monolithic structure, a protein capacity can be accurately derived from the data obtained by measuring

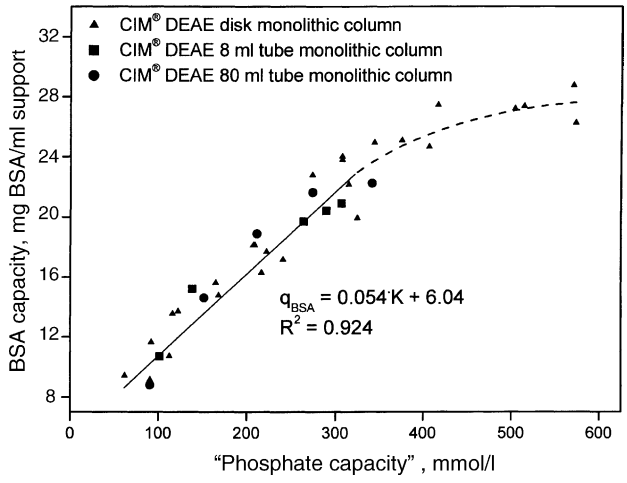

Fig. 11. Correlation of BSA dynamic capacity and "phosphate capacity" for CIM DEAE disk, 8 and 80 ml tube monolithic columns. BSA capacity was determined by frontal analysis, using 1 mg/ml (disks) and 3 mg/ml (tubes) BSA in 20 mM Tris–HCl buffer solution, pH 7.4 and flow rate 3 ml/min (disks), 16 ml/min (8 ml tubes) and 160 ml/min (80 ml tubes). The capacity at 50% of absorbance measured at 280 nm was calculated. "Phosphate capacity" was calculated from time of the pH transient using Eq. (8). Method: 0.5 M phosphate buffer solution, pH 6.8 (5 min), 20 mM phosphate buffer solution, pH 6.8 (2 h). Flow rate: 10 column volumes per minute (disks) and 1 column volume per minute (tubes). Detection: absorbance at 210 nm.

the time of the pH transient. The latter method is even more informative, since the amount of ion-exchange groups can be determined in a wider interval.

5. Conclusions

A new fast, simple, non-destructive and non-toxic method for determining the amount of ion-exchange groups on resins was developed by measuring a pH transient formed using a concentration step change of adsorbing buffering species. The method can be used for anion as well as for cation exchangers. Due to its non-invasivity is especially convenient for determining the amount of the ionic groups on monolithic columns, but it can also be used for particle bed columns as well. Because the method causes no contamination, it can serve as a quality control method for produced columns as well as for monitoring the chromatographic properties of columns during their use. Furthermore, since it allows characterization and traceability of a monolithic column's properties, this method can form the basis for a cGMP test of monolithic columns, which is required by the pharmaceutical industry.

6. Nomenclature

A	charged form of a buffer (or salt) species
C_A	liquid phase concentration of buffer species A (mol/l)
C_2	concentration of low concentration buffer solution (mmol/l)
dq_A/dC_A	slope of the adsorption isotherm for buffer species A
k	constant, defined by column porosity and solute/ion exchanger system ($=(1-\alpha)f'(C_A)/\alpha$) (l/mol)
K	"phosphate capacity" (mmol/l)
L	column length (cm)
q_A	adsorbed phase concentration of buffer species A (mol/l stationary phase)
q_t	total ionized functional group concentration in the stationary phase, also total ionic capacity of the resin (mol/l stationary phase)
t(pH)	transit time for a pH value (s)
v_{fluid}	interstitial or linear velocity (cm/s)
V_c	column volume (ml)

Greek symbols

α	column porosity (void volume) fraction
ε	internal particle porosity
ϕ_V	volume flow rate (ml/min)
θ_A	fractional coverage of buffer species A

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