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## Research Article

# Capillary electrochromatographic separation of proteins on a column coated with titanium dioxide nanoparticles

A TiO<sub>2</sub> nanoparticle (TiO<sub>2</sub> NP)-coated open-tubular column for the capillary electrochromatographic separation of proteins is described. The surface chemistry of the TiO<sub>2</sub> NPs on the inner wall of the fused silica was significantly affected by the running buffer. By varying of the phosphate buffer pH, only cathodic EOF was indicated. The results showed that TiO<sub>2</sub> NPs are existed as a complexed form with the buffer ligand. Good separation of conalbumin (ConA), apo-transferrin (apoTf), ovalbumin (OVA), and BSA could be achieved with phosphate buffer (40 mM, pH 8.0) and an applied voltage of 15 kV. Five peaks of glycoisoforms of OVA were observed under these conditions. In comparison with the retention behavior of the analytes on the bare fused-silica column, the new column's high resolving power seems to be predominantly derived from the ligand exchange of the analytes with the phosphate adsorbed onto the TiO<sub>2</sub> NPs. The method was also used to separate egg-white proteins. Both acidic and basic proteins in egg white were separated in a single run. The microheterogeneities of OVA could also be found in it. The separation efficiency for the main peak of OVA in egg white was around 10 000 plates/m.

**Keywords:** Capillary electrochromatography / Protein / Stationary phase / Titanium dioxide nanoparticle  
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## 1 Introduction

Many peptides and all proteins contain hydrophobic residues. They carry net charges at any pH except their intrinsic *pI*. The combination of charged and hydrophobic moieties makes them an ideal class of analytes to exploit the inherent advantage of CEC [1–4]. The columns used for CEC are usually packed [5, 6], monolithic [7–10], or open-tubular [11–13]. The separation mode can be RP or normal phase, and based on ion exchange, hydrophilic interaction, or size exclusion. However, major impediments to the application of CEC in proteomics are the irreversible adsorption of peptides and proteins to naked silica capillary walls and/or packing materials, and electrostatic interactions between charged groups on amino acid residues and charged packings that are required for

producing EOF [1–4]. In other words, peptide and protein separation by CEC has started to leave its infancy, yet is still young enough to provide many exciting research opportunities [14].

Titanium dioxide has been the focus of numerous investigations in recent years, in areas such as catalysis, photography, electronics, optics, and biotechnology. Particularly due to its hydrolytic stability at extreme pH, higher *pI* value than silica, and amphoteric nature, it has been used as an alternative to silica as a column packing material in HPLC. The use of titanium dioxide as a sorbent in normal phase, RP, and ion-exchange LC has been reported [15–18]. Though its applications in HPLC have been widely investigated, little attention has been paid to its applicability in CE and CEC. Tsai *et al.* [19] were the first to prepare a titanium dioxide-coated column by the sol-gel method for the CE separation of proteins. Fujimoto [20] employed titanium dioxide and an ODS-modified column for the CE and CEC separation of carbohydrates and proteins. Recently, an ordered, mesoporous titanium dioxide film was introduced and used to coat a capillary by means of the sol-gel technique for the CE separation of alkaloids in a nonaqueous system [21].

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**Abbreviations:** apoTf, apo-transferrin; ConA, conalbumin; OVA, ovalbumin; TiO<sub>2</sub> NPs, TiO<sub>2</sub> nanoparticles

Alkylphosphates and phosphonates have been shown to form ordered self-assembled monolayer on the surface of metal oxide, such as tantalum oxide, niobium oxide, and titanium oxide [22, 23]. In particular, titanium dioxide is known to be employed for the extraction of phospholipids [24] and phosphopeptides [25, 26], as well as for the separation of phosphorus-containing compounds [27]. Recently, Chen and Chen [28] reported the use of  $\text{Fe}_3\text{O}_4/\text{TiO}_2$  core-shell nanoparticles as affinity probes for the mass spectrometric analysis of phosphopeptides. In addition, aromatic carboxylic acids have been shown to affect the dispersion properties of  $\text{TiO}_2$  nanoparticles ( $\text{TiO}_2$  NPs) [29].

We have prepared  $\text{TiO}_2$  NP-coated capillaries for the open-tubular CEC separation of oligopeptides [30]. The surface properties of the prepared columns are significantly affected by the composition of the mobile phase, and are therefore very attractive for our purpose. Here we describe our attempts to further critically evaluate the potential applicability of the  $\text{TiO}_2$  NP-coated column for the CEC separation of proteins of similar molecular weights or  $pI$ 's.

## 2 Materials and methods

### 2.1 Apparatus

All experiments were carried out in a laboratory-built unit. It consisted of a  $\pm 30$  kV high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA) and a UV-Visible detector (Model L-4200, Hitachi, Japan). Electrochromatograms were recorded and processed with a Peak-ABC chromatography workstation ver 2.11 (JiTeng Trading Pte, Singapore) running on the Windows XP operating system. Fused-silica capillaries of 50  $\mu\text{m}$  id and 375  $\mu\text{m}$  od were purchased from Polymicro Technologies (Phoenix, AZ, USA).

### 2.2 Reagents and chemicals

Most chemicals were of analytical reagent grade and from Merck (Darmstadt, Germany). Purified water (18  $\text{M}\Omega\text{-cm}$ ) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions. Conalbumin (ConA, from chicken egg white and substantially iron-free), human apo-transferrin (apoTf), ovalbumin (OVA, from chicken egg white), BSA, and lysozyme (from chicken egg white) were obtained from Sigma (St. Louis, MO, USA). Perchloric acid, benzyl alcohol, phosphoric acid, sodium phosphate (monobasic, dibasic, and tri-

basic), and Tris were purchased from Merck; formic acid, L-glutamic acid, 1-propanol, PEG (PEG 8000, average MW = 8000), sodium hydroxide, and hydrochloric acid were purchased from Acros (Geel, Belgium); and titanium (IV) isopropoxide was purchased from Fluka (Buchs, Switzerland).

Stock solutions of the proteins (5 mg/mL) were prepared in pure water and stored in the refrigerator. They were diluted to 1 mg/mL prior to use. All solvents and solutions for CEC analysis were filtered through a 0.45  $\mu\text{m}$  PTFE (Millipore), cellulose acetate membrane (Whatman, Middlesex, UK) or mixed cellulose ester membrane (Advantec MFS, Pleasant, CA, USA).

### 2.3 Preparation of a $\text{TiO}_2$ NP-coated open-tubular column

The nanosized  $\text{TiO}_2$  particles and the  $\text{TiO}_2$  NP-coated column were prepared following the procedures described earlier [30]. The inner wall of the fused-silica capillary was treated with 1.0 M NaOH, followed by rinsing with pure water, flushing with 1.0 M HCl, pure water, and then methanol. After being dried in a GC oven at 110°C for 1 h under nitrogen flow, the concentrated NPs stabilized by PEG 8000 were introduced into the capillary column under a nitrogen flow of  $100 \text{ kg}\cdot\text{m}^{-1}\text{s}^{-2}$  for 10 min. The column was then plugged with GC septa and reacted at 150°C for 24 h. The resulting column was flushed successively with methanol, 0.1 M NaOH, and pure water for 10 min to remove unreacted material.

### 2.4 Capillary electrochromatographic conditions

Before analysis, the coated capillaries were preconditioned with the running buffer. They were rinsed with methanol, pure water, and buffer between runs at 1 or 2 min intervals. The samples were injected by the hydrodynamic mode. The EOF was measured with benzyl alcohol. The samples were detected by measurement of UV light absorption at 214 nm.

### 2.5 Egg-white protein preparation

Chicken eggs were obtained from the local supermarket. The egg white and egg yolk were separated. The egg white was then diluted with Tris buffer (20 mM, pH 7.4) in a 1:20 ratio and filtered through a 0.2  $\mu\text{m}$  mixed cellulose ester membrane before use.

### 3 Results and discussion

The feasibility of protein separation on the TiO<sub>2</sub> NP-coated open-tubular column was evaluated using OVA, BSA, Con A, and apoTf as model analytes. The physical and chemical properties of these proteins are listed in Table 1. Their hydrophobic parameters are comparable; the *pI* values of OVA and BSA as well as apoTf and ConA are similar, as are the molecular weights of ConA and apoTf. The proteins are more hydrophobic and contain more active groups than do peptides. Hence it was anticipated that they would not be easy to separate.

**Table 1.** Physical and chemical properties of proteins

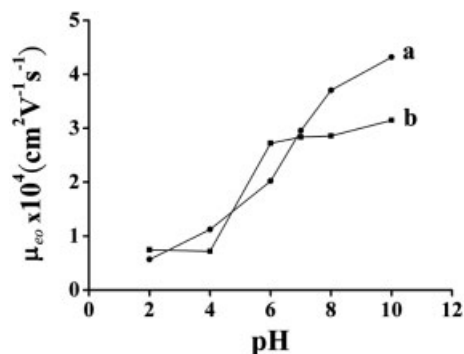
Protein (sources)	MW	<i>pI</i>	<i>R</i> <sup>a)</sup> (nm)	Hydrophobic parameter (cal/mol) <sup>b)</sup>
OVA (chicken egg white)	45 000	4.7	2.3	980
BSA (bovine plasma)	66 000	4.7	2.6	1000
apoTf (human)	77 000	6.2	5	930
ConA (chicken egg white)	77 000	6.3	2.8	980
Lysozyme (chicken egg white)	14 600	~11		890

a) Spherical radius, calculated by assuming that all the proteins are spherically shaped.

b) [39].

#### 3.1 pH of the mobile phase

The TiO<sub>2</sub> NP-coated capillary exhibited an exceptional surface property which was dependent on buffer composition and pH [30]. In phosphate buffer, only a cathodic EOF was demonstrated (Fig. 1). The EOF increases sharply from pH 4 to 6, then levels off in the pH range of 6–8, and only slightly increases at higher pH (Fig. 1). The *pK<sub>a</sub>*

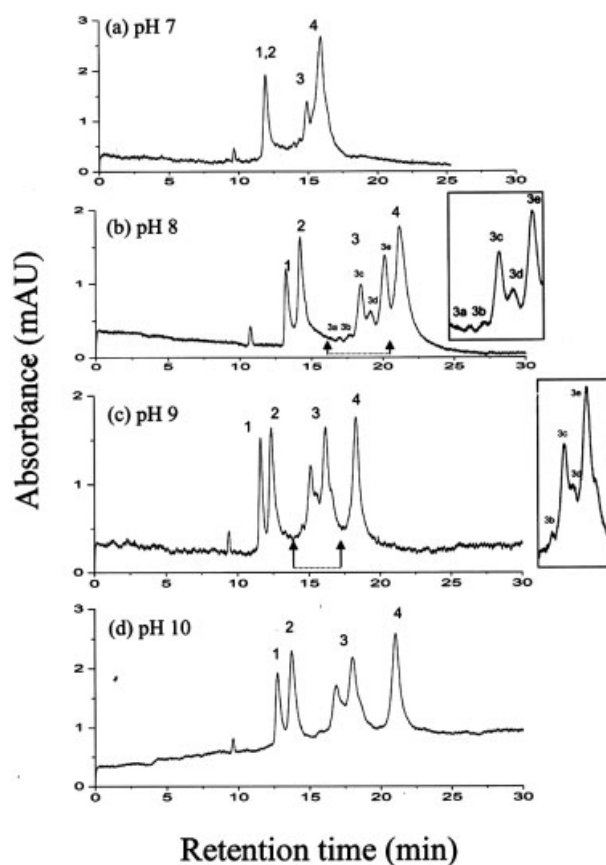


**Figure 1.** Effect of buffer pH on the EOF. Column dimension, 70 (50) cm × 50 μm id; marker, benzyl alcohol; hydrodynamic injection (10 cm, 2 s); phosphate buffer (40 mM); voltage, +15 kV; detection, 214 nm. (a) Bare fused-silica capillary (b) TiO<sub>2</sub> NP-coated column.

values of titanium dioxide are 2.60, 5.50, and 9.00 [34]. The cathodic EOF indicated that TiO<sub>2</sub> NP does not exist as the following free species, >Ti–OH<sub>2</sub><sup>+</sup>, >Ti–OH, >Ti–O<sup>–</sup>, or >Ti<sub>2</sub>=O<sup>–</sup>, but a complexed form in the presence of phosphate buffer.

With phosphate buffer (40 mM) of pH 6, no peak was observed within 2 h, irrespective of whether the sample was injected from the cathode or anode. By varying the pH of the phosphate buffer (40 mM) from 7 to 10, the electrochromatograms presented in Fig. 2 were produced.

At pH values greater than *pI*, analytes carry the negative charge, while at pH values less than *pI*, analytes carry the positive charge. Con A and apoTf have similar *pI* values (ca. 6.3) and molecular weight, resolution at pH 7 is not easy, in spite of the separation mechanism being based on ligand exchange with phosphate adsorbed on TiO<sub>2</sub> NP bonded phase or electrophoretic mobility difference. A



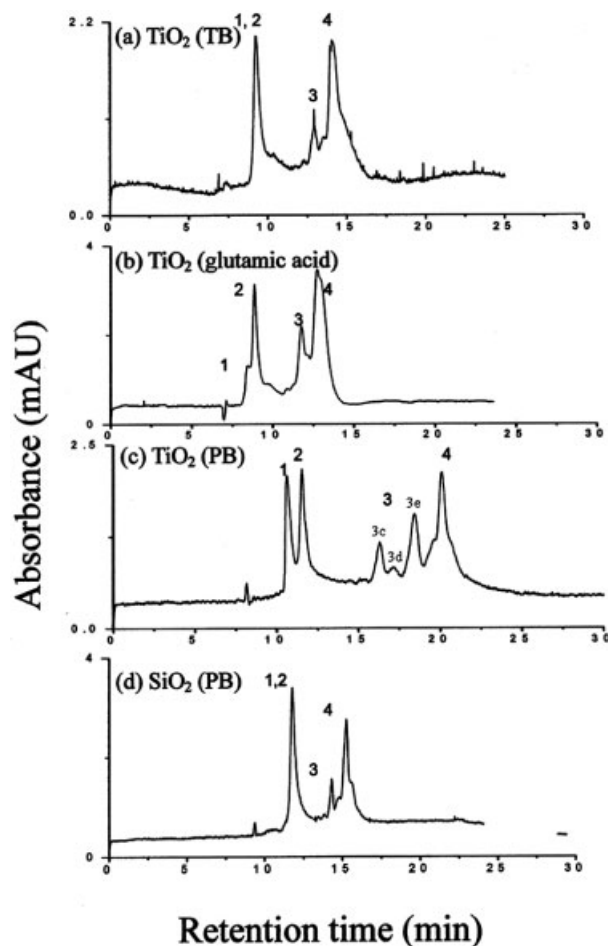
**Figure 2.** Separation of proteins at different pHs. Column: TiO<sub>2</sub> NP-coated column, 70 (50) cm × 50 μm id; sample concentration: 1 mg/mL each; hydrodynamic injection (10 cm, 10 s); phosphate buffer (40 mM); voltage, +15 kV; detection, 214 nm. Peak identification: (1) ConA; (2) apoTf; (3) OVA (a, b, c, d, and e: glycoisomers of OVA); (4) BSA.

partial resolution was seen for the pair of OVA and BSA (Fig. 2a). As the pH was increased to 8, more than four analytes could be separated. Five glycoisoforms of OVA were observed (Fig. 2b). When the pH was further increased above 8, a greater EOF (Fig. 1) made the analytes migrate slightly faster, but only four isoform peaks were observed (Fig. 2c). When the pH reached to 10, only two isoform peaks were seen (Fig. 2d). As the pH was increased to values far from the proteins' *pI* values, difference between their effective charges will be less. Hence, the recognition ability of the TiO<sub>2</sub> NP bonded phase to the glycoisoform becoming meager is rational.

OVA is a glycoprotein of avian egg white. It is 385 amino acids in length and has two potential sites for asparagine-linked glycosylation. Its structure is very heterogeneous [31]. Accordingly, the splitting of the OVA peak seems reasonable. The maximum number of OVA glycoforms observed was five at pH 8; fewer peaks were observed at the other pHs. Although these isoform peaks were not baseline separated, the result suggests that this column is a highly promising device for the separation of glycoprotein isoforms. Further work is needed for a more detailed understanding of its function. Legaz and Pedrosa [31] have reported the separation of OVA glycoforms by CE with borate buffer, but it required the addition of polyamines, such as spermidine and spermine. The addition of SDS increased the resolution of the three main peaks of OVA. Che *et al.* [32] have investigated glycoforms in turkey and chicken egg OVA by CE. Different results were indicated, but borate buffer with putrescine additive was necessary for complete separation. Pacáková *et al.* [33] reported that the separation of OVA isoforms could be achieved using the additives of  $\alpha$ ,  $\omega$ -diamine alkanes or *bis*(2-aminoethyl)amine. In our case, OVA glycoisoforms could be separated using only phosphate buffer. Because OVA is a phosphorylated protein and phosphate can self-assemble onto the surface of TiO<sub>2</sub> particle [22, 23], we reasoned that ligand exchange of the analyte with the phosphate buffer adsorbed on the coated TiO<sub>2</sub> NPs might be the basis for such an effect.

### 3.2 Effect of the composition of the mobile phase

To further understand the contribution of the stationary phase, different types of buffer at pH 8 were tested (Fig. 3). The chelating affinity toward the central metal ion decreases in the order, phosphate > glutamate > Tris buffer. In Tris buffer, ConA and apoTf coeluted (Fig. 3a). A slight resolution of ConA and apoTf was seen in glutamate buffer (Fig. 3b). Much better separation was achieved using phosphate buffer (Fig. 3c). We also compared the TiO<sub>2</sub> NP-coated column with the bare fused-



**Figure 3.** Effect of buffer composition on the separation of proteins at pH 8. Column: 60 (40) cm  $\times$  50  $\mu$ m id (a, b, and c) TiO<sub>2</sub> NP-coated column (d) bare fused-silica column; hydrodynamic injection (10 cm, 10 s); voltage, +15 kV; detection, 214 nm; mobile phase (40 mM): (a) Tris buffer; (b) glutamate buffer; (c) and (d) phosphate buffer. Peak identification: (1) ConA; (2) apoTf; (3) OVA (c, d, and e: glycoisoforms of OVA); (4) BSA.

silica column (Fig. 3d). Not only a better separation for conA and apoTf, but also several peaks for OVA were observed on the TiO<sub>2</sub> NPs-coated column. When Figs. 3c and 2b are compared, we can observe that improved resolution was seen when the column length was increased. Folkers *et al.* [22] reported that on titanium dioxide, phosphonic acids form more stable monolayers than either hydroxamic or carboxylic acids. Oliva *et al.* [34] have studied the adsorption property of HSA on colloidal TiO<sub>2</sub> particle without the addition of buffer to avoid possible interference with the protein molecules. They concluded that the interaction forces might be electrostatic, hydrogen bonding, and ligand exchange. Of course, the proteins investigated in our work should have similar interaction force with the TiO<sub>2</sub> NP-coated column.

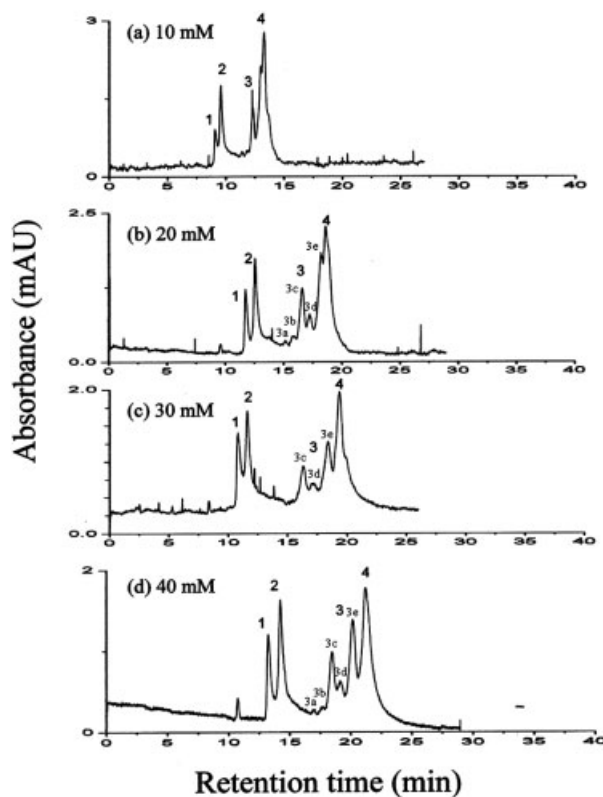
But one thing that is eminently clear is that the main separation mechanism might be due to the ligand exchange of the analyte with the phosphate adsorbed onto the TiO<sub>2</sub> NPs, not directly from the complexation with the bare TiO<sub>2</sub> NPs.

### 3.3 Effect of phosphate concentration

Among the mobile phases studied, phosphate buffer was the most effective at pH 8 (Fig. 3). When the buffer concentration was increased over a range from 10 to 40 mM, a longer retention time was observed (Fig. 4). Meanwhile, the resolution for the glycoisoforms was clearly improved. Since there is a competition between phosphate buffer and the analytes binding onto the surface of the bonded TiO<sub>2</sub> NPs, higher concentrations of phosphate will shift the equilibrium. But at this situation, all analytes migrate against the cathodic EOF. The net effect resulted in a slower migration to the detection window when the buffer concentration is progressively increased.

### 3.4 Reproducibility of the separation

When phosphate buffer (40 mM, pH 8.0) was used as the mobile phase, five measurements of the separation were performed. The RSDs (%) for the retention time (min) of ConA, apoTf, OVA, and BSA were 5.50, 3.18, 4.92, and 5.93, respectively (Table 2). Only the main peak of OVA was used to assess the reproducibility of the separation. By comparison with the RSDs (1.21–1.51%) reported by Hsieh *et al.* [30], who used oligopeptide analytes and phosphate buffer (40 mM, pH 6.0), the inferior reproducibility we observed might be due to selfassembly of phosphorylated proteins on the surfaces of the TiO<sub>2</sub> NPs [22, 23].



**Figure 4.** Electrochromatograms for the separation of proteins at different concentrations of phosphate buffer. Conditions as described in Fig. 2, except buffer pH 8. Peak identification: (1) ConA; (2) apoTf; (3) OVA (a, b, c, d, and e: glycoisoforms of OVA); (4) BSA.

### 3.5 Real sample analysis

Egg-white proteins have been analyzed by CE with a phospholipid bilayer-coated column [35]. Recently, Paredes *et al.* [36] used anion-exchange chromatography

**Table 2.** Run to run repeatability in the separation of a synthetic sample mixture, and quantitation of OVA in egg white with TiO<sub>2</sub> NP-coated open-tubular column<sup>a)</sup>

Analyte <sup>b)</sup>	Retention time (min) (RSD%)	Graphical standard addition method for OVA <sup>c)</sup>		
		Linear equation ( $x$ , concentration/mg/mL; $y$ , peak area/mAU · s)		Coefficient of determination, $r^2$ (0.9299)
ConA	13.36 (5.50)			
apoTf	14.11 (3.18)			
OVA	18.02 (4.92)	Slope ( $3.52 \times 10^4$ )	Intercept ( $2.29 \times 10^5$ )	
BSA	20.53 (5.93)			

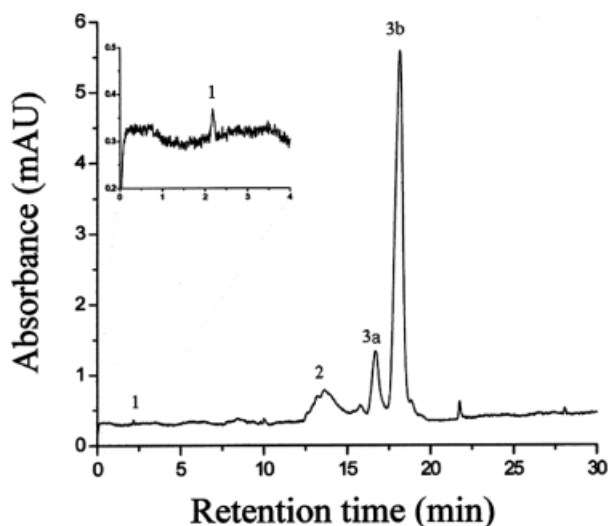
a) Column: 70 (50) cm × 50 μm id TiO<sub>2</sub> NP-coated column; voltage, +15 kV; detection, 214 nm; phosphate buffer (40 mM, pH 8.0).

b) Hydrodynamic injection (10 cm, 10 s) for the synthetic sample mixture,  $n = 5$ .

c) Hydrodynamic injection (15 cm, 10 s) for OVA spiked in egg white.

based on poly(glycidyl methacrylate-co-ethylendimethacrylate) for the fractionation of egg-white protein. Möller *et al.* [37] employed isoelectric membranes with differences of 0.025 pH units to resolve three protein isoforms from egg white.

We also evaluate the feasibility of the TiO<sub>2</sub> NP-coated column for the separation of egg-white proteins. The main protein constituent is OVA (54%). The others are ConA (12%), ovomucoid (11%), ovomucin (3.5%), lysozyme (3.4%), ovomucoprotein (1.5%), ovoflavoprotein (0.8%), ovomacroglobulin (0.5%), avidin (0.5%), among smaller fractions of other proteins [38]. After filtration and appropriate dilution, egg white was injected directly into the TiO<sub>2</sub> NP-coated capillary in phosphate buffer (40 mM, pH 8.0). The peaks were identified by the standard addition method. The elution order was lysozyme (*pI* ~11) > ConA (*pI* ~6) > OVA (*pI* ~5) (Fig. 5). Since lysozyme carries a positive charge, it was eluted first. Due to the lower abundance of lysozyme compared with the other analytes, its signal was rather small. The ConA was eluted in a broad peak. This might be due to coelution of other constituents. The average separation efficiencies of OVA (only for the main peak) based on five consecutive runs was around 10 000 plates/m. With graphical standard addition method, four different amount of OVA standard samples over the range from 2 to 10 mg/mL were spiked in egg white with 20-fold dilution. The linear equation was shown in Table 2. The magnitude of the intercept on the *x*-axis is the concentration of OVA in the diluted egg white. The amount of OVA in the original egg white was determined to be approximately 0.26 g/mL.



**Figure 5.** Electrochromatogram of the separation of proteins in egg white. Conditions as in Fig. 2, except sample injection (15 cm, 10 s); mobile phase: phosphate buffer (40 mM, pH 8.0). Peak identification: (1) lysozyme; (2) ConA; (3) OVA (a and b: glycoisomers).

## 4 Concluding remarks

The *pI* values of OVA and BSA as well as apoTf and ConA are similar, as are the molecular weights of ConA and apoTf (Table 1). The TiO<sub>2</sub> NP-coated open-tubular column exhibited an exceptional surface property which was dependent on buffer composition and pH. In the presence of phosphate buffer (40 mM, pH 8.0), the TiO<sub>2</sub> NP-coated column forms a stable surface for the CEC separation of the mixture of ConA, apoTf, OVA, and BSA. Ligand exchange seems to be the main basis for the separation. As we know, the separation of glycoisomers is not easy [31–33]. At its best, the column shows great promise in the separation of OVA glycoisomers, although resolution between peaks of glycoisomers remains far from being acceptable, and further improvement is still needed. We also demonstrated that the prepared column can be used for the analysis of both acidic (ConA and OVA) and basic (lysozyme) proteins in egg white in a single run at physiological pH. Glycoisomers were also found in this complex matrix sample. The results indicate that the column has great potential for use in proteomic applications.

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