Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1025 (2004) 209–216
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Enrichment of proteinaceous materials on a strong cation-exchange diol silica restricted access material: protein–protein displacement and interaction effects

Olga Willemsen [a], Egidijus Machtejevas [b,*], Klaus K. Unger [b]

[a] *Organic Technology Department, Kaunas University of Technology, Radvilėnų pl. 19, 3028 Kaunas, Lithuania*
[b] *Institut fuer Anorganische Chemie und Analytische Chemie, Johannes Gutenberg-Universitaet, Duesbergweg 10-14, 55099 Mainz, Germany*

Received 6 February 2003; received in revised form 10 October 2003; accepted 16 October 2003

Abstract

A study of size exclusion and enrichment of proteins employing strong cation-exchange diol silica restricted access material (SCX-RAM) under saturation conditions is presented. Experiments were carried out with bacitracin, protamine, ribonuclease, lysozyme and bovine serum albumin as individual proteinaceous analytes as well as comprehensive binary mixtures and with human urine samples. Protein size dependent capacity features of the SCX-RAM column was observed. Bacitracin demonstrated the highest capacity followed by protamine while adsorption capacities of both ribonuclease and lysozyme were found smaller by a factor of 10. Applying binary protein samples occurring displacement effects were apparent: proteins with strong cationic properties displaced those already adsorbed by the bonded cation-exchange ligands. Bacitracin was displaced in all binary mixture experiments in particular by protamine. Furthermore, the binary mixtures displayed increased adsorption for some proteins due to complex formation. Lysozyme and ribonuclease showed double capacity values when paired with bacitracin. Both phenomena, displacement and enhanced adsorption occurred in the saturated state and led to changes in the urine composition during sample preparation. Injecting urine samples the relative proportions of fractions changed from 4 up to more than 20 times, due to the differences of the protein adsorption capacities on the SCX-RAM column. Analysing urine samples the SCX-RAM column provided extensive long-term stability.

Keywords: Restricted access media; Sample preparation; Proteins; Peptides

1. Introduction

The analysis of complex biological samples such as plasma, serum, saliva, urine, etc., requires robust, precise and fast methods [1–7]. The success of such analyses is largely dependent on the quality of the sample preparation [4].

During the last two decades, a variety of new sample preparation techniques have been developed [8,9]. Among them, efficient solid phase extraction and pre-column techniques have become popular due to their high analyte recoveries and reproducibility, together with the advantage of automation [8,10].

Restricted access materials (RAMs) have been developed for the clean-up of biological samples; in particular, RAMs have been used for the separation of large biomolecules and extraction of the low-molecular weight analytes. A classification of different types of RAMs has been made by Boss and Rudolphi [11] according to the working principle. Practical aspects of using RAM as pre-column materials have also been discussed [12,13].

In this work, a RAM was tested with external diol groups and internal sulphuric acid groups abbreviated below as SCX-RAM (SCX: strong cation-exchange). This silica-based RAM possesses pores of 6 nm with a molecular weight (M_r) exclusion limit of approximately 15,000. Thus, when a complex biological sample is injected into such a RAM column, the charged analytes small enough to penetrate the pores will be trapped and enriched on the cation-exchange surface, while the large molecular weight constitutes will be excluded with the dead volume of the column.

Račaitytė et al. [14] have shown that this type of RAMs is highly suitable for on-line extraction of the target peptides

* Corresponding author. Tel.: +49-6131-3925927; fax: +. +49-6131-3922710.
E-mail address: egidijus@ak-unger.chemie.uni-mainz.de (E. Machtejevas).

0021-9673/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2003.10.072

for their analysis in complicated biological samples (i.e. plasma). Račaitytė et al. [14] coupled a SCX-RAM column to an HPLC–electrospray ionisation MS system. The plasma samples were injected without pre-treatment into this system. High sensitivity and short analysis time were the advantages of the developed method.

Wagner et al. [15] have also demonstrated that this SCX-RAM can be successfully employed for the separation of the low-molecular-weight proteinaceous fraction of biofluids in order to obtain their protein map. A SCX-RAM column was incorporated for this purpose on-line into a chromatographic system. Extracted samples were separated on two type HPLC columns and analysed off-line by MS. Such a chromatographic system is useful for proteomics due to the fast analysis and high resolving power of small proteins ($M_r < 15{,}000$) and can therefore complement the two-dimensional gel electrophoresis.

An analogue RAM to the one used in this work but with alkyl functionalities on the inner surface (alkyldiol silica, ADS), was characterised by several authors. Schaefer and Lubda [16] stressed that the ADS column is a powerful tool for the fast and efficient sample clean-up in an on-line mode, which allows one to fully automate the chromatographic system.

Mišl'anova and Hutta [17] investigated the influence of direct injections of different biological samples onto the performance of an HPLC system incorporating an ADS and an analytical RP column. They observed that on-line extraction of chiral drugs with the ADS column decreases the retention times of the analytes up to 25% and increases asymmetry factors of the peaks by up to 5%. Nevertheless, peak areas and selectivity remain constant.

Yu et al. [18] compared the chromatographic behaviour of an ADS and a semipermeable surface (SPS) precolumns in the course of direct plasma injections. Both types of the RAM demonstrated long life-times when they were properly washed.

The objective of this study is to investigate the protein size exclusion and enrichment of low molecular weight proteinaceous samples on the SCX-RAM. Phenomena which occur in the SCX-RAM columns at high loads, i.e., protein–protein displacement and protein–protein interactions are presented. Standard proteins and peptides of different molecular weights were chosen to investigate these phenomena by frontal analysis. In addition, urine samples were utilised to monitor these effects performing peptide mapping.

2. Experimental

2.1. Chemicals

Potassium dihydrogenphosphate, orthophosphoric acid (85%), sodium chloride and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Acetonitrile was of HPLC grade (Fisher Scientific, Loughborough, UK). Water was prepared with a water purification system model Milli-Q (Millipore, Bedford, MA, USA). Bovine serum albumin, initial fractionation by heat shock, was purchased from Sigma–Aldrich (Steinheim, Germany); lysozyme, from chicken egg white and protamine, from salmon, were obtained from Sigma (St. Louis, MO, USA); bacitracin was purchased from Serva (Heidelberg, Germany); Ribonuclease, from bovine pancreas, was purchased from Merck. All samples were filtered through Millex-HV filter units of 0.45 μm pore diameter (Millipore). All mobile phases were degassed by sparging with helium prior to use.

The SCX-RAM (25 μm particle diameter and about 6 nm pore diameter) was a research product provided by D. Lubda (LSP MDA, Merck). It was packed into 25 mm × 2 mm and 4 mm i.d. stainless steel columns.

2.2. Instrumentation

A two-dimensional HPLC system, composed of an isocratic and a gradient system equipped with a diode array detector with the loop-transfer between the dimensions, was set-up (Fig. 1). A micro-pump (Bischoff 2200 Micropump, Leonberg, Germany) was connected to a six-port injection valve (Model 7125, Rheodyne, Berkeley, CA, USA) equipped with a 5 ml injection loop for introducing the protein and salt solutions. The injection valve was connected to the SCX-RAM column (25 mm × 2 mm i.d.), which was in turn connected to a six-port switching valve (Model 7000, Rheodyne). The switching valve was equipped with a 20 μl loop. The eluate of the SCX-RAM column was transferred

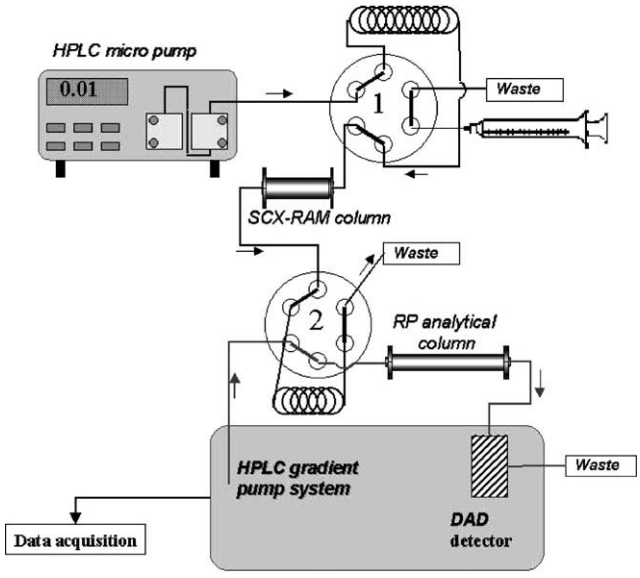

Fig. 1. A scheme of the two-dimensional chromatographic set-up for RP analyses of fractions of the SCX-RAM column eluate during its saturation and elution: position 1 of the switching valve: SCX-RAM loading and washing as well as analysis of the sample on the RP column were performed. In the picture: 1, injection valve; 2, switching valve.

by the isocratic pump via the switching valve to the waste either through the 20 μl loop (position 1 of the valve 2) or directly (position 2 of the valve 2).

The gradient system (HP 1090 system of Agilent, Waldbronn, Germany) was also connected to the six-port switching valve. Its eluents were transferred to a Chromolith SpeedROD RP-18e column (50 mm × 4.6 mm i.d.; Merck) either directly (valve 2, position 1) or via the injection loop (valve 2, position 2). In time the valve was switched from position 1 to position 2, a fraction of the SCX-RAM eluate from the loop was transferred onto the Chromolith SpeedROD RP-18e. After a few seconds, the valve was switched back for the loop loading while the transferred fraction was analysed on the Chromolith SpeedROD RP-18e.

2.3. Chromatographic conditions

2.3.1. Saturation of the SCX-RAM column

The SCX-RAM column was equilibrated with potassium phosphate buffer (15 mM, pH 3). A solution of a single protein or a mixture of two proteins prepared in the same buffer was pumped through the column at a constant flow-rate of 0.3 ml/min. Once the front of the sample solution had reached a plateau, the non-adsorbed sample was washed from the column with the buffer. After completion of the washing, the adsorbed proteins were eluted with a 1 M sodium chloride solution in the phosphate buffer at a flow-rate of 0.1 ml/min. After introduction of the salt solution, the isocratic pump was stopped every 30 s (0.05 ml) to transfer a fraction of the SCX-RAM column eluate to the Chromolith SpeedROD RP-18e column for analysis. After analysis of the fraction and column equilibration, elution of the proteins from the SCX-RAM column was continued. After 0.5 ml was eluted from the SCX-RAM column, the pump was stopped in time corresponding to 0.6, 0.7 and 1.0 ml of elution volume, respectively. After this point, RP analysis was performed every 5 min (0.5 ml) without interrupting the protein elution from the SCX-RAM column.

Adsorptions of individual proteins were studied as well as of mixtures of two proteins at equal concentrations (see Table 1).

2.3.2. RP analysis of the SCX-RAM column eluate

Analyses were performed on a Chromolith SpeedROD RP-18e column, using 0.1% TFA in water (solution A) and 0.1% TFA in acetonitrile (solution B) at a flow-rate of 2 ml/min. The gradient programmes chosen for each experiment are described in Table 1. After each run the Chromolith SpeedROD RP-18e column was washed for 0.5 min with solution B and then reconditioned with the initial composition of the mobile phase. UV detection was performed at 214 and 275 nm wavelengths.

RP analyses of the SCX-RAM column eluate were performed during both its saturation and the elution steps. The concentrations of the proteins in the SCX-RAM eluate were calculated from the peak areas and then plotted versus the volume of the eluate (Fig. 2). The amounts of the proteins adsorbed on the SCX-RAM column were calculated by integrating these elution profiles. Applying this method, elution profiles for each protein in the mixture of two were obtained and the SCX-RAM column capacities for each protein separately were calculated.

Table 1
Concentrations of the protein solutions used for saturation of the SCX-RAM column and programmes of the linear gradients used for the RP analysis of the column effluents

Protein(s)	Concentration (mg/ml)	Gradient conditions[a]
BSA	0.5	0 min: 30% B; 2 min: 45% B
BSA-LYS	1.0	0 min: 34% B; 2 min: 45% B
BSA-RNase	1.0	0 min: 25% B; 2 min: 50% B
BSA-PROT	1.5	0 min: 7% B; 2.5 min: 45% B
BSA-BAC	1.5	0 min: 23% B; 3 min: 45% B
LYS	1.0	0 min: 34% B; 2 min: 45% B
LYS-RNase	1.0	0 min: 25% B; 1 min: 33% B; 3 min: 40% B
LYS-PROT	1.5	0 min: 7% B; 2 min: 45% B
LYS-BAC	1.5	0 min: 25% B; 0.1 min: 30% B; 2 min: 33% B; 3.5 min: 40% B
RNase	1.0	0 min: 25% B; 1.5 min: 35% B
RNase-PROT	1.5	0 min: 7% B; 1.5 min: 40% B
RNase-BAC	1.5	0 min: 26% B; 3 min: 28% B[b]
PROT	1.5	0 min: 7% B; 2 min: 30% B
PROT-BAC	1.5	0 min: 7% B; 2 min: 45% B
BAC	1.5	0 min: 23% B; 2 min: 35% B

[a] RP analysis flow-rate was 2.0 ml/min. After each gradient analysis, the column was flushed with solution B for 30 s.

[b] Programme used for RP analysis during the SCX-RAM column saturation step. Since the concentration of material eluted from the SCX-RAM column was several times higher than that of loaded and peaks were overlapping, the gradient programme was prolonged from 3 to 5 min in order to obtain a base line separation.

2.3.3. Experiments with human urine

Ten times diluted, filtered urine samples were applied onto the SCX-RAM column at a flow-rate of 0.3 ml/min. After an appropriate washing they were transferred by a salt gradient to an analytical SCX column at a flow-rate of 0.5 ml/min (BioAssist S, 50 mm × 4.6 mm i.d., Tosoh Biosep, Stuttgart, Germany) and separated. A linear gradient was used from 0 to 50% of 1 M sodium chloride solution in the run buffer (19 mM sodium phosphate pH 2.5/methanol, 95:5, v/v) in 20 min, and then to 100% in the next 5 min. The analytical SCX column was reconditioned with the run buffer for 5 min. The SCX-RAM column was used in the same system analysing urine samples for the period of 1 month (~150 injections) with run-to-run reproducibility below 5% R.S.D. and below 10% R.S.D. for the relative fractions.

3. Results and discussion

Five proteins were chosen as model compounds: bacitracin (BAC), $M_r = 1420$, isoelectric point (pI) = 8.8, pro-

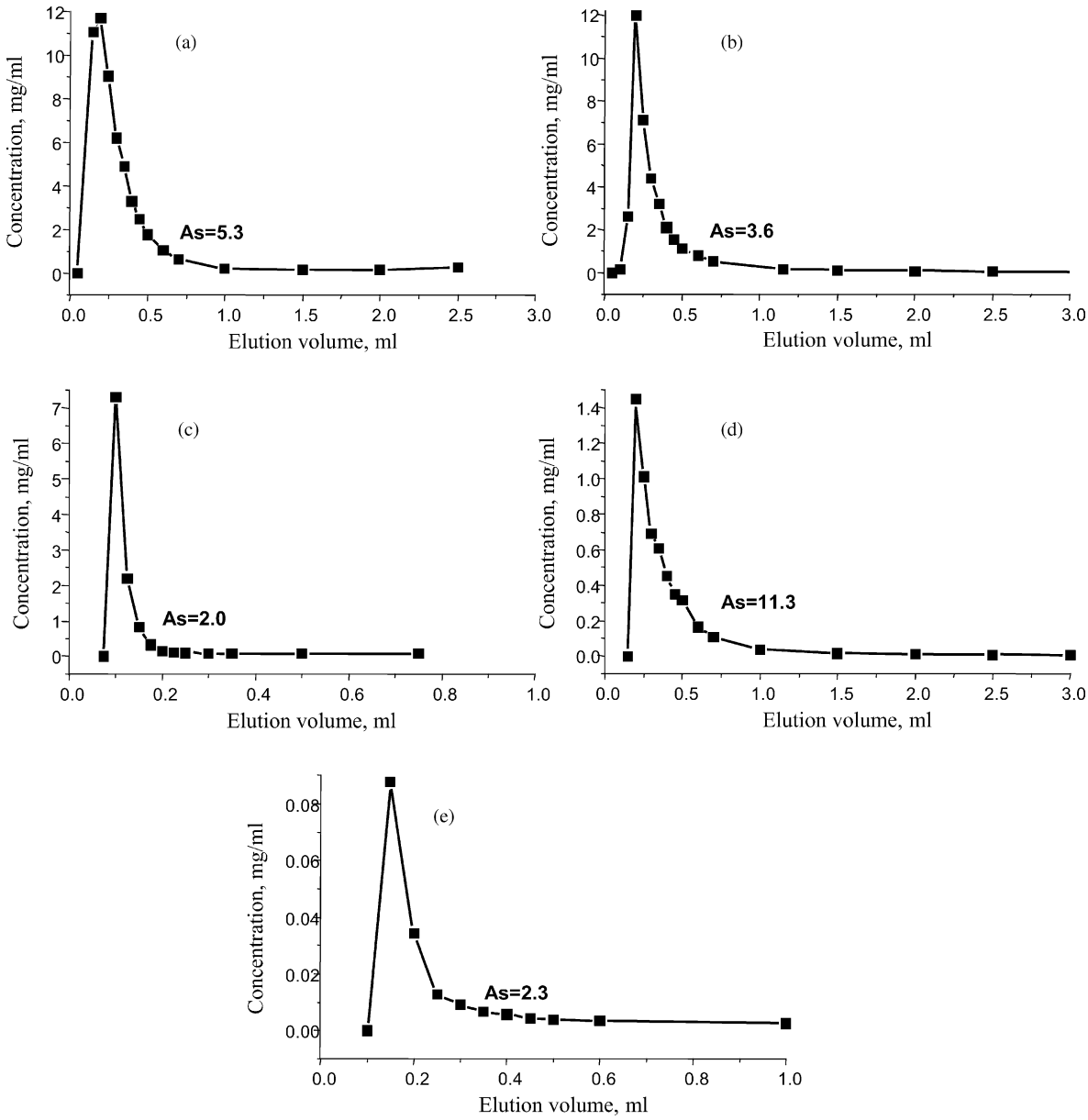

Fig. 2. Elution profiles of individual proteins on the SCX-RAM column: (a) BAC; (b) PROT; (c) RNase; (d) LYS; (e) BSA. Elution profiles were obtained by measuring the concentration of proteins in the effluent of the SCX-RAM column during the elution and plotting them versus the volume of the effluent. The asymmetry factors (A_s) of the profiles shown in the plots were calculated at 10% of their height. *Conditions*: The SCX-RAM column (2.5–2 mm i.d.) was saturated with a protein solution (see Table 1) at a flow-rate of 0.3 ml/min and after this washed with 15 mM phosphate buffer, followed by desorption with 1 M sodium chloride solution in the same buffer at a flow-rate of 0.1 ml/min. The elution profiles of the adsorbed protein were obtained performing RP-analysis of separate fractions of the SCX-RAM columns eluate during desorption and then plotting these results versus the volume of the eluate.

tamine (salmine) (PROT), $M_r = 4200$, p$I = 13.3$ (calculated for salmine A1), ribonuclease (RNase), $M_r = 13700$, p$I = 8.0$, lysozyme (LYS), $M_r = 14300$, p$I = 10.5$–11.0, and bovine serum albumin (BSA), $M_r = 68{,}000$, p$I = 4.8$ [19,20]. These proteins differ in their size and pI values. The smallest ones are BAC and PROT. RNase and LYS are proteins of similar molecular weight, which is close to the exclusion limit of the tested restricted access material ($M_r \sim$ 15,000). BSA was chosen as a model protein which should not penetrate the pores of the SCX-RAM.

The experiments were performed at pH 3. At such a low pH, the molecular structures of LYS and RNase are stable [21]. BSA changes its conformation at pH below 4 [22]; the molecule partly unfolds becoming thinner and longer (the hydrodynamic axial ratio of 9). Thus, more functional groups of the molecule, including the hydrophobic ones, become available for the interaction with the stationary phase and proteinaceous molecules. BAC is a dodecapeptide and does not possess a complicated structure [23].

Table 2
Basic properties of the proteins [20,23,24]

Protein	Amount of basic amino acids				Ratio N/M_r ($\times 10^{-3}$)
	Arg	His	Lys	Total (N)	
BAC	–	1	1	2	1.4
PROT	21	–	–	21	5
RNase	5	4	11	20	1.5
LYS	11	1	6	18	1.3
BSA	26	17	60	103	1.5

All the proteins are basic except BSA. PROT is the most basic among them (Table 2). All the proteins have positive net-charges at pH 3 and, they can be trapped electrostatically within the SCX-RAM pores. The negative charges of the proteins are negligible at this pH since the pK_a of the acidic amino acids are about 4.5 [25].

3.1. Loading and elution of solutions of individual proteins on the SCX-RAM column

As is evident from the data presented in Table 3, BAC shows the highest capacity on the SCX-RAM column followed by PROT. The adsorption capacity of both RNase and LYS is by a factor of 10 smaller than that of the small proteins. The adsorption of BSA is negligibly small, as expected. The same was found concerning ADS [25]. The fact that BSA unfolds below pH 4 could lead to the possibility that the molecule partly enters a pore and interacts with the cation-exchange groups of the inner surface of the RAM.

Fig. 2 displays the elution profiles of the individual proteins obtained by eluting them with a 1 M NaCl solution in a phosphate buffer at pH 3 after saturation of the SCX-RAM column. Substantial tailing is observed as monitored by the asymmetry factor when measured at 10% of their peak height. The asymmetry factor appears to be a function of the protein capacity as well as the affinity of the protein to the SCX-RAM surface. It increases proportionally with the protein capacity (i.e., from BSA and RNase to PROT and further to BAC). Nevertheless, in the case of RNase and LYS, though the areas of these two profiles are almost equal, the RNase profile maximum is about five times higher than that of LYS and the asymmetry factor of the LYS profile is more than 5.5 times higher as compared to the profile of RNase.

Table 3
Capacity of different proteins on the SCX-RAM as a function of their molecular weight

Protein	M_r	SCX-RAM capacity	
		mg/ml	mmol/ml
BAC	1420	107.6	75.8
PROT	4200	71.0	16.9
RNase	13700	10.1	0.73
LYS	14300	10.2	0.71
BSA	68000	0.3	0.004

Conditions: The SCX-RAM column (2.5–2 mm i.d.) was saturated with a protein solution (see Table 1) at a flow-rate of 0.3 ml/min and after this washed with 15 mM phosphate buffer, followed by desorption with 1 M sodium chloride solution in the same buffer at a flow-rate of 0.1 ml/min. The amount of the adsorbed protein was determined performing RP-analysis of separate fractions of the SCX-RAM columns eluate during desorption, then plotting these results versus the volume of the eluate and integrating the obtained elution profiles.

The LYS (pI = 10.5–11) elution profile broadening may be explained by a stronger binding when compared to RNase (pI = 8), which depends on the number of charged functional groups attaching the protein to the SCX-RAM inner surface, the Z-value [26,27]. Another reason for the band broadening could be due to the unfolding of the LYS molecules. Some proteins can unfold upon interaction with ion-exchange supports and, therefore, attach more strongly to the surface via an increased number of accessible functional groups [28–31].

Sane et al. [32] examined the chromatographic behaviour of LYS and RNase at the surfaces of stationary phases. They found that rigid RNase molecules are stable and there was little perturbation of their secondary structure on a strong cation-exchanger. LYS, in contrast, unfolds adsorbed on the stationary phase. The unfolding becomes stronger under the column saturation conditions. This occurs due to intermolecular interaction or even aggregation of the protein [32].

Protein molecules tend to unfold when their residence time on ion-exchange columns is prolonged; this is accompanied by peak broadening [29,30,32,33]. This may be also the case in the present study, because loading and washing of the SCX-RAM column before the elution took approximately 20–30 min.

3.2. Loading and elution of protein mixtures on the SCX-RAM column

By knowing the SCX-RAM column capacities for the single proteins, one could assume that in binary mixtures they will decrease to halves. The following experiments show that in reality the picture became much more complicated due to the difference of the natures of the protein.

3.2.1. Proteins of the size close to the exclusion limit of the RAM: protein–protein interactions

RNase and LYS exhibited a similar behaviour in pairs with other proteins and peptides under the SCX-RAM column saturation conditions (Fig. 3). In pairs with BSA, the amount of adsorbed LYS and RNase decreased significantly (see Section 3.2.3). In the other experiments, the capacity of the SCX-RAM showed an increase. When the sample contained both LYS and RNase, the amount of adsorbed RNase was 47% of its SCX-RAM capacity, for it whereas the amount of LYS dropped only 14% compared to its SCX-RAM capacity. In the experiment with the pair RNase/PROT, the adsorbed amounts of both proteins were about 80% of their SCX-RAM capacities. In pairs with BAC, both LYS and RNase were adsorbed in amounts significantly higher than in

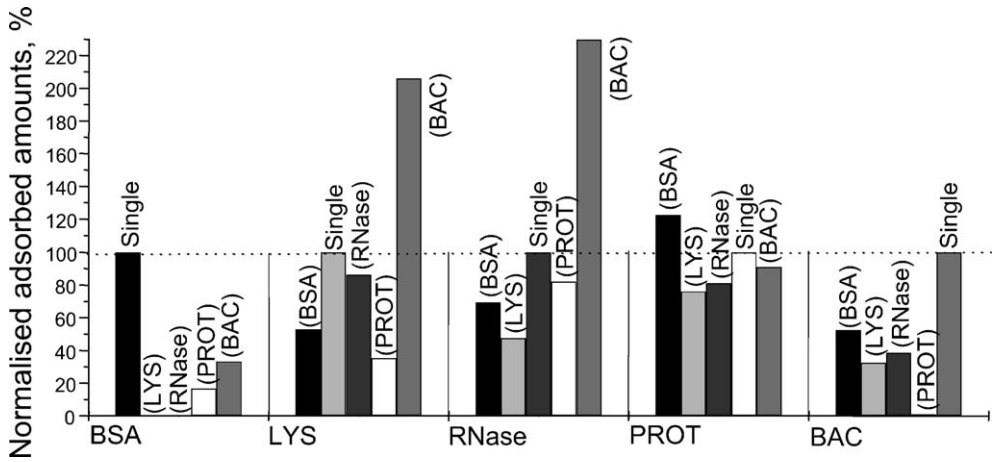

Fig. 3. The changes in adsorption capacity of the proteins in binary solutions on the SCX-RAM column. The relative amounts of the capacities are given in groups for each protein separately (abscissa axis). The second protein of the binary sample is written in brackets above the corresponding column. The capacities of the RAM for each protein alone were normalised to 100%. *Conditions*: The SCX-RAM column was saturated with a solution of an individual protein or a mixture of two proteins (see Table 1) at a flow-rate of 0.3 ml/min and after this washed with 15 mM phosphate buffer, followed by desorption with 1 M sodium chloride solution in the same buffer at a flow-rate of 0.1 ml/min. The amount of the adsorbed proteins was determined performing RP-analysis of separate fractions of the SCX-RAM columns eluate during desorption, then plotting these results versus the volume of the eluate and integrating the obtained elution profiles.

the experiments with the single proteins (2.1 and 2.3 times, correspondingly) (Table 4).

These experiments revealed the ability of the proteins to interact with other proteins or peptides already adsorbed on the surface. Such a phenomenon of protein–protein interaction was observed in preparative chromatography [34–38]. It is in the nature of proteins to form complexes [5,6], but in solution the probability of interaction is low. However, when the surface of a stationary phase is saturated, the concentration of proteins there is high. Then the proteins present in the solution can form complexes with the molecules already adsorbed on the stationary phase. Xu and Regnier [39] have shown that proteins can interact more strongly with molecules of different species than with their own. They prepared unmodified weak ion exchangers, as well as modified weak ion exchangers with different proteins. In their investigations, LYS especially showed a tendency for strong interaction with the proteinaceous adsorbed on the stationary phases.

Table 4
Comparison of individual protein capacity on SCX-RAM column (2.5–2 mm i.d.) to their capacity in pairs with BAC

Second protein	Capacity for single protein (mol)	Capacity for proteins in binary mixture (mol)		Excess of the second protein (mol)
		Second protein	BAC	
BSA	0.0014	0.0005	12.5	−0.0009
LYS	0.22	0.46	7.7	0.24
RNase	0.23	0.53	9.2	0.30
PROT	5.3	4.7	0.3	−0.6

Capacity for BAC alone on SCX-RAM column was 23.8 mol. *Conditions*: The SCX-RAM column was saturated with a solution of an individual protein or a mixture of two proteins (see Table 1) at a flow-rate of 0.3 ml/min and after this washed with 15 mM phosphate buffer, followed by desorption with 1 M sodium chloride solution in the same buffer at a flow-rate of 0.1 ml/min. The amount of the adsorbed proteins was determined performing RP-analysis of separate fractions of the SCX-RAM columns eluate during desorption, then plotting these results versus the volume of the eluate and integrating the obtained elution profiles.

3.2.2. Small proteins: protein–protein displacement

Under the SCX-RAM column overloading conditions PROT and BAC, the two smallest of the tested proteins, behaved very differently in the binary solutions with other proteins. The amount of adsorbed BAC in the presence of other proteins decreased compared to the capacity of the column for this peptide: 1.9, 2.6 and even 3.1 times in the experiments with BSA, RNase and LYS, respectively (Table 4). The amount of adsorbed PROT, in contrast, varied insignificantly when compared to the experiment with the single peptide (Fig. 3).

PROT interacted with SCX-RAM stronger than other tested proteins. It even displaced BAC when the column was saturated with a mixture of two; the amount of adsorbed BAC was almost 80 times lower than in the experiment with the single peptide. In the presence of PROT, the amount of adsorbed LYS was 35% of its column capacity. Such an effect of competitive adsorption and exchange processes at a surface is also observed for the blood proteins and is called the Vroman effect [40]. If the most abundant proteins interact weakly with the surface of the blood vessels they can be displaced by the less abundant but more strongly interacting proteins [40].

PROT has the highest affinity for the SCX-RAM, because this nuclear peptide is highly cationic (Table 2). Thus is due to the fact that Salmine A1, a PROT of Salmon, is built of 32 amino acids and contains 21 arginine residues (pK_a = 12) [20]. In contrast to bigger proteins, the PROT charges

are neither distributed over the large surface, nor they can be hidden within the folded molecule. Therefore, a larger number of its charges can interact simultaneously with the stationary phase.

Both PROT and BAC formed complexes with RNase and LYS (see previous section). Since the amount of PROT adsorbed on the SCX-RAM column varied insignificantly and was close to its column capacity, we suppose that PROT exchanged considerably also RNase and LYS on the SCX-RAM surface. But RNase and LYS could be adsorbed on the PROT molecules due to the protein–protein interaction. PROT also interacted with BSA, the amount of adsorbed PROT in the presence of BSA was 1.2 times higher than without it.

3.2.3. BSA: blocking the access to the SCX-RAM pores

Frontal analyses and desorption of BSA in pairs with LYS, RNase, PROT and BAC were performed. The amount of BSA adsorbed in pairs with PROT and BAC decreased approximately six and three times, respectively, compared to the SCX-RAM capacity for BSA alone (Fig. 3). In the experiments with LYS and RNase, the amount of adsorbed BSA was below the quantification limit.

Despite of the relatively low amount of BSA adsorbed on the SCX-RAM column, the adsorption capacities for the other proteins/peptides in its presence also decreased (Fig. 3): 1.9, 2 and 1.5 times in the case of BAC, LYS and RNase, respectively. Most probably, the large molecules of BSA block the entrance to the pores. As mentioned above the experiment with PROT was different due to protein–protein interaction.

3.2.4. Analysis of urine samples

Urine contains trace amounts of proteins and peptides originating from blood plasma, the kidneys and the urogenital tract. Excessive proteins in urine are usually indicative of disease and can be classified in various ways [41]. Therefore, urine has enormous potential for non-invasive detection of protein biomarkers for the diagnosis and clinical monitoring of human disease.

Cation-exchange chromatography of urine samples was performed after on-line extraction of the samples on the SCX-RAM column. Fig. 4 presents several chromatograms corresponding to different volumes of the urine samples. The profiles of the chromatograms varied depending on the loaded amounts of the samples. These chromatograms were relatively divided into fractions a, b and c, as shown in the Fig. 4. The relative areas of these peaks were in the ratio of 1:4:2.3 when injecting 250 μl of urine sample, 1:2.6:0.85 when injecting 90 μl of urine sample, and went down to 1:1.1:0.11 when 10 μl of human urine was loaded. The proportions changed from 4 up to more than 20 times because of the differences in the SCX-RAM column capacity for proteins of different size, probable protein complexation and displacement.

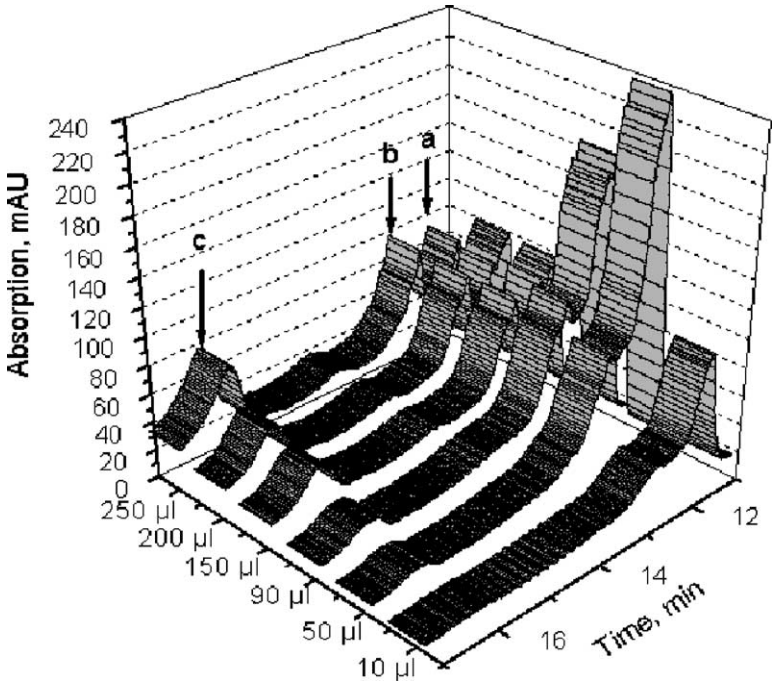

Fig. 4. The dependence of the chromatogram fractions peak heights on the amount of urine sample injected. *Conditions*: Ten times diluted urine samples were purified and preconcentrated on the SCX-RAM column at a flow-rate of 0.3 ml/min then transferred by salt gradient to the analytical SCX column at flow-rate of 0.5 ml/min (BioAssist S, 50 mm × 4.6 mm i.d., TOSOH BIOSEP GmbH, Stuttgart, Germany) and separated. A step gradient was used from 0 to 50% of 1 M sodium chloride in run buffer 19 mM sodium phosphate pH 2.5/methanol 5% (v/v) in 20 min, then increased to 100% in 5 min and reconditioned with the run buffer for 5 min. Detection was performed at 214 nm UV.

The probability of obtaining the real concentrations of the peptides and proteins increases with the decrease of the sample amount. Nevertheless, decreasing concentration further leads to the detection of the single sample components becoming impossible, as they disappear below the detection limit. In order to avoid the changes in protein concentrations during extraction of the urine samples on the SCX-RAM, the size of the column should be increased.

4. Conclusions

It has been demonstrated that a proteinaceous sample can undergo an alteration in its composition during the sample preparation step if the SCX-RAM column is overloaded. Thus, incorrect conclusions may be drawn from the analysis of such a sample.

Though the tested restricted access material has an exclusion limit of $M_r \sim 15{,}000$, its capacity for proteins under this limit depends on their molecular weight. Proteins of molecular weight M_r of 13,000–14,000 have capacities of ∼10 mg/ml of SCX-RAM, which is ∼10 times lower than the capacity for $M_r = 1000$ proteins. Therefore, the SCX-RAM columns become saturated much earlier with proteins of size close to $M_r = 15{,}000$, whereas smaller proteins continue to adsorb. Thus, the relative amounts of the bigger proteins on the SCX-RAM surface decrease and the relative amounts of the smaller ones increase.

Large proteins with a molecular weight several times higher than $M_r = 15{,}000$ can have some affinity to restricted access materials by partially penetrating the pores. Though the amount of the adsorbed large protein is small, it noticeably reduces the capacity of the SCX-RAM column, presumably by blocking access to the pores. The adsorbed proteins of M_r several times higher than 15,000 can also influence the sample clean up procedure by forming complexes with other proteins.

It was observed that on the strong cation-exchange surface of the restricted access material, proteins with strong cationic properties can displace ones with weaker cationic properties. Certain proteins tend to interact with others and, after the precolumn was saturated, they can still adsorb onto other proteins.

Since proteins have flexible structure elements, they can unfold upon interaction with the ion-exchange surface. The interaction between proteins and the surface then becomes stronger and they can be adsorbed irreversibly. Therefore, long residence times of unknown proteinaceous samples on the columns should be avoided.

Acknowledgements

We thank Mr. Dieter Lubda, LSP Merck KGaA, Darmstadt, Germany for supplying the research samples of SCX-RAM and Dr. Thomas Patrick Hennessy of University Mainz, Germany for his helpful and valuable discussions.

References

[1] K.L. Williams, Electrophoresis 20 (1999) 678.
[2] A. Padney, M. Mann, Nature 405 (2000) 837.
[3] F. Regnier, A. Amini, A. Chakraborty, M. Geng, J. Ji, L. Riggs, C. Sioma, S. Wang, X. Zhan, LC–GC Int. 19 (2001) 200.
[4] F. Lottspeich, Angew. Chem. Int. Ed. 38 (1999) 2476.
[5] C. Gavin, M. Bösche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, et al., Nature 415 (2002) 140.
[6] Y. Ho, A. Gruhler, A. Heilbut, G.D. Bader, L. Moore, S.-L. Adams, et al., Nature 415 (2002) 180.
[7] A. Kumar, M. Snyder, Nature 415 (2002) 123.
[8] M. Gillar, E.S.P. Bouvier, B.J. Compton, J. Chromatogr. A 909 (2001) 111.
[9] G. Theodoridis, I.N. Papadoyannis, Mickrochim. Acta 136 (2001) 199.
[10] D.J. Anderson, Anal. Chem. 65 (12) (1993) 434R.
[11] K.-S. Boss, A. Rudolphi, LC–GC Int. 15 (1997) 602.
[12] K.-S. Boos, C.-H. Grimm, Trends Anal. Chem. 18 (3) (1999) 175.
[13] R.E. Majors, K.-S. Boos, C.-H. Grimm, D. Lubda, G. Wieland, LC–GC Int. 14 (1996) 554.
[14] K. Račaitytė, E.S.M. Lutz, K.K. Unger, D. Lubda, K.S. Boos, J. Chromatogr. A 890 (2000) 135.
[15] K. Wagner, T. Miliotis, G. Marko-Varga, R. Bischoff, K.K. Unger, Anal. Chem. 74 (2002) 809.
[16] C. Schaefer, D. Lubda, J. Chromatogr. A 909 (2001) 73.
[17] C. Mišl'anova, M. Hutta, J. Chromatogr. B 765 (2001) 167.
[18] Z. Yu, D. Westerlund, K.-S. Boos, J. Chromatogr. B 704 (1997) 53.
[19] S. Budavary (Ed.), The Merck Index, 11th ed., Merck & Co., Whitehouse Station, NJ, 1989, pp. 888, 1253, 1305, 1325.
[20] http://us.expasy.org/sprot/.
[21] Y. Liu, M. Sturtevant, Biochemistry 35 (1996) 3059.
[22] G.-L. Friedli, Doctoral Thesis, 1996, http://www.friedli.com/research/PhD/PhD.html.
[23] D. Voet, J.G. Voet, C.W. Pratt, Fundamentals of Biochemistry, upgrade ed., Wiley, New York, 2002, p. 487.
[24] PDB, http://www.pdb.
[25] E. Karlsson, L. Rydén, J. Brewer, in: J.-C. Janson, L. Rydén, Protein Purification, VCH, New York, 1989, p. 428.
[26] C. Bratt, C. Linberg, G. Marko-Varga, J. Chromatogr. A 909 (2001) 279.
[27] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, F.E. Regnier, J. Chromatogr. 266 (1983) 3.
[28] M.A. Rounds, F.E. Regnier, J. Chromatogr. 283 (1984) 37.
[29] W. Kopaciewicz, M.A. Rounds, F.E. Regnier, J. Chromatogr. 318 (1985) 157.
[30] X.-M. Lu, A. Figueroa, B.L. Karger, J. Am. Chem. Soc. 110 (1988) 1978.
[31] S.C. Goheen, B.M. Gibbins, J. Chromatogr. A 890 (2000) 73.
[32] S.U. Sane, S.M. Cramer, T.M. Przybycien, J. Chromatogr. A 849 (1999) 149.
[33] S.C. Goheen, J.L. Hilsenbeck, J. Chromatogr. A 816 (1998) 89.
[34] R.C. Castels, C.B. Castels, M.A. Castillo, J. Chromatogr. A 775 (1997) 73.
[35] Y.-L. Li, N.G. Pinto, J. Chromatogr. A 658 (1994) 445.
[36] A. Velayudhan, Cs. Horváth, J. Chromatogr. A 663 (1994) 1.
[37] S.R. Gallant, A. Kundu, S.M. Kramer, J. Chromatogr. A 702 (1995) 125.
[38] Y.-L. Li, N.G. Pinto, J. Chromatogr. A 702 (1995) 113.
[39] W. Xu, F.E. Regnier, J. Chromatogr. A 828 (1998) 357.
[40] C.H. Bamford, S.L. Cooper, T. Tsuruta (Eds.), The Vroman Effect, VSP, Zeist, The Netherlands, 1992, p. 192.
[41] T. Marhall, K.M. Williams, Anal. Chem. Acta 372 (1998) 147.