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Optimization of peptide and protein separation with a monolithic reversed-phase column and application to arsenic-binding studies

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ABSTRACT

A separation method for a mixture of eight sulfur-containing peptides and proteins characterized by a wide molar mass (1–18.4 kDa) and pI range (4.5–10.7) was developed onto a monolithic phenyl phase. Based on the first optimization steps that revealed an increase of the acetonitrile content to 45 vol.% as sufficient for the elution of all biomolecules and the addition of the ion pairing reagent trichloroacetic acid (TCA) as preferable over the eluent additives formic acid or ammonium acetate buffer, the critical variables TCA concentration, gradient time, and eluent flow rate were optimized using a Box-Behnken experimental design. To achieve optimum values for separation factors of all peak pairs, a TCA content of 0.025% (m/v), a gradient time of 10 min, and a flow rate of 3.5 mL min^{-1} were selected. Arsenic binding studies were undertaken under conditions optimized with respect to the crucial separation factor of the nonapeptides vasotocin (Vtc) and vasopressin (Vpr) in a shortened gradient time of 7.5 min. A complete separation of phenylarsenic-substituted and unmodified forms of these peptides allowed the calculation of both consumptions and apparent equilibrium constants *K* from HPLC–UV peak areas. The nonapeptide consumptions by the reaction with phenylarsine oxide (PAO) increased from 7% up to 100% in dependence on the molar ratio of the reaction components. Due to an enhanced UV absorption of the phenylarsenic-substituted biomolecules, the calculation of apparent equilibrium constants led to increasing *K* values with rising PAO molarities from 9.6×10^5 to 1.2×10^8 in case of Vtc and from 2.2×10^6 to 1.4×10^9 in case of Vpr. For α-lactalbumin, a consumption of $59.2 \pm 6.1\%$ by the reaction with molar excesses of PAO varying from 1.4 to 21 can be derived from the chromatograms. The quantitative evaluation of the reaction of the small protein aprotinin with PAO was hindered by a pronounced peak broadening that occurred after reduction of the disulfide bridges.

1. Introduction

A quantitative analysis of binding equilibria possesses a high significance for toxicological and pharmaceutical questions because the quantitative part of a biomolecule population can be determined which is affected by binding to environmental pollutants or drugs. Therefore, binding constants are regarded as fundamental characteristics for toxicity and efficiency of biochemically active compounds. Various analytical techniques offer the possibility for quantitative measurements of biomolecule–ligand interactions [1] including separation techniques such as capillary electrophoresis (CE) and liquid chromatography (LC), mass spectrometric techniques in combination with soft ionization variants such as electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), measurements of the surface plasmon resonance (SPR), the nuclear magnetic resonance (NMR) and the circular dichroism (CD) as well as potentiometric and microcalorimetric titrations and some others (see Ref. [1]). Sometimes, especially for rather complex interaction systems, binding constants are difficult to determine [2]. In such cases, consumption numbers which describe the degree of the binding formation provide more reliable data concerning the binding equilibria. One possibility to estimate the consumption of a biomolecule by a reaction with a drug or poison consists in the chromatographic separation of the unreacted biomolecule from its modified reaction product. Further, LC-based methods show some advantages such as analysis in the solution phase, relatively low costs when using UV detection, and they offer the possibility to investigate samples containing several biomolecules in mixtures. Further, low biomolecule concentrations are sufficient to perform binding experiments by means of LC. Up to now, LC-based binding studies are restricted to size exclusion columns. In the diverse techniques, either both interacting species, namely ligand and protein, or one of them, were included into the eluent solution [3,4]. Therefore, these size exclusion chromatographic (SEC) analyses are restricted to one biomolecule–ligand pair. Mixtures of

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biomolecules cannot be investigated in this way regarding their binding properties. SEC provides more native separation conditions than reversed phase liquid chromatography (RP-LC) but the low separation performance was not sufficient to completely differentiate reactants and products emerging from arsenic reactions with different sulfur-containing biomolecules [5]. In order to get distinct signals of the original unreacted proteins and their arsenic-substituted forms for quantitative evaluation of the binding equilibria, a coupling of SEC to ESI-MS was established [5]. However, mass spectrometric binding studies are complicated by some drawbacks such as the transfer of the analytes in the gas phase, competing effects in the ionization process, and unknown ionization efficiencies for protein–ligand complexes. In comparative investigations, large differences in binding constants determined by means of the SEC–ESI-MS coupling and by direct ESI-MS measurements without chromatographic coupling were observed [5,6]. Because reversed phase liquid chromatography exhibits a much higher chromatographic resolution for peptide and protein separations, this type of separation techniques was now implemented for qualitative and quantitative analysis of arsenic-binding biomolecules. Cysteine-containing peptides and proteins were chosen for which condensation reactions took place with an organic arsenic compound under reducing conditions [5,6]. The nonapeptides vasotocin (Vtc, M = 1.05 kDa, pI ≈ 9.84), vasopressin (Vpr, M = 1.084 kDa, pI ≈ 9.84), and oxytocin (Otc, M = 1.007 kDa, pI ≈ 6.01) possess one structure-stabilizing disulfide bridge each, whereas the small protein aprotinin (Apr, M = 6.5 kDa, pI = 10.5) exhibits three constitutive disulfide bridges. The milk proteins α-lactalbumin (α-Ltb, M = 14.2 kDa, pI = 4.5) and β-lactoglobulin (β-Ltg, M = 18.4 kDa, pI = 5.28) have eight and four cysteine residues linked to four and two disulfide bridges, respectively. α-Ltb pertains to the group of metalloproteins and binds Ca and Zn ions amongst others. In the native structure of β-Ltg, an additional non-reactive thiol group is located in a hydrophobic slot. Two additional proteins, cytochrome c (Cyt c, M = 12.4 kDa, pI ≈ 10.0–10.5) and lysozyme (Lys, M = 14.6 kDa, pI = 10.7), for which no arsenic interactions could be detected using ESI-MS were included in the samples as inalterable reference points.

Slow diffusional mass transfer rates especially for larger molecules like peptides and proteins within the stagnant mobile phase inside the pores of conventionally packed columns constrain fast and efficient LC separations [7]. In contrast, the analyte mass transfer in mesopores of monolithic columns is driven by convection and the mobile phase can unimpededly pass through the macropores both enabling high separation efficacies due to diminished peak broadening and short separation times due to low flow resistance. For instance, a mixture of five standard proteins was separated at a flow rate of 10 mL min^{-1} in less than 30 s [7]. For these reasons, a monolithic reversed phase chromatographic column was chosen in the current work instead of a packed column. A monolithic column composed of an organic polymer (polystyrene–divinylbenzene, PS–DVB) was preferred over a silica-based monolith because organic monoliths are better suited for separation of macromolecules whereas silica monoliths are favored for separation of smaller analytes [8]. A 30% higher separation performance for peptides and an increased recovery were achieved on a PS–DVB monolithic capillary column compared to a packed capillary with pore sizes of 5 μm [9]. Similarly, silica monoliths provided superior separation efficiencies at simultaneously reduced analysis times in comparison to packed capillaries [10] and were successfully employed for separation of polypeptide mixtures as well as of very complex tryptic protein digests [11–13]. A further advantage of monolithic materials consists in their easier fabrication opposite to their particulate counterparts [14]. As an alternative to the monoliths, long columns packed with pellicular stationary phases with large particle diameters generate very high peak capacities for tryptic digest peptides [15]. Similarly to monolithic materials, the effect of a faster mass transfer is exploited to maximize peak capacities.

In the current study, the method development was focused on the optimization of the separation of a mixture containing the eight selected peptides and proteins characterized by a wide molar mass range (1–18.4 kDa) as well as a wide pI range (4.5–10.7) onto the monolithic phenyl phase by varying the course of the gradient elution, the flow rate, and the addition of an ion pairing reagent. This simultaneous analysis of both smaller peptides and larger proteins in one chromatographic run on a phenyl stationary phase poses a novelty because no comparable methods could be found in the literature. The resulting RP-LC separation method was then applied for the separation of reaction mixtures containing the original peptides and proteins as well as their arsenic-substituted reaction products. From these measurements, quantitative data concerning the binding equilibria were deduced and critically discussed.

2. Experimental

The monolithic reversed phase column ProSwift® RP-2H, 4.6 × 50 mm, filled with a PS–DVB polymeric support material coated with a phenyl phase (Dionex, Sunnyvale, CA), was assembled in an Agilent 1100 series liquid chromatograph equipped with a binary pump, a degasser, autosampler, and a variable wavelength detector. Eluents were vacuum-filtrated through cellulose acetate membrane filters (pore size 0.45 μm, Whatman GmbH, Germany) or polyamide filters (grade: 290, Sartorius AG, Germany) in case of enlarged acetonitrile (ACN) content because cellulose acetate was unstable in presence of high ACN concentrations. The filtrated eluents were degassed in an ultrasonic bath for 20 min.

All proteins were purchased as lyophilized powders in a purity ≥ 85% from Sigma–Aldrich (Steinheim, Germany). The nonapeptides were obtained as acetate salts in a purity ≥ 97% from Sigma–Aldrich as well. 10 mg mL^{-1} stock solutions of peptides and proteins in deionized water were stored at −18 °C. For HPLC measurements, 0.5 mg mL^{-1} and 0.1 mg mL^{-1} dilutions for single component measurements and for mixed samples, respectively, were prepared freshly in deionized water. According to our previous work [6,16], for arsenic binding studies, the biomolecules were reduced with tris(carboxyethyl)phosphine (TCEP, purchased as 0.5 M hydrochloride solution from Sigma–Aldrich). For mixed samples, the peptides and proteins were incubated for 10 min with a 10-fold molar excess of the reducing agent related to the sum concentration of all biomolecules present in the sample in order to ensure a complete reduction and therewith to obviate an impact of the reduction degree on the arsenic–thiol reaction. For further 10 min, the reduced biomolecules were incubated with different amounts of the organic arsenic compound phenylarsine oxide (PAO, ≥97% purity from Sigma–Aldrich). 25 μL of the samples were injected into the LC system. A detection wavelength of 215 nm was chosen.

Chemicals needed for eluent preparation were purchased from Merck, Darmstadt, Germany (formic acid and acetic acid, both in p.a. quality), J. T. Baker B.V., Deventer, Holland (ammonium acetate, for HPLC), Sigma–Aldrich, Steinheim, Germany (trichloroacetic acid, >99.5%), or from VWR, Darmstadt, Germany (acetonitrile, HPLC gradient grade). The pH values in the different eluents were measured in the aqueous phase with a HI 9321 microprocessor pH meter (Hanna Instruments).

The peptide and protein separation was optimized by variation of the water–acetonitrile eluent composition which comprised the variation of the formic acid content between 0.01 and 0.5 vol.%, the addition of 50 and 100 mM ammonium acetate buffer providing pH 3.5 or 4.5, and the addition of different amounts (0.01–0.1%

m/v) of trichloroacetic acid. Type and duration of gradient elution were modified and the flow rate was varied between 0.5 and 4 mL min^{-1}.

After completion of the most important optimization steps regarding the eluent composition, a Box-Behnken experimental design was created using STATGRAPHICS® Centurion XV in which three main factors, namely TCA concentration, gradient time, and flow rate, were varied over three steps in a total of 15 measurements to gain optimum parameters for the selected chromatographic target value of separation factors of all pairs of adjacent peaks. For evaluation of the experimental design and finding out the optimum parameters regarding the separation factors, response area diagrams were created which describe the desirability (values between 0 and 1) as a measure for the optimum in dependence on the determining factors. Because several separation factors of various adjacent peaks had to be optimized, the individual target functions were summarized in one complex target function referred to as desirability reflecting the geometric mean of all individual target functions.

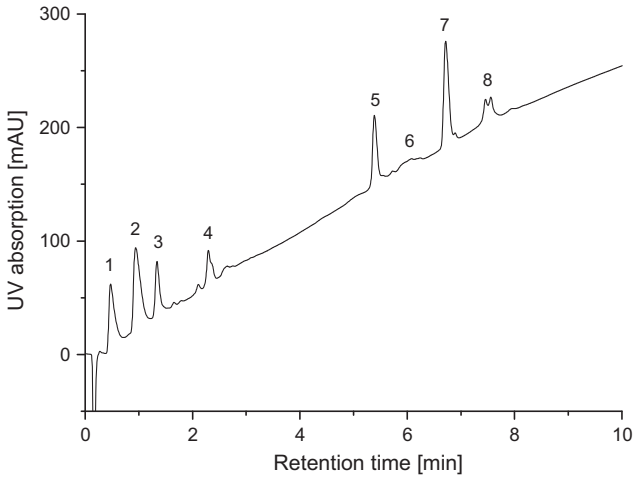

Fig. 1. Separation of an eight-component peptide–protein mixture on a monolithic phenyl phase under optimized elution conditions concerning the separation factors. Mobile phase A: H_2O/ACN (95/5, v/v); B: H_2O/ACN (5/95, v/v); +0.025% (m/v) TCA in A and B. Linear gradient from 1% B to 50% B in 10 min. Flow rate: 3.5 mL min^{-1}. *Components*: (1) vasotocin, (2) vasopressin, (3) oxytocin, (4) aprotinin, (5) cytochrome c, (6) lysozyme, (7) α-lactalbumin, and (8) β-lactoglobulin.

3. Results and discussion

3.1. Optimization of the combined peptide and protein separation on the phenyl phase of a monolithic column

Under starting conditions chosen for optimization of the combined peptide and protein separation (eluent A: 94.9% H_2O/5% ACN/0.1% HCOOH, v:v:v; eluent B: 4.9% H_2O/95% ACN/0.1% HCOOH, v:v:v; linear gradient elution from 1% B to 75% B in 30 min; flow rates of 0.5 or 2 mL min^{-1}) a coelution of the nonapeptides, a strong tailing of aprotinin, and an incomplete separation of Cyt c and Lys were observed. It was found that it suffices for the elution of all proteins to increase the portion of eluent B to 50% instead of 75%. A slight improvement of the nonapeptide separation was achieved by inserting a stage holding 1% B at the beginning of the gradient elution from 0 to 3 min. In contrast, the separation of the proteins eluting in the middle retention time area, Cyt c, and Lys, could not be improved by inserting a second stage between 10 and 11 min holding 35% B. A pH change from 3.2 to 2.3 in the aqueous phase of eluent A caused by varied formic acid content from 0.01 to 0.5 vol.% delivered no enhancement of the separation efficiency.

The addition of a buffer component (50 and 100 mM ammonium acetate, pH 3.5 and 4.5) to the eluents deteriorated the separation performance compared to the addition of formic acid. At this point it must be kept in mind that the pH value which was measured in the aqueous phase of the eluents changes upon addition of an organic modifier such as ACN or methanol [17,18]. Therefore, the ionization degree of the analytes which occurs during the LC separation cannot be correctly derived from the mentioned pH values of the aqueous eluent phases. The efficiency of peptide and protein separation was considerably improved by the presence of >0.01% (m/v) TCA instead of HCOOH in both eluents.Because the separation effect in RP-LC is based on differing hydrophobic properties of the analytes, an increased hydrophobicity, which is achieved by the binding of an ion-pairing reagent on ionic side chains of the biomolecules, can improve the separation efficiency. The effects of hydrophobicity and concentration of ion-pairing reagents on retention and selectivity in peptide reversed phase separations were elucidated in [19].

Because very strong interactions exist between the individual variables such as flow rate, gradient time, and eluent strength that influence the peak capacity of gradient elution RP-LC, optimization procedures are complicated [20]. Multivariate optimization strategies can help to find optimum separation conditions [20]. The continuative combined optimization of the TCA concentration, the flow rate, and the gradient duration was conducted by the help of a Box-Behnken experimental design. A general increase of the separation factors of all peak pairs was recorded with rising flow rate at fixed gradient time or, equally, with prolonged gradient duration at fixed eluent flow rate. The last observation is in agreement with other works where longer gradient times produced larger peak capacities [20]. Thereby, the peak capacities increased faster with prolonged gradient times at slower eluent flow indicating a strong interaction between the variables flow rate and gradient time. In contrast to the firstly mentioned observation for the monolithic column, maximum peak capacities for peptide separations on a packed RP column (3.5 μm particle size) were achieved at intermediate flow rates [20]. The optima of the influencing variables afford the complete separation of eight biomolecules characterized by widely differing molar masses and pI values in 8 min (Fig. 1). Also the structurally very similar three nonapeptides appear as baseline-resolved peaks. ß-Ltg splits up into three conformers. The reason for this chromatographic behaviour can be ascribed to a formation of different denatured structures during the separation process caused by the altering composition of the mobile phase [21]. Only the Lys peak shows a broad, plain profile. Larger proteins elute later from the column than smaller peptides due to a greater area of hydrophobic domains that interact with the stationary phenyl phase. Furthermore, the basic peptide Vpr elutes earlier than the more acidic Otc because the former exhibits a more positively charged state at the acidic pH conditions provided by the eluents and therefore a weaker interaction with the stationary phase. Generally, acidic and denaturing conditions are favored for reversed phase separations of proteins because hydrophobic areas are exposed to the surface due to defolding leading to an increased affinity to the unpolar stationary phase.

Because the optimization for the separation factors was based on a mean value of the target functions of all peak pairs (see Section 2), a further optimization was undertaken directed towards the crucial separation factor of the nonapeptides Vtc and Vpr. Under the obtained conditions of the gradient elution (0.025% (m/v) TCA, gradient time 7.5 min, flow rate 3.5 mL min^{-1}), the baseline resolution of all remaining biomolecules was ensured. The mentioned separation conditions were than used for the implementation of arsenic binding studies (see Section 3.3).

3.2. Linearities and detection limits of the RP-LC–UV method for peptides and proteins

The detection limits of bioanalytical methods are important features for samples of biological origin which are characterized by generally low analyte concentrations. Moreover, detection limits as well as linearities of the peak area dependence on the analyte concentration are crucial parameters for the capability and quantitative evaluation of binding studies.

Using the gradient elution parameters optimized for the separation factors (see Section 3.1), calibration lines were recorded for selected biomolecules in the following concentration ranges including seven calibration points: 10–100 μM for Vpr (R^2 = 0.996); 2–30 μM for Apr (R^2 = 0.982); 1–15 μM for Cyt c, Lys, α-Ltb, β-Ltg, each (R^2 = 0.984 for Cyt c, 0.983 for Lys, 0.993 for α-Ltb, 0.980 for β-Ltg). The standard deviation, *s*, of the variance of the measured peak areas about the regression line was determined and the peak area at the limit of detection, PA_{LOD}, was calculated according to the following equation:

$$PA_{LOD} = 3s \quad (1)$$

The relative standard deviation caused by the manual peak integration remained under 1%. The following concentration values for the detection limits resulted: 12.2 μM for Vpr regarded as representative for the other nonapeptides Vtc and Otc, 4.1 μM for Apr, 2.0 μM for Cyt c, 1.9 μM for Lys, 1.25 μm for α-Ltb, and 1.9 μM for β-Ltg.

3.3. Arsenic binding studies of sulfur-containing peptides and proteins by RP-LC

In former studies [5,6,16], condensation reactions of the organic arsenic compound phenylarsine oxide with different cysteine-containing peptides and proteins in their reduced state were observed by ESI-MS. Since the formed arsenic–sulfur bindings are very stable in contrast to bindings of other arsenic species, reactions with PAO can serve as an easily manageable probe for method development concerning the analysis of arsenic-binding biomolecules. A separation of some phenylarsenic-substituted peptides and proteins from their unsubstituted original forms succeeded using the developed RP-LC method (Section 3.1) onto a monolithic column (Fig. 2). With rising molarities of the arsenic compound, the peak areas of the reactants decreased whereas the peak areas of the reaction products increased. To achieve similar peak areas with the UV detection, the various biomolecules were measured in different molarities but in same mass concentrations of 0.1 mg mL^{-1}. Already after reduction of the biomolecules, some changes occur in the chromatogram (compare both lower chromatograms in Fig. 2) especially in the region of shorter retention times. Only Cyt c and both β-Ltg isoforms were not affected regarding their retention behaviour. Both nonapeptides shift to a somewhat higher retention time accompanied by a peak flattening. For Apr, the most noticeable effects were observed. The distinct peak of oxidized Apr smears over a broad retention time range after reduction of this small protein. α-Ltb splits into two smaller peaks compared to the oxidized state. The reduction process leads to a conformational change of the biomolecules since intramolecular structure-stabilizing disulfide bridges were broken. Definite baseline-separated product peaks emerging from the reaction with PAO arised for both nonapeptides. Thereby, the phenyl-arsenic substituted Vpr appeared as a double-peak. Even though the phenylarsenic-modification of Apr led to a somewhat more pronounced peak shape opposite to the reduced protein species, a quantitative evaluation was not feasible. Peak shape as well as retention time of Cyt c and β-Ltg did not change after reduction and incubation with PAO. For Cyt c, no arsenic bindings were

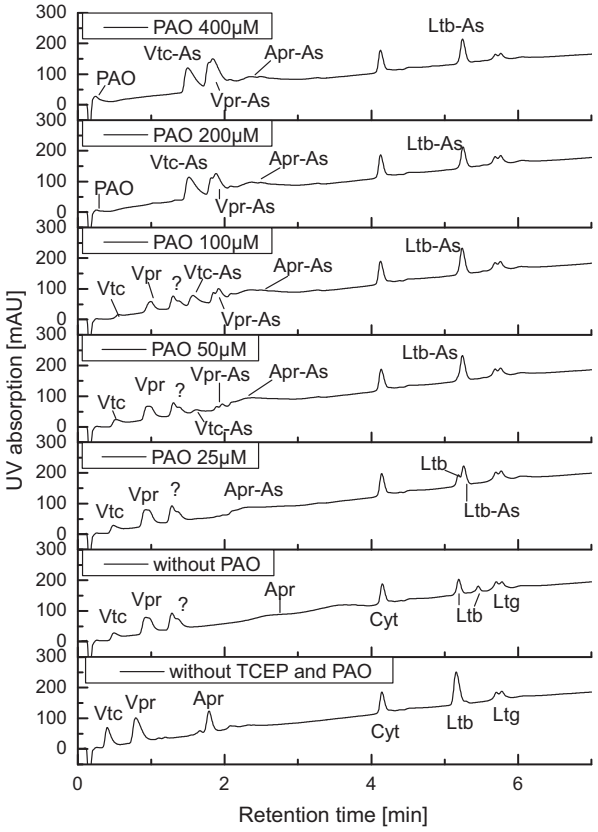

Fig. 2. Chromatograms of reaction mixtures containing six biomolecules (95 μM Vtc, 95 μM Vpr, 30.8 μM Apr, 8 μM Cyt, 7 μM Ltb, 5.4 μM Ltg), the reducing agent TCEP (2 mM) and phenylarsine oxide (PAO) in different molarities. Mobile phase A: H_2O/ACN (95/5, v/v); B: H_2O/ACN (5/95, v/v); +0.025% (m/v) TCA in A and B. Linear gradient from 1% B to 50% B in 7.5 min. Flow rate: 3.5 mL min^{-1}.

detected using direct ESI-MS measurements of PAO-containing Cyt c samples. In case of β-Ltg, arsenic bindings were observed in ESI mass spectra but the reaction equilibrium was widely located at the side of the unmodified protein. Possibly, the chromatographic resolution in the current RP-LC method is not sufficient to separate the arsenic-containing and the original form of this relatively large protein due to an only small mass and structure modification. The chromatogram obtained in presence of 25 μM PAO exhibits the uncomplete separation of phenylarsenic-substituted and unmodified α-Ltb. The described effects were confirmed in RP-LC measurements of the individual biomolecules after incubation with TCEP and PAO in addition to the injection of mixed samples. But the unidentified peak at 1.3 min appeared solely in chromatograms of the mixed samples possibly indicating an aggregation of two of the reduced biomolecules. The detection limit of unbound PAO (approx. 5 μM), which elutes near the dead volume of the column, was exceeded in reaction mixtures originally containing at least 200 μM PAO. The dependence of the HPLC–UV peak areas of reactants and products on the molar ratio of the arsenic compound to the biomolecule reflecting the reaction degree is depicted for single component measurements in Fig. 3. For both nonapeptides, the degree of the reaction with the arsenic compound can be easily monitored by the HPLC–UV measurements. In case of α-Ltb (Fig. 3c), the peak area ratio between the unbound and the arsenic-bound form firstly decreased and then fluctuated around a mean ratio corresponding to a consumption of the starting protein of 59.2 ± 6.1% by the reaction with 1.4-fold up to 21-fold molar excesses of PAO (calculated according to Eq. (3), see below). In reaction mixtures containing several biomolecules which compete for the reaction with the arsenic compound (Fig. 2) a complete

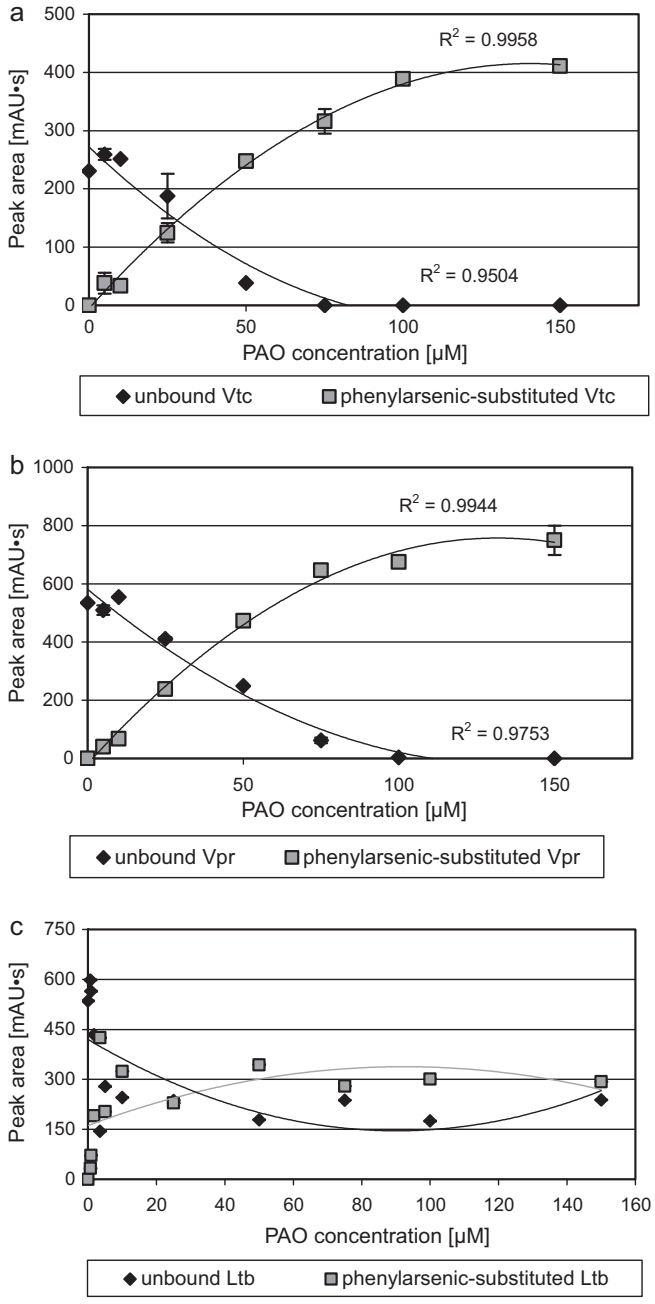

Fig. 3. Dependence of HPLC–UV peak areas of reaction products and starting biomolecules on the PAO concentration in single component measurements of (a) Vtc, (b) Vpr, and (c) Ltb. Standard deviations from three repeated measurements are indicated.

consumption of α-Ltb appeared at molar PAO:protein ratios higher than 7.1. For theregarded arsenic-binding biomolecules, no time-dependent changes of the peak ratios of starting components and reaction products were observed in a time range of two hours. In these samples, the binding equilibrium in the original aqueous solution is measured presuming that the binding dissociation is characterized by a slower kinetics than the time which is needed for passing the chromatographic column.

Using the peak areas shown in Fig. 2 for reaction mixtures (RM) and in Fig. 3 for single component (SC) measurements, the consumptions of the nonapeptides by the reaction with PAO were calculated in two ways according to the following equations:

$$\text{Consumption} = \frac{PA_{PhAs\text{-}BM,eq} \times 100\%}{(PA_{PhAs\text{-}BM,eq} + PA_{BM,eq})} \quad (2)$$

$$[BM]_{eq} = f \times PA_{BM,eq} \quad (3a)$$

$$[PhAs - BM]_{eq} = [BM]_0 - [BM]_{eq} \quad (3b)$$

$$\text{Consumption} = [PhAs - BM]_{eq} \times 100\,\%/[BM]_0 \quad (3c)$$

with $PA_{PhAs\text{-}BM,eq}$ = peak area of the phenylarsenic-substituted product; $PA_{BM,eq}$ = peak area of the starting biomolecule;

$[BM]_{eq}$ = equilibrium concentration of the starting biomolecule; f = calibration factor for the dependence of the UV-detected peak area on the biomolecule concentration; $[PhAs\text{-}BM]_{eq}$ = equilibrium concentration of the phenylarsenic-substituted product; $[BM]_0$ = starting concentration of the biomolecule.

The calculation way presented in Eq. (3) is exclusively based on the peak areas of the starting biomolecules. For single component measurements, a lower consumption resulted from calculation way 2 compared to calculation way 1 at all tested PAO/peptide ratios (Table 1). Moreover, except for one sample (Vtc, PAO:Vtc ratio = 0.53), the consumption calculated according to Eq. (3) remains under the maximal peptide consumption that is possible at a complete consumption of the arsenic compound. In contrast, the comparison of the consumptions which were calculated from the peak areas of the reaction products (Eq. (2)) with the maximally possible consumption highlights a weak point of this kind of quantitative evaluation: in some cases, larger peptide consumptions were indicated by the measurements than they could take place assuming a complete PAO consumption. These findings can be ascribed to an enhanced UV absorption of the phenylarsenic-containing reaction products because they have incorporated an aromatic ring system. If peptides and proteins with an unknown UV absorptivity should be quantitatively analysed by HPLC–UV, their molar extinction coefficients can be predicted by calculation of the sum of the molar extinction coefficients of amino acids and peptide bonds contributing to the UV absorption of the complete amino acid chain [22]. The molar extinction coefficients of several peptides and proteins that were predicted in this way agreed with measured values within error ranges of <11–25%. According to this approach, in order to predict the molar extinction coefficients of the reaction products considered in the current arsenic binding study, the molar extinction coefficients of the unsubstituted peptides and proteins must be extended by the molar extinction coefficient of the phenylarsenic moiety which can be deduced from the UV absorption of free PAO. However, the effect of the spatial structure change of the biomolecules induced by the phenylarsenic substitution cannot be taken into account. Further, the eluent composition prevailing at the retention time of the reaction products must be considered for estimation of the molar extinction coefficients. The assumed UV absorption enhancement of the reaction products also caused an increasing trend for the apparent K values calculated according to Eq. (4) from 9.6×10^5 and 2.2×10^6 at a PAO/peptide molar ratio of 0.05 to 1.2×10^8 and to 5.0×10^7 at a 10-fold higher molar ratio in case of Vtc and Vpr, respectively. For Vpr, the K value further increased to 1.4×10^9 at an equimolar ratio with PAO.

$$K = \frac{[\text{Peptide-As}(C_6H_5)]_{eq} \cdot [H_2O]}{[\text{Peptide}]_{eq} \cdot [PAO]_{eq}} \quad (4)$$

with $[\text{Peptide-As}(C_6H_5)]_{eq}$ = peak area (peptide-As(C_6H_5); $[\text{Peptide}]_0/\Sigma$ (peak area (peptide-As(C_6H_5); peak area (peptide));

$[\text{Peptide}]_{eq}$ = peak area (peptide); $[\text{peptide}]_0/\Sigma$ (peak area (peptide-As(C_6H_5); peak area (peptide)); $[PAO]_{eq} = [PAO]_0 - [\text{peptide-As}(C_6H_5)]_{eq}$ and $[H_2O] = 55.56 \times 10^6\ \mu\text{mol L}^{-1}$.

The comparison of peptide consumptions by PAO in single component measurements and in separations of mixtures containing four biomolecules which compete for the reaction with PAO (see Fig. 2) shows that a generally lower consumption resulted in the reaction mixtures if calculation way 1 is used (Table 1). Both in SC and RM samples, the consumption values obtained from both

Table 1
Consumptions of the nonapeptides Vtc and Vpr by the reaction with PAO calculated from peak areas of reactants and products (calculation way 1 (C.w. 1) explained in Eq. (1)) or from peak areas of reactants (calculation way 2 (C.w. 2) explained in Eq. (2)) obtained from HPLC–UV measurements of single components (SC, containing the peptide and PAO) and reaction mixtures (RM, containing four arsenic-binding biomolecules and PAO).

Molar ratio of PAO to the peptide	Peptide consumption [%] based on HPLC–UV peak areas								Maximally possible peptide consumption[a] [%]
	Vtc				Vpr				
	SC		RM		SC		RM		
	C.w. 1	C.w. 2	C.w. 1	C.w. 2	C.w. 1	C.w. 2	C.w. 1	C.w. 2	
0.05	12.8	0	0	0	7.3	0	0	0	5.3
0.1	11.8	0	0	0	11.0	4.7	0	0	10.5
0.25	39.9	18.7	0	64.3	36.7	23.1	2.7	23.0	25
0.53	86.6	83.3	29.2	65.4	65.5	53.4	17.4	39.4	53
1.0	100	100	89.4	89.2	99.5	99.3	55.7	63.9	100
2.0	100	100	100	92.0	100	100	97.3	97.5	100

[a] Assuming a complete consumption of the applied PAO amount.

calculation ways were similar at the higher applied PAO amounts where a complete consumption of the peptides was reached. In the reaction mixtures, a larger consumption of the starting peptide was observed by using calculation way 2 partially exceeding the maximally possible consumption. This finding can be presumably ascribed to the assumed adduct formation of the reduced biomolecules mentioned above that is indicating by the peak eluting at 1.3 min. Because of this, the calculation of the consumption by the decrease of the peak area of the starting peptide (Eq. (3)) is no good choice in case of reaction mixtures since a higher consumption for the reaction with PAO is pretended. In general, Vtc reacted with PAO in a larger extent than Vpr. A nearly complete consumption which exceeded the detection limit of the starting peptide of 12 μM was reached in SC measurements at an equimolar ratio of the starting components. This indicates a high reactivity.

In the previously performed SEC separations, *K* values of reaction systems could be calculated by coupling to ESI-MS but not by using the UV detection because reactants and products could not be completely separated owing to the restricted separation performance [5]. But the mass spectrometric detection requires a substantially more laborious method development than simple UV detection. Moreover, the ionization of the analytes is strongly influenced by the sample composition. ESI-MS measurements with direct injection of reaction systems revealed a pronounced decrease of *K* values with rising initial reactant concentrations [2]. This system-dependent behaviour of the calculated apparent equilibrium constants corresponds to the inverse trend observed in the current RP-LC–UV analyses. Compared to the SEC-MS coupling, the novel RP-LC method affords the complete separation of the original and the arsenic-bound forms at least for the smaller biomolecules on the monolithic phenyl phase. However, the drawback of unknown detection sensitivities for the arsenic-substituted reaction products persisted.

4. Conclusion

A fast separation of a mixture of peptides and proteins characterized by a wide molar mass and pI range was achieved with a monolithic reversed phase column and an acetonitrile/water gradient supported by an ion pairing reagent. The optimized method could be applied to the separation of phenylarsenic-substituted species of biomolecules from their unmodified forms. For nonapeptides and for α-Ltb, the peak areas obtained by UV detection could be quantitatively evaluated in terms of calculation of consumptions and apparent binding constants. To improve the quantitative results, the molar absorptivity of the reaction products can be approximatively predicted by summing up the molar absorptivities of the starting biomolecule and of PAO. For Apr and β-Ltg, the separation was not sufficient to completely differentiate arsenic-bound and unbound forms. In order to afford a separate detection of reactants and products, a RP-LC–ESI-MS coupling method should be established. Even though the sample can be injected as aqueous solution, the eluent exerts a denaturing influence on the proteins during the separation process because of the ACN and TCA additives. To solve these problems, non-denaturing separation methods such as capillary zone electrophoresis with aqueous buffer systems or hydrophobic interaction chromatography could serve as a further alternative to investigate the arsenic binding behaviour of peptide and protein mixtures.

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References

[1] Z. Chen, S.G. Weber, Trends Anal. Chem. 27 (2008) 738.
[2] A.C. Schmidt, S. Steier, J. Mass Spectrom. 45 (2010) 870.
[3] D.J. Winzor, Anal. Biochem. 383 (2008) 1.
[4] J. Gailer, W. Lindner, J. Chromatogr. B 716 (1998) 83.
[5] A.C. Schmidt, B. Fahlbusch, M. Otto, J. Mass Spectrom. 44 (2009) 898.
[6] A.C. Schmidt, M. Neustadt, M. Otto, J. Mass Spectrom. 42 (2007) 771.
[7] S. Xie, R.W. Allington, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 865 (1999) 169.
[8] F. Svec, C.G. Huber, Anal. Chem. 78 (2006) 2101.
[9] J. Zhang, S.L. Wu, J. Kim, B.L. Karger, J. Chromatogr. A 1154 (2007) 295.
[10] F.C. Leinweber, D.G. Schmid, D. Lubda, K.H. Wiesmüller, G. Jung, U. Tallarek, Rapid Commun. Mass Spectrom. 17 (2003) 1180.
[11] T.P. Hennesy, R.I. Boysen, M.I. Huber, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A 1009 (2003) 15.
[12] H. Pham-Tuan, L. Kaskavelis, C.A. Daykin, H.-G. Janssen, J. Chromatogr. B 789 (2003) 283.
[13] V.V. Tolstikov, A. Lommen, K. Nakanischi, N. Tanaka, O. Fiehn, Anal. Chem. 75 (2003) 6737.
[14] N.W. Smith, Z. Jiang, J. Chromatogr. A 1184 (2008) 416.
[15] X. Wang, W.E. Barber, P.W. Carr, J. Chromatogr. A 1107 (2006) 139.
[16] A.C. Schmidt, J. Koppelt, M. Neustadt, M. Otto, Rapid Commun. Mass Spectrom. 21 (2007) 153.
[17] S. Espinosa, E. Bosch, M. Rosés, J. Chromatogr. A 964 (2002) 55.
[18] X. Subirats, E. Bosch, M. Rosés, J. Chromatogr. A 1121 (2006) 170.
[19] M. Shibue, C.T. Mant, R.S. Hodges, J. Chromatogr. A 1080 (2005) 68.
[20] X. Wang, D.R. Stoll, A.P. Schellinger, P.W. Carr, Anal. Chem. 78 (2006) 3406.
[21] K.D. Nugent, W.G. Burton, T.K. Slattery, B.F. Johnson, L.R. Snyder, J. Chromatogr. 443 (1988) 381.
[22] B.J.H. Kuipers, H. Gruppen, J. Agric. Food Chem. 55 (2007) 5445.