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Alternative high-performance liquid chromatographic peptide separation and purification concept using a new mixed-mode reversed-phase/weak anion-exchange type stationary phase

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Abstract

This article describes a new complementary peptide separation and purification concept that makes use of a novel mixed-mode reversed-phase/weak anion-exchange (RP/WAX) type stationary phase. The RP/WAX is based on *N*-(10-undecenoyl)-3-aminoquinuclidine selector, which is covalently immobilized on thiol-modified silica particles (5 μm, 100 Å pore diameter) by radical addition reaction. Remaining thiol groups are capped by radical addition with 1-hexene. This newly developed separation material contains two distinct binding domains in a single chromatographic interactive ligand: a lipophilic alkyl chain for hydrophobic interactions with lipophilic moieties of the solute, such as in the reversed-phase chromatography, and a cationic site for anion-exchange chromatography with oppositely charged solutes, which also enables repulsive ionic interactions with positively charged functional groups, leading to ion-exclusion phenomena. The beneficial effect that may result from the combination of the two chromatographic modes is exemplified by the application of this new separation material for the chromatographic separation of the N- and C-terminally protected tetrapeptide *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide from its side products. Mobile phase variables have been thoroughly investigated to optimize the separation and to get a deeper insight into the retention and separation mechanism, which turned out to be more complex than any of the individual chromatography modes alone. A significant anion-exchange retention contribution at optimal pH of 4.5 was found only for acetate but not for formate as counter-ion. In loadability studies using acetate, peptide masses up to 200 mg could be injected onto an analytical 250 mm × 4 mm i.d. RP/WAX column (5 μm) still without touching bands of major impurity and target peptide peaks. The corresponding loadability tests with formate allowed the injection of only 25% of this amount. The analysis of the purified peptide by capillary high-performance liquid chromatography (HPLC)-UV and HPLC–ESI-MS employing RP-18 columns revealed that the known major impurities have all been removed by a single chromatographic step employing the RP/WAX stationary phase. The better selectivity and enhanced sample loading capacity in comparison to RP-HPLC resulted in an improved productivity of the new purification protocol. For example, the yield of pure peptide per chromatographic run on RP/WAX phase was by a factor of about 15 higher compared to the standard gradient elution RP-purification protocol.

Keywords: Liquid chromatography; Synthetic peptide separation; Stationary phase; Mixed-mode chromatography; Reversed-phase/weak anion-exchange material; *N*-(10-Undecenoyl)-3-aminoquinuclidine

1. Introduction

In recent years, synthetic peptides of variable length have become increasingly demanded in particular by the pharmaceutical industry and various research institutions owing to their exceptional potential and broad use as drugs, drug transporters, diagnostics, radio-pharmaceuticals, synthetic vac-

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cines, bioactive research compounds, building blocks, structural probes, analytical standards, and so forth [1]. They have to be therefore synthesized in huge numbers and extended mass range in quantities ranging from microgram to kilogram scale, with several thousands of tons produced in total worldwide every year.

To do so, solid-phase synthesis concepts are usually utilized since its ground-breaking introduction by Merrifield [2,3], if they have to be produced on milligram to gram scale only. This strategy minimizes the number and amounts of side products, because reagents are applied in huge excess and can be washed off afterwards. However, if the peptide has to be supplied in gram to kilogram quantities, the solid-phase synthesis concept becomes too expensive due to the high costs of resins and excessive use of reagents. Therefore, the "old-fashioned" solution-phase synthesis is preferred in that case, which of course leads typically to a lower final quality of the crude product in terms of purity.

In general, the synthesis provides not only the target peptide, but also impurities resultant from multiple coupling due to deprotection, failed coupling (deleted sequences), hydrolysis of protected side chains (e.g. *t*-butyl ester of Glu), imide formation, deamidation (e.g. of Gln, Asn side chains), racemization (yielding epimers or diastereomers), oxidation, S–S exchange, β-elimination, and so forth. After the initial standard clean-up procedures, these impurities are often still present. Hence, a final chromatographic purification step is demanded to end up with the required purity. Nowadays, this is normally performed by gradient elution reversed-phase high-performance liquid chromatography (RP-HPLC) with acetonitrile as organic modifier (containing 0.1% trifluoroacetic acid), using normally octadecyl-modified silica (ODS) as stationary phase [4–6]. Although this method offers in principle good selectivity and high efficiency, it unfortunately often fails in particular for very hydrophilic or also very hydrophobic peptides, as well as structurally closely related peptides that are often present as impurities. Such side products appear in the RP-HPLC chromatograms as accompanying minor peaks in close vicinity to the front or tailing end of the main component peak, being insufficiently resolved. If it comes to preparative separations, where overloading is mandatory to achieve sufficient productivity, they tend to co-elute with the main component. This is particularly critical and disturbing when the peptide has to be produced with drug quality (typically less than 0.1% impurity).

A number of alternative purification concepts has been therefore suggested to overcome the aforementioned selectivity problems [7], but are in fact under-estimated and under-utilized, when compared to gradient elution RP-HPLC. Such alternative methods include ion-exchange chromatography, particularly employing strong cation-exchange materials [8–10] (and references therein), hydrophobic interaction chromatography (HIC) [11] and hydrophilic interaction chromatography (HILIC) [12], and for peptides only to minor extent, also size-exclusion chromatography (SEC) [13] and affinity chromatography [14] (these two latter techniques are however widely employed for larger peptides and proteins). Moreover, also molecularly imprinted polymers have been tailored for peptide separation [15–17].

Another often used strategy is, on the other side, the combination of two different columns with orthogonal selectivity principles, such as RP and ion-exchange, either in series or as two individual chromatographic steps, which is especially advantageous for the resolution of complex mixtures. Analytically, this is nowadays frequently realized in on-line 2D-chromatography concepts with column switching techniques in proteomic studies [18]. On a preparative scale, such an on-line coupling of different columns operating by orthogonal selectivity principle is more difficult to realize and therefore seldom exploited. On contrary, the combination of two chromatographic steps in series is avoided, if possible, to save eluents and time, as well as guarantee higher yields. Blending of two types of different materials, such as RP- and ion-exchange particles (either SCX or SAX), in a single column has been suggested as alternative and such column is commercially available with tradename Duet from Hypersil.

We herein propose the use of a bivalent separation material with two distinct binding domains in a single chromatographic interactive ligand, which integrates the two orthogonal separation principles of ion-exchange and reversed-phase chromatography. Such obtained mixed-mode reversed-phase/ion-exchange separation media are supposed to exhibit selectivities towards peptide impurities that differ strongly from those of conventional RP-type stationary phases. They may therefore be considered as powerful complementary separation concepts, leading eventually to better product qualities. The presence of the ion-exchange retention increment is supposed to improve the loading capacity, as was previously shown for a pure ion-exchange separation principle. This, in turn, would have a positive effect on the productivity of peptide purification methods that make use of such separation material.

Actually, mixed-mode mechanisms are not a completely new concept [34,35]. Several examples of synthesis of mixed-mode reversed-phase/ion-exchange phases on silica supports are reported in the literature [22,35–45], many of them with applicability for purification of proteins, peptides, and nucleic acids.

Mixed-mode RP/ion-exchange phases (most often RP/SCX, seldom RP/WAX or RP/SAX) have previously been developed for reversed-phase capillary electrochromatography, with the aim to achieve stable and strong electroosmotic flow (EOF) [19–23]. In such a concept, the ion-exchange site has the function of providing the charge for EOF generation, when they are operated under conditions that avoid strong attractive ionic interaction (non-interactive or repulsive mode of ion-exchangers), i.e. RP/SCX are employed for acids and RP/SAX for bases. The separation is then based on reversed-phase partitioning and electrophoretic migration differences.

Generally, also organic polymer-based ion-exchangers may have a significant hydrophobic non-specific reten-

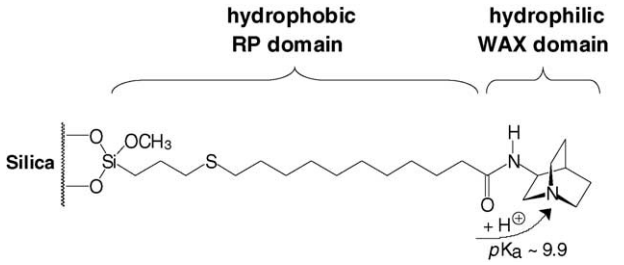

Fig. 1. Structure of mixed-mode reversed-phase/weak anion-exchange (RP/WAX) type stationary phase.

tion increment that originates from the polymer backbone or spacers, i.e. the utilized linker chemistry. Polymer-based ion-exchangers, such as those based on poly(styrene-divinylbenzene) or polymethacrylate, should be mentioned here [24]. The same may apply to ion-exchange materials that have been prepared by coating of polyelectrolytes with adjusted hydrophobicity onto a chromatographic support [25]. The mixed-mode reversed-phase/anion-exchange material that we are presenting in this report is a typical brush-type phase, which consists of a selector immobilized onto thiol-modified silica gel (Fig. 1). The weak anion-exchange (WAX) site is located on the outer surface of the lipophilic layer and is linked to the hydrophilic silica support via a lipophilic spacer with polar embedded amide and sulfide groups.

The objective of the present study is to demonstrate the potential of the mixed-mode reversed-phase/weak anion-exchange (RP/WAX) stationary phase shown in Fig. 1 for the HPLC separation of peptides. In particular, we wish to illustrate the usefulness of the concept and the mechanism of the new stationary phase for the chromatographic separation of *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide. This diagnostic N- and C-terminally protected tetrapeptide is a value-added product, being produced in multi-hundred gram batches.

By the optimization of the experimental variables such as pH, buffer strength, and organic modifier content of the mobile phase, we will outline the retention and separation mechanism of this new system. In order to illustrate the applicability of this method for preparative scale separations, a loadability study in the zonal elution chromatography mode under overload conditions (batch chromatography mode) has been performed and the quality of the separately collected fractions has been controlled by capillary HPLC-UV and HPLC–ESI-MS using C-18 stationary phases. The results should allow assessing the value of this new method in comparison to standard RP-HPLC method.

2. Experimental

2.1. Materials

The synthesis of the mixed-mode reversed-phase/weak anion-exchange type stationary phase (Fig. 1) is described elsewhere [26]. Briefly, the *N*-(10-undecenoyl)-3-amino-quinuclidine selector was covalently bonded to thiol-modified Kromasil 100, 5 μm (Eka Chemicals, Bohus, Sweden) by radical addition reaction. The selector coverage, as determined by elemental analysis, was calculated to be 0.47 mmol/g stationary phase. The RP/WAX stationary phase was packed into stainless-steel HPLC columns of either 250 mm or 150 mm length and 4 mm i.d. The RP-analysis was performed with a Beckman Ultrasphere (ODS) column, 5 μm, 150 mm × 4.6 mm i.d. For the capillary HPLC, a C18 column Zorbax SB, 5 μm, 150 mm × 0.5 mm i.d. was employed. A Superspher RP-18e column, 5 μm, 125 mm × 3 mm i.d. (Merck KGaA) was used for the LC–MS experiments.

The peptides *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide and *N*-acetyl-Ile-Glu-Gly-Lys-*p*-nitroanilide were synthesized in several 100 g batches by FMOC chemistry utilizing solution-phase protocols (piCHEM, Graz, Austria) and obtained as unpurified crude products. Unless otherwise stated, samples for chromatography were prepared in acetonitrile–water (10:90; v/v) at concentrations of around 0.45 mg/ml. For loadability studies, higher concentrations (up to 0.5 g/ml) were prepared, which required sonication to obtain a homogeneous solution. All samples and solutions were stored under refrigeration at 10 °C.

All the other chemicals utilized for HPLC were analytical grade and supplied by Sigma-Aldrich (Vienna, Austria), except the solvents, which were HPLC-grade and from Merck KGaA (Darmstadt, Germany).

2.2. Instrumentation

A LaChrom HPLC (pump L-7100, autosampler L-7200, interface D-7000, UV detector L-7400) and a column thermostat Jetstream 2 Plus (Alltech, Unterhaching, Germany) were used for gradient elution RP-HPLC and for Engelhardt and Tanaka tests of RP-18 and RP/WAX columns. The peptide separation and purification was carried out with a modular isocratic HPLC Hewlett Packard Series 1050 (interface 35900E, UV–vis detection).

The analysis of the purified fractions was carried out by gradient elution capillary RP-HPLC using an Agilent 1100 Series capillary HPLC, and by on-line LC–MS using an API 365 triple quadrupole mass spectrometer (PE Sciex, Thornhill, ON, Canada). pH measurements were obtained with a pH-Meter 540 GLP (WTW, Weilheim, Germany).

The void volume marker was uracil (0.16 mg/ml in acetonitrile–water 80:20; v/v). The RP/WAX column was regenerated after the semi-preparative runs of the loadability study with a solution of 2% triethylamine in methanol, as well as methanol or acetonitrile gradient up to 100% to elute possibly trapped components (e.g. multiply negatively charged species and hydrophobic compounds, respectively).

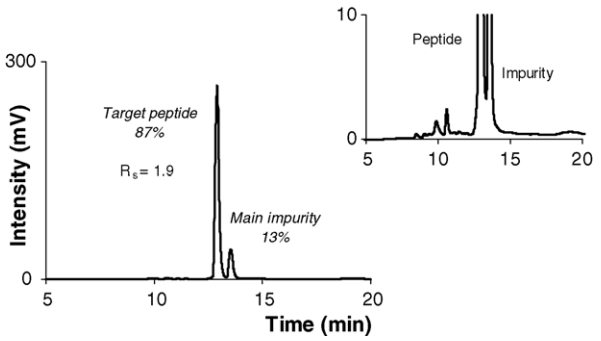

Fig. 2. Separation of target peptide *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide from its main impurity by gradient elution RP-HPLC using a C18 column (5 μm, 150 mm × 4.6 mm i.d.). Experimental conditions: mobile phase, 0.1% TFA in water (A) and 0.1% TFA in ACN (B), gradient from 5 to 60% B in 20 min, flow-rate, 1 ml/min; temperature, 25 °C; detection wavelength, 316 nm. The enlargement on the right side shows that some other impurities are also present in low concentrations.

3. Results and discussion

3.1. Failure of reversed-phase purification scheme

The N- and C-terminally protected target tetrapeptide *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide is synthesized by FMOC chemistry in a nine-step synthesis in solution. After purification with the standard work-up protocol, this peptide still contains a major side product that closely elutes with the target product in RP gradient elution chromatography (Fig. 2). In addition, there are a number of minor side products detectable in the crude product.

Although the resolution capability of gradient RP-HPLC seems to be sufficient for analytical purposes, it turned out to be to a large extent inadequate for up-scaling and preparative application. Upon overloading, which is mandatory in preparative scale chromatography in the batch mode to achieve reasonable productivity, overlapping of peptide and impurity peaks occurs. Furthermore, while the impurity elutes after the peptide in analytical scale, it shifts to the front end of the main peak with increasing amounts loaded onto the column. Thereby, the impurity co-elutes with the major mass pulse of the target peptide, what makes the process to a certain extent useless in terms of loadability and productivity. For example, the mass balance of the collected pure fractions gave a yield of 43% pure peptide product of the 300 mg crude peptide that have been injected onto a 250 mm × 20 mm i.d. RP-column.

To escape from such problems that often may arise, we propose to develop complementary peptide purification materials and methods, which may afford enhanced selectivity (α) values. This is particularly important for preparative separations, because the loadability increases proportionally with $(\alpha-1)^2/\alpha^2$ and thus the productivity roughly with $(\alpha-1)^3/\alpha^3$ [27]. From this, it becomes evident that optimization of the stationary phase selectivity is the most appropriate way to improve productivity. We envisaged and developed the mixed-mode RP/WAX stationary phase (Fig. 1) as a powerful complementary sorbent to replace RP-purification protocols. The results for the peptide Ac-Ile-Glu-Gly-Arg-*p*-nitroanilide and its Lys-analogue (replacing Arg by Lys) will be outlined in the following discussion.

3.2. Characteristics of new RP/WAX stationary phase

The interactive chromatographic ligand of the brush-type mixed-mode RP/WAX stationary phase (Fig. 1) consists essentially of two distinct modular binding domains: a comparably hydrophilic anion-exchange site and a hydrophobic alkyl-thioalkyl-chain that links the anion-exchange site to the chromatographic support.

Some of the properties of the RP/WAX phases are summarized in Table 1. The pK_a of the quinuclidinyl WAX-site is about 9.9. In the protonated state, it represents a hydrophilic charged center. This site, together with the polar embedded amide and sulfide groups, make the stationary phase compatible with 100% aqueous eluents (without collapsing of the ligands).

The chromatographic ligand (selector) is bonded onto the surface of spherical silica particles with 100 Å average pore diameter (Kromasil 100, 5 μm) and surface area of ca. 340 m^2/g. Such a narrow pore diameter still ensures diffusional access of small peptides (e.g. the presently investigated protected tetrapeptide) to the inner pore surface, but may need to be replaced by wide pore supports in case of large peptides. As dedicated material for the present peptides, the large surface area is of special interest and guarantees a high selector loading (about 470 μmol/g stationary phase) and thus reasonable sample loadability.

Through the utilized bonding chemistry, i.e. radical addition of the selector to thiol-modified silica, a relatively dense hydrophobic layer is obtained. Since only about 50% of the total thiol groups are modified with selector, remaining reactive thiols have been capped with 1-hexene. However, no

Table 1
Properties of RP/WAX stationary phase compared to representative RP-phase

Property	RP/WAX[a]	RP18[b]
pK_a (quinuclidine)	9.89[c]	–
Selector loading (mmol/g)	0.47	–
Surface area (m^2/g)	340	na
Selector coverage (μmol/m^2)	1.4	na
% Carbon	14.50	12.00
Hydrophobicity α_{CH_2}[d]	1.40	1.80
Shape selectivity[e]	3.30	1.50

Engelhardt test [28]: mobile phase, methanol–water (49:51; w/w); temperature, 40 °C; flow rate, 1 ml/min; detection wavelength, UV 254 nm. Tanaka test: mobile phase, methanol–water (632:200; w/w); temperature, 30 °C; flow rate, 1 ml/min; detection wavelength, UV 254 nm. na—not available.

[a] For structure, see Fig. 1.
[b] Beckman Ultrasphere C18.
[c] Calculated using Advanced Chemistry Development (ACD) software.
[d] Engelhardt test (selectivity between ethylbenzene and toluene).
[e] Tanaka test (selectivity between triphenylene and *o*-terphenyl).

significant increase in carbon loading could be found by elemental analysis. A representation of the RP/WAX stationary phase would consist of a superficial WAX surface and an interior lipid layer formed by the strands of the alkyl-thioalkyl chains, which keep an approximate calculated distance of ca. 11 Å from each other.

While the carbon content of the RP/WAX stationary phase largely matches that of typical reversed-phase C18 sorbents (e.g. 14.5% C versus 12% C of a Beckmann Ultrasphere C18), the chromatographic characterization of the hydrophobicity by the Engelhardt test revealed a methylene selectivity (separation factor between ethylbenzene and toluene) of 1.4 [26], which is comparable to the hydrophobicity of a C8 rather than a C18 stationary phase (e.g. α_{CH_2} is 1.80 for Beckmann Ultrasphere C18). This lower hydrophobicity, however, can be easily compensated by using mobile phases with lower organic modifier content. The Tanaka test also indicated improved shape selectivity as determined by the separation factor between triphenylene and *o*-terphenyl (Table 1) describing the ability to distinguish between planar and non-planar solutes [26]. The apparently improved steric recognition seems to originate, however, from additional retention increments arising from electrophilic interactions of the planar aromatic triphenylene with sulfide and/or amide groups which are not existing or weaker for the non-planar *o*-terphenyl.

3.3. RP/WAX mechanism

In accordance with the bimodal chemical surface character of the stationary phase, a mixed chromatographic separation mechanism may be the outcome through the complex interplay of reversed-phase, ion-exchange, and ion-exclusion retention increments. The relative contribution of each mechanism depends on the hydrophobicity and charge character of solutes, as well as on experimental conditions, mainly mobile phase composition.

It is convenient to describe the reversed-phase retention by adopting the linear solvent strength theory. According to this theory, plots of $\log k$ versus percent fraction of organic modifier (φ) in the eluent (Eq. (1)) drop linearly as the modifier content is increased, due to weakening of hydrophobic interactions.

$$\log k = \log k_w - S\varphi \quad (1)$$

whereby the constant $\log k_w$ is characteristic of the lipophilicity of the solute and S is related to the hydrophobic contact area (which is proportional to the accessible molecular surface area) of the solute.

On the other hand, the ion-exchange process is governed by electrostatic interactions between peptide and ion-exchange site of the sorbent. A net-charge retention model has been often utilized to explain the presence and absence of ion-exchange interactions for multiply charged solutes. Thus, an anion-exchange process may be superimposed upon the hydrophobic (reversed-phase) retention contribution, if the mobile phase pH is above the p*I* of the peptide. However, deviations from the net-charge model were frequently found for multiply charged species like peptides and proteins, which were assumed to occur most probably due to charge asymmetry, since only a fraction of the solute interacts with the stationary phase [29]. Therefore, ion-exchange interactions may also happen at pH-values at which no ion-exchange process is expected according to the net charge model.

Although more sophisticated models based on electrical double-layer theory have been developed [30,31] (due to the criticism that, in a strict thermodynamic sense, electrostatic interactions do not follow the stoichiometric law), the simple empirical stoichiometric displacement model [29,32,33] will be utilized in the following discussion. According to this model, plots of $\log k$ versus log counter-ion concentration (C) are linear (Eq. (2)) [29],

$$\log k = \log K_z - Z \log C \quad (2)$$

wherein K_Z is a constant related to the ion-exchange equilibrium constant and the ion-exchange capacity and Z is related to the ratio of the valencies of solute ion (s) and counter-ion (c) ($Z = s/c$). This empirical linear relationship between $\log k$ and $\log C$ is a valuable tool to assess the existence of an ion-exchange process. Examination of the slope Z allows to derive the number of charges involved in the ion-exchange, whereby for a monovalent counter-ion the slope is representative for the charge on the solute.

In addition, between positively charged solute moieties and the selector quinuclidinium ring, repulsive ionic interactions may be in action too. This may lead, under appropriate elution conditions, to an ion-exclusion mechanism for positively charged species, which then elute even before the neutral unretained void marker. Hence, overall a complex interplay of individual attractive and repulsive peptide-sorbent interactions may exist, which offers more variables for optimization of the separation than any of the individual retention mechanisms alone and allows a delicate fine-tuning of selectivities between the target peptide and peptide impurities.

All mechanisms can be, to some extent, independently governed and varied by the mobile phase conditions such as pH, ionic strength (counter-ion concentration and type, which are the main variables for balancing the anion-exchange and ion-exclusion processes), and type and percentage of organic modifier (primary variable for reversed-phase retention increment). The results of these optimization studies are discussed in the following paragraphs to give insight into the retention and separation mechanisms of the mixed-mode RP/WAX phase for the present separation example.

3.4. Optimization of the RP/WAX separation

3.4.1. pH-dependence

For the ion-exchange process and the RP-retention, the ionic states of both peptide and stationary phase have to be

considered. Therefore, the pH of the eluent is supposed to be the major influential variable.

Although N- and C-terminally protected, the target peptide *N*-Ac-Ile-Glu-Gly-Arg-*p*-nitroanilide has amphoteric nature due to the acidic Glu residue (p$K_a \sim 4.25$) and to the basic Arg side chain (p$K_a \sim 13.2$). Accordingly, the p*I* will be around 8.7 and a significant negative peptide net-charge, as supposed to be required to enable strong anion-exchange retention, will hence be obtained only at pH-values above 9. Conversely, weak anion-exchangers, such as the present investigated phase based on the quinuclidine ring (p$K_a \sim 9.9$, ACD software), should be used at weakly acidic pH, where they exhibit maximal exchange capacity. Therefore, at first glance it seems to be unlikely that an ion-exchange mechanism is at work in the useful weakly acidic pH-range of the mobile phase.

The observed experimental behavior in terms of pH-profile of retention (Fig. 3) is difficult to interpret due to the complex mechanism and it appears to be impossible to deconvolute the individual RP and WAX retention increments. We will, therefore, adhere to a more phenomenological discussion here. Within the investigated pH range (2.7–6.5), the retention factors of the target peptide, as well as of the main impurity, constantly increased with increasing pH. This behavior may be easily explained by the reversed-phase retention mechanism: the hydrophobicity of the peptide increases rapidly when the mobile phase pH is approximating its p*I*, and thus the reversed-phase retention increment is increasingly enhanced when the eluent pH is changed from 2.7 to 6.5.

On the other hand, also the ionic interactions of the carboxylate group with the WAX-site might lead to similar effect. With increasing pH, the dissociation of the carboxylic side chain increases and thus ionic interaction might be assumed to get stronger, which would also contribute to the increase of *k*-values. Since the net-charge is still significantly below the p*I*, such a contribution would most likely only exist, if the positive charge of the guanidinium group is shielded and thereby weakened by ion-pairing effects, as could be envisioned for acetate counter-ions. At this point, however, this is merely a hypothesis.

The major impurity, which has not yet been fully identified but contains the structural element *N*-Ac-Ile-X-X-Arg-*p*-NA (X-X has the mass of Glu-Gly +50 amu), exhibited a very similar pH-tendency. However, at pH below 4 it eluted before the void marker, which is indicative for ion-exclusion phenomena (Fig. 3b, inset). On the basis of this chromatographic behavior, it may be assumed that the impurity possesses an additional positive charge. This was supported by the fact that the ESI-MS provided only stable ions in the positive mode and could be monitored solely as doubly charged species (Fig. 13) (molecular mass of 685 was obtained by MALDI).

Maximum selectivity between the target peptide and its impurity was found at a pH around 4, and optimal resolution at around 4.5 (Fig. 3c). Since gradient elution RP-HPLC provided poor selectivity under acidic and slightly basic eluent conditions, a selectivity contribution arising from charge differences and ionic interactions with the WAX-domain may be postulated for the present separation system with the mixed-mode RP/WAX stationary phase.

3.4.2. Counter-ion effects

The above data do not unequivocally prove that the target peptide is retained by an ion-exchange mechanism. Studies on the counter-ion effect should in contrast provide this information. A linear dependency between $\log k$ and $\log C$ with negative slope would according to the empirical relationship of Eq. (2) be an indicator for an ion-exchange process, and the slope of such a plot should indicate the number of charges involved in this ion-exchange process. Fig. 4 shows that the retention of target peptide decreases with increasing ammonium acetate concentration in the eluent, yielding largely a linear inverse relationship between $\log k$ and $\log C$, which complies with Eq. (2) and is typical for the ion-exchange process.

However, the slope resulting from the ratio of the charge on the solute ion and counter-ion is rather small ($Z = \sim 0.1$), which indicates only a weak, but significant ionic interac-

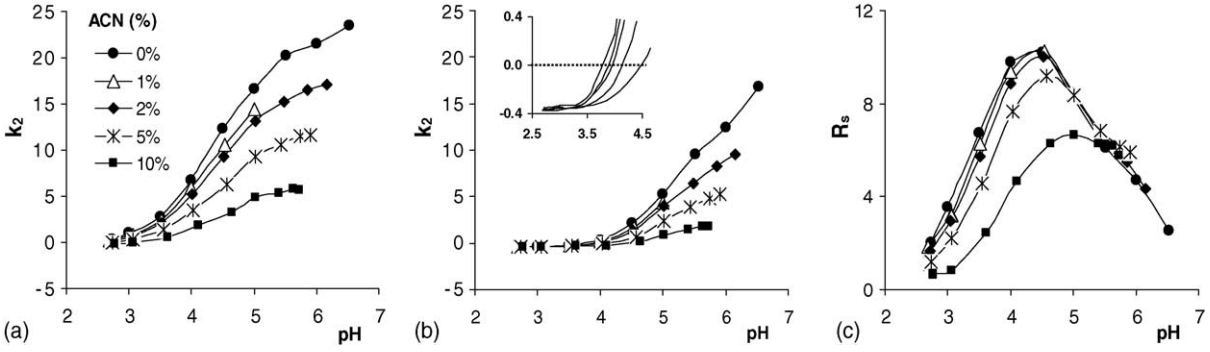

Fig. 3. Influence of pH in dependence of organic modifier concentration on retention factor of target peptide (k_2) (a), retention factor of its main impurity (k_1) (b) and resolution R_s (c) for RP/WAX separation. Experimental conditions: stationary phase, RP/WAX (5 μm, 150 mm × 4 mm i.d.); mobile phase, 1% (v/v) acetic acid containing 0–10% ACN, pH 2.7–6.5 adjusted with ammonia; flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm. The enlargement over (b) shows the negative values of k_1, which are indicative of the ion-exclusion phenomena.

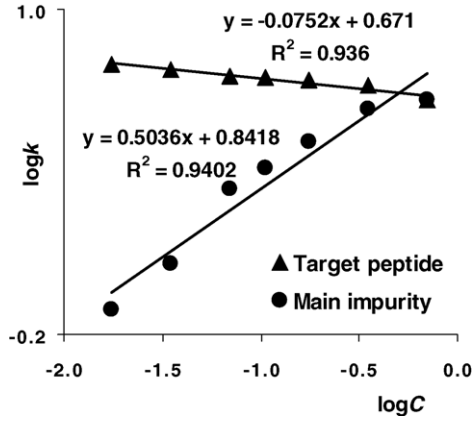

Fig. 4. Plots of $\log k$ vs. $\log C$, where C is the acetic acid concentration (M), for the separation of target peptide from its main impurity on RP/WAX. Experimental conditions: stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, acetic acid (0.0175–0.70 M) containing 10% ACN, pH 4.5 adjusted with ammonia; flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm.

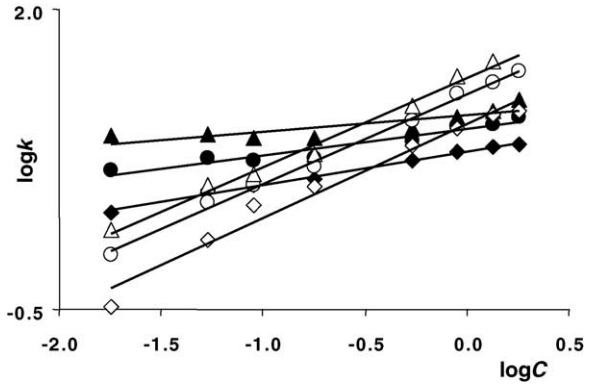

Fig. 5. Plots of $\log k$ vs. $\log C$, where C is the formic acid concentration (M), for the separation of target peptide from its main impurity on RP/WAX at three different pH-values. Experimental conditions: stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, formic acid (0.0180–1.8 M) containing 10% ACN, pH adjusted to 4.5 (▲, △), 4.0 (●, ○), or 3.5 (♦, ◊) with ammonia; flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm. Closed symbols, target peptide; open symbols, main impurity.

tion contribution. In contrast, the main impurity shows an increase of retention, when the ammonium acetate concentration is increased. Such a tendency might be expected if repulsive ionic interactions are active. Higher ionic strength causes a thinner double layer around the solute ion and the surface-bound ion (i.e. the ion-exchange site) and thus a lower potential. This of course weakens the actual electrostatic repulsion between positively charged ion-exchanger and (presumably) positively charged peptide impurity. If the electrostatic repulsion is weaker, the hydrophobic retention increment may become more important and even dominant. Consequently, if the ionic interactions are diminished at high ionic strength, similarly poor selectivity as observed on typical RP18 stationary phase is also seen here.

From a practical point of view, ammonium formate buffer would be preferred over ammonium acetate, as it can be more easily removed by lyophilization. Fig. 5, however, reveals some profound differences in the behavior of acetate and formate counter-ions. Both impurity and peptide show positive slope, i.e. absence of ion-exchange. Like for the impurity, a repulsive ionic interaction mode seems to be active also for the target peptide. The difference between the two distinct counter-ions could be explained by an ion-pairing mechanism: acetate is a better ion-pairing agent than formate and more tightly interacting with the guanidinium group of the Arg side chain, what reduces the effective positive charge on this moiety. Therefore, an ion-exchange mechanism may be established with acetate, what does not happen with formate. This would be in agreement with the both distinct behaviors as discussed.

3.4.3. Isocratic elution

With focus on later preparative scale application, an isocratic elution, if appropriate, was thought to be the method

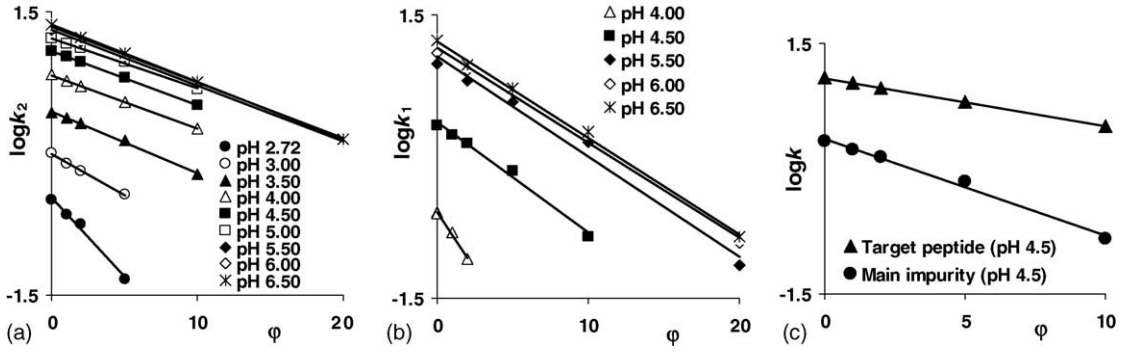

Fig. 6. Plots of $\log k$ vs. acetonitrile percentage (φ), in dependence of pH, for target peptide (a) and its main impurity (b). Experimental conditions: stationary phase, RP/WAX (5 μm, 150 mm × 4 mm i.d.); mobile phase, 1% (v/v) acetic acid containing 0–20% ACN, pH 2.7–6.5 adjusted with ammonia; flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm. In (c), only the peptide and impurity curves at pH 4.5 are shown, illustrating the change of selectivity with the organic modifier content.

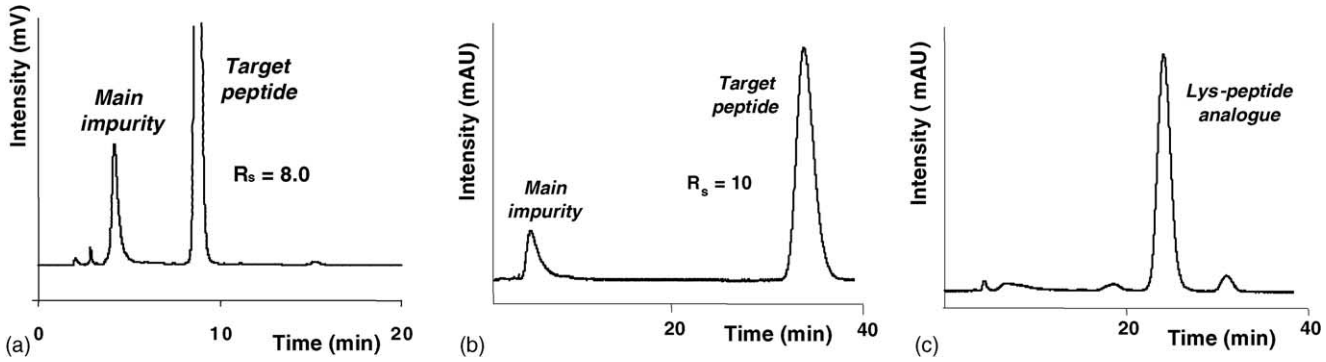

Fig. 7. Optimized separations of *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide (a/b) and the Lys-peptide analogue *N*-acetyl-Ile-Glu-Gly-Lys-*p*-nitroanilide (c) using RP/WAX with hydro-organic mobile phase (a/c) and purely aqueous eluent (b). Experimental conditions: (a) stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, ACN–water–1 M formic acid pH 4.5 (20:70:10; v/v/v) (pH adjusted with ammonia); (b) stationary phase, RP/WAX (5 μm, 150 mm × 4 mm i.d.); mobile phase, 1% (v/v) aqueous acetic acid, pH 4.5 adjusted with ammonia, no ACN; (c) stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, formic acid (0.0180 M) containing 10% ACN, pH 4.5 adjusted with ammonia. Other conditions: flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm.

of choice regarding eluent savings and avoiding extra-time required for re-conditioning in gradient elution mode. Therefore, we primarily investigated the isocratic mode and studied the effect of organic modifier content in dependence of the pH. The relationship between retention factor and percentage of acetonitrile is fully in agreement with the linear solvent strength theory for both target peptide and impurity (Fig. 6).

Fig. 7a depicts the chromatogram of an optimized isocratic separation of the *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide peptide from its major impurity, which gives a resolution of about 8. In order to demonstrate the applicability of the present RP/WAX stationary phase with purely aqueous mobile phases, the same separation employing such conditions is depicted in Fig. 7b (R_s = 10). It is evident that selectivity and resolution are vastly improved compared to RP-HPLC (cf. Fig. 2, R_s = 1.9), what clearly emphasizes the beneficial effect of combining both RP and WAX mechanisms. Moreover, the peptide analogue, which has Arg replaced by Lys and is also an added-value product, can be separated by the same method (Fig. 7c).

3.4.4. Gradient elution

The elution conditions for the RP/WAX purification of a new peptide mixture are difficult to predict, due to the complex separation mechanism. Moreover, anticipating that peptide impurities might vary substantially in their elution behavior in relation to the target product, three generic gradient methods have been tested, which might be useful for an initial screening, mainly if late-eluting impurities are present. The first of these gradient methods consists of an organic modifier (acetonitrile) gradient at constant ionic strength (Fig. 8a), in the second procedure the counter-ion concentration is linearly increased at constant organic modifier percentage (salt gradient) (Fig. 8b), and finally the third method combines the two former ones, i.e. mixed modifier and counter-ion gradients (Fig. 8c).

The best results were as expected accomplished with the modifier gradient (Fig. 8a), which we therefore suggest as generic method for the RP/WAX stationary phase. Therefore, a linear gradient from 5 to 70% acetonitrile in 20 or 30 min at a constant total ionic strength of 100 mM formate

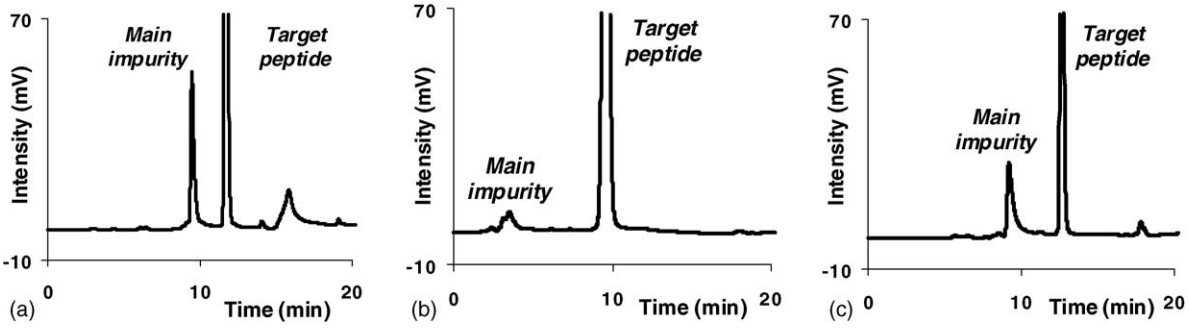

Fig. 8. Gradient elution chromatograms of *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide peptide using RP/WAX (5 μm, 250 mm × 4 mm i.d.) and ACN gradient (a), buffer gradient (b), or mixed ACN and buffer gradients (c). Experimental conditions: stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, bi-distilled water (A), 1 M formic acid, pH 4.5 adjusted with ammonia (B), and ACN (C); (a) linear gradient from 5 to 70% C in 20 min, B constant at 10%; (b) linear gradient from 5 to 50% B in 20 min, C constant at 20%; (c) linear gradient of B and C in 20 min from (A:B:C = 95/0/5) at 0 min to (A:B:C = 10/20/70) at 20 min. Each run was followed by isocratic elution with final composition for 5 min. Other conditions: flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm.

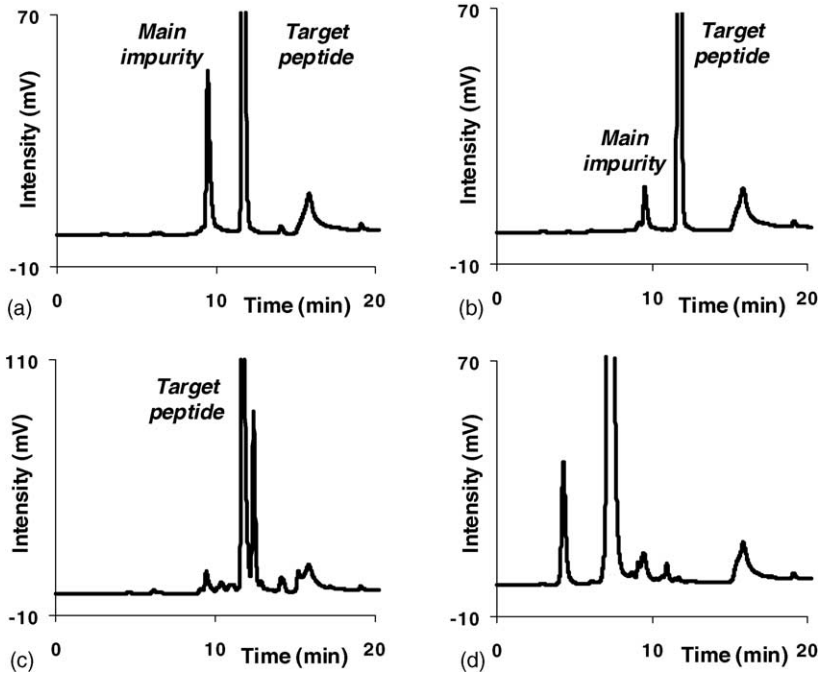

Fig. 9. Gradient elution chromatograms of three different batches of *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide peptide (a/b/c) and of product of failed peptide synthesis (d) using RP/WAX and ACN gradient. Experimental conditions: same as in Fig. 8a.

or acetate buffer (pH between 4 and 6 adjusted with ammonia) is suggested as preliminary test (Fig. 9). If the detection is performed at lower wavelengths (e.g. 210 nm), it is advisable to reduce the buffer concentration to 50 or even 20 mM to minimize UV interference, or even better replace the organic buffers by inorganic phosphate buffer.

Depending on the spectrum of side products, the other two gradient methods may also be appropriate and provide better selectivity. Particularly when multiply negatively charged compounds are present in the sample, a high counter-ion concentration may be required. Replacing the univalent counter-ion by a bivalent one and adopting the buffer gradient elution technique are recommended in that case.

3.5. Loadability studies in the batch chromatography mode

In order to preliminarily assess the mass loading capacity of the mixed-mode RP/WAX phase, an analytical column (5 μm, 250 mm × 4 mm i.d.) was loaded with increasing masses (1–200 mg) of crude peptide, as obtained from the synthesis protocol. Ammonium acetate was used as buffer and the system was operated under isocratic conditions (for conditions, see Fig. 10).

As can be seen in Fig. 10, the analytical column easily tolerates mass loadings up to 200 mg still without touching bands between major impurity and target peptide. This is exceptionally remarkable and typical for ion-exchange systems. However, when ammonium acetate was replaced by the more volatile ammonium formate, the loading capacity dropped significantly, e.g. from more than 200 mg to approximately 50 mg in batch chromatography process (Fig. 11). It appears that by omission of the ion-exchange retention contribution, as observed with formate buffer (positive slope of $\log k$ versus $\log C$, Fig. 5), the loadability falls off substantially. With regards of high loading capacity, acetate buffer is therefore the preferred choice for the given separation.

3.5.1. Purity control of collected fractions

The fractions of the semi-preparative separations corresponding to impurities and target peptide were collected separately and re-chromatographed to check their purity and the

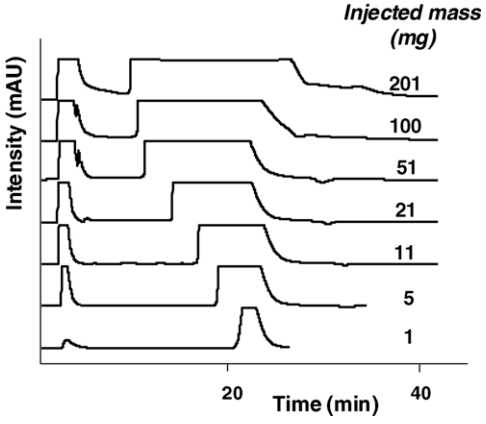

Fig. 10. Loadability studies with *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide peptide on RP/WAX using acetate buffer. Experimental conditions: stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, acetic acid (0.0175 M) containing 10% ACN, pH 4.5 adjusted with ammonia; flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm.

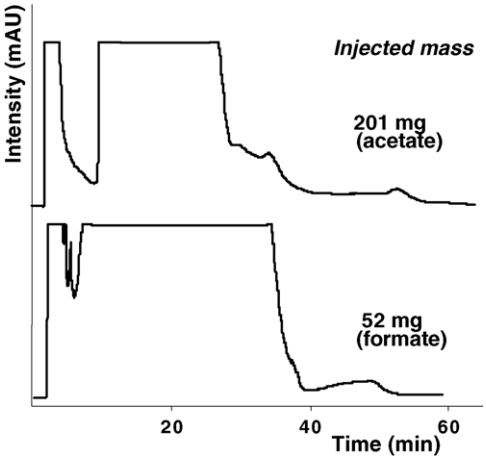

Fig. 11. Comparison of loadabilities of *N*-acetyl-Ile-Glu-Gly-Arg-*p*-nitroanilide peptide on RP/WAX using acetate and formate buffers. Experimental conditions: stationary phase, RP/WAX (5 μm, 250 mm × 4 mm i.d.); mobile phase, acetic acid (0.0175 M) or formic acid (0.0180 M) containing 10% ACN, pH 4.5 adjusted with ammonia; flow-rate, 1 ml/min; room temperature; detection wavelength, 316 nm.

success of the chromatographic purification. Fig. 12 shows the chromatograms obtained in a capillary HPLC using gradient elution reversed-phase with UV detection for the following fractions: (a) non-purified raw peptide, (b) purified target peptide, and (c) combined fractions removed during RP/WAX semi-preparative purification. It is clearly seen that a single purification step on the new RP/WAX stationary phase yields a peptide product with good quality. Considering only the main impurity (it also contains *p*-nitroanilide and has therefore a comparable extinction coefficient to the target peptide), whose elimination was our objective, there was a reduction from 4% to less than 0.03%.

The high purity of the target peptide fraction was also confirmed by on-line gradient elution RP-HPLC coupled to

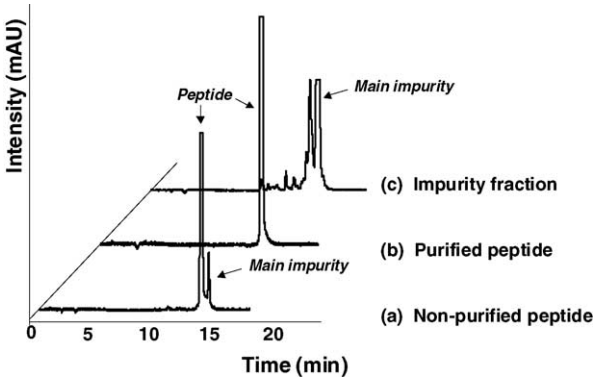

Fig. 12. Purity control by gradient elution capillary RP-HPLC-UV of non-purified peptide and collected fractions from semi-preparative chromatography of peptide on mixed-mode RP/WAX stationary phase. (a) Crude peptide, (b) purified target peptide, and (c) collected fractions of main impurities. Experimental conditions: stationary phase, C18 column (5 μm, 150 mm × 0.5 mm i.d.); mobile phase, 0.1% TFA in water (A), 0.1% TFA in ACN (B), linear gradient from 5 to 60% B in 20 min; temperature, 25 °C; flow-rate, 15 μl/min; detection wavelength, 316 nm.

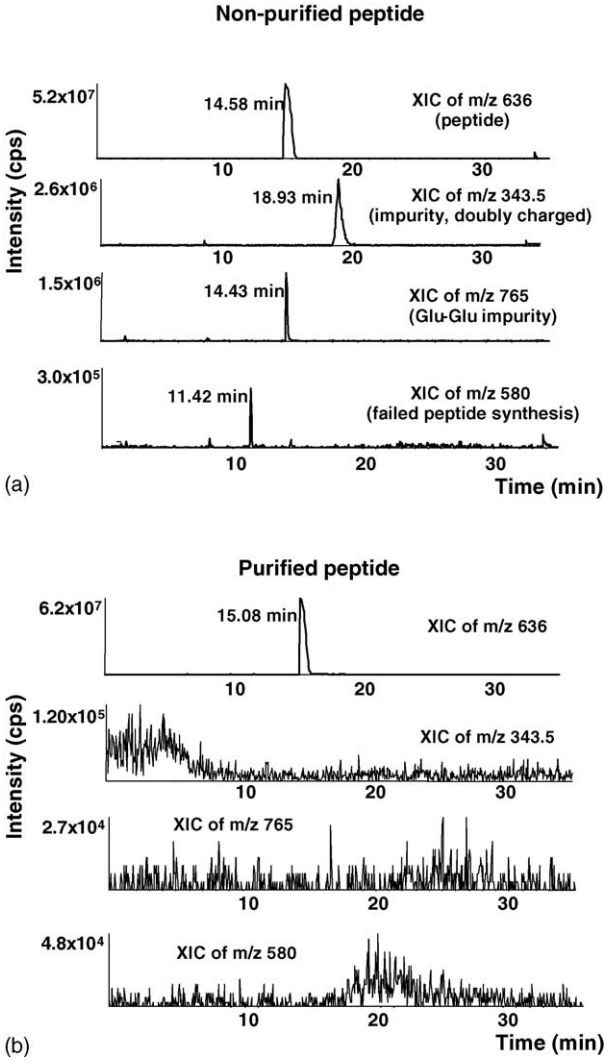

Fig. 13. Purity control by gradient elution RP-HPLC with on-line ESI-MS detection (positive ion mode) of non-purified peptide and collected fractions from semi-preparative chromatography of peptide on mixed-mode RP/WAX stationary phase. (a) Crude peptide, and (b) purified target peptide. From top to bottom: extracted ion chromatograms (XIC) of target peptide ($[M+H]^+$ 636), main impurity as doubly charged species ($[M+H]^+$ 343.5), other impurity containing -Glu-Glu- ($[M+H]^+$ 765), and impurity correspondent to peptide of failed synthesis in the sixth step ($[M+H]^+$ 580). Experimental conditions: stationary phase, RP-18 column (5 μm, 125 mm × 3 mm i.d.), mobile phase, 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B), linear gradient from 5 to 60% B in 20 min; flow-rate, 0.5 ml/min; temperature, 25 °C; detection wavelength, 316 nm.

ESI-MS (positive ion mode). Formic acid was used instead of trifluoroacetic acid (TFA), since the latter has a detrimental effect on the ionization efficiency due to ion-pairing effect with positively charged groups. Fig. 13 shows the results for (a) the non-purified and (b) purified peptide samples. The following extracted ion chromatograms (XIC) are depicted from top to bottom: $[M+H]^+$ 636 (target peptide) $[M+H]^+$ 343.5 (main impurity as doubly charged species, the mass of 685 was also found by MALDI) $[M+H]^+$ 765 (another impurity containing -Glu-Glu- instead of Glu in the peptide structure),

and $[M + H]^+$ 580 (complete failure of peptide formation in the 6th synthesis step in another synthesis batch, Fig. 9d). After RP/WAX purification, the signals from the impurities disappeared and were amounting to less than their detection limit by ESI-MS.

Herewith, it could be convincingly demonstrated that the single purification step with the new mixed-mode RP/WAX stationary phase afforded a highly pure peptide. The procedure removed not only the main peptide impurity, but also other minor impurities, even when the applied sample load of raw peptide on the analytical column (250 mm × 4 mm i.d.) was as high as 200 mg.

4. Conclusions

We have shown herein that the novel peptide purification concept utilizing a new mixed-mode reversed-phase/weak anion-exchange type stationary phase has great potential for peptide separations by high-performance liquid chromatography. The new selector, covalently bonded to the surface of thiol-modified silica, features at least two distinct binding domains in a single interactive ligand: the surface-near anion-exchange site of the quinuclidinium ring and the hydrophobic alkyl chain with polar embedded sulfide and amide groups, which links the former to the support and represents the reversed-phase domain.

Hence, two separation modes can be combined in a single separation unit, which may lead to selectivities that are complementary to conventional standard gradient elution RP-HPLC separations and may outperform the latter. Thereby, specific separation problems may be solved, since selectivity is based on hydrophobicity and charge differences as well. The individual retention increments may be to some extent independently balanced by experimental variables such as pH, counter-ion type, ionic strength, as well as organic modifier type and content. More variables are available for fine-tuning of the separation than in RP-HPLC, which is however accompanied by a more complex mechanism as the major trade-off of the method. In general, weakly acidic conditions are employed and volatile buffers like ammonium acetate may also be utilized.

The great capability of the new purification concept has been exemplified by its application to a given peptide separation problem. It was found that the new mixed-mode reversed-phase/weak anion-exchange separation mechanism offers better selectivity and resolution in the purification of peptides with acidic or basic side chains compared to the traditional separation by reversed-phase HPLC (gradient of ACN containing TFA as ion-pairing agent), which translates into better quality of the purified peptide in terms of spectrum of minor impurities.

In accordance with ion-exchange systems, the present stationary phase exhibited an enhanced mass loading capacity over RP columns in the semi-preparative batch chromatographic mode, which might be attributed to the substantial contribution of ion-exchange mechanism which is known to possess increased saturation capacities. Both better selectivity and higher saturation capacity may have contributed to this enhanced sample loading capacity, which, in turn, results in an improved productivity of the new purification protocol. For example, the yield for the given peptide after RP/WAX purification was by a factor of about 15 higher compared to the RP purification protocol (ca. 65 mg/g RP/WAX versus 4 mg/g RP).

The chemistry of RP/WAX synthesis is simple and cheap enough to enable its up-scaling. This was already performed in a 100 g scale on 10 μm particles, which showed essentially identical separation and loadability as the 5 μm material. Finally, also the batch-to-batch (including column-to-column repeatabilities) were tested and found to be satisfactory (RSD < 6% for peptide retention time, three different batches packed into three columns, 10 HPLC injections). All these data, in particular the high loadability, the vastly improved productivity in the semi-preparative batch chromatography, and the high purity of the obtained peptide, are indicators for a high potential of the RP/WAX separation system in preparative scale applications.

Acknowledgements

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