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Replacement of acetonitrile by ethanol as solvent in reversed phase chromatography of biomolecules

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ABSTRACT

Acetonitrile, which is a by-product of acrylonitrile synthesis, is the commonly used solvent in ion-pair reversed phase chromatography. In consequence of the decreasing demand for acrylonitrile due to the financial crisis, a worldwide shortage of acetonitrile is observed. Therefore, the aim of this study was to establish ion-pair reversed phase chromatographic assays using alternative eluents for acetonitrile and to decrease costs incurred hereby. We compared the performance of ion-pair reversed phase chromatography using acetonitrile with the alternative eluents methanol, ethanol and *n*-propanol, using monolithic reversed phase C5 as well as C18 chromatography columns. We used triethylammonium acetate (TEAA) and tetrabutylammonium sulfate (TBA) as representative cationic ion-pair reagents and trifluoroacetic acid (TFA) as representative anionic ion-pair reagent. For covering a large field of applications, we fractionated representative low, middle and high-molecular weight biomolecules, in particular dinucleoside polyphosphates, peptides, proteins and tryptic digested human serum albumin. Whereas the chromatographic characteristics of both methanol and *n*-propanol were partly insufficient, ethanol was characterised equally or partly even better in the matter of elution strength and separation quality compared to the eluent water–acetonitrile. In conclusion, ethanol is an appropriate alternative for acetonitrile in ion-pair reversed phase chromatography of biomolecules.

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1. Introduction

Isolation, purification and quantification of biomolecules are the essential procedures in current biochemical and medical research. Ion-pair reversed phase chromatography is a common method for separation and purification of biomolecules, both for low molecular weight and highly charged biomolecules like dinucleoside polyphosphates and for middle and high-molecular weight ampholytic biomolecules like peptides and proteins [1–4]. Therefore, ion-pair reversed phase chromatography is used for desalting steps to avoid an interference of salt in the following physiological/pathophysiological characterisation steps, chromatographic purification and mass-spectrometric analysis. Furthermore, in reversed phase chromatography of ionic substances the use of ion-pair reagents is applied in order to mask their charge and allow their retention on the hydrophobic resin. For instance, the ion-pair reagent tetrabutylammonium sulfate (TBA) is commonly used for chromatographic quantification of highly negatively charged low molecular weight biomolecules like dinucleoside polyphosphates [3]. The positively charged ion-pair reagent TBA enhances the hydrophobicity of the complex by its four butylene groups. Unfortunately, TBA is not removable by lyophilisation in the presence of negatively charged substances like dinucleoside polyphosphates because of its low vapour pressure. Therefore, the use of TBA does not allow subsequent mass-spectrometric analysis of the biomolecules of interest. The volatile, positively charged ion-pair reagent triethylammonium acetate (TEAA) is an appropriate alternative for TBA in reversed phase chromatography followed by mass-spectrometric analysis [5], but the resulting chromatographic resolution is reduced compared to TBA. Trifluoroacetic acid (TFA) is a well-suited ion-pair reagent in ion-pair reversed phase chromatography of amphoteric molecules like peptides and proteins (e.g., [4,6–8]). The protons of TFA are masking anionic amino acid residues and the anionic part of TFA trifluoroacetate interacts with cationic amino acid residues. Because of its volatility, TFA is removable by lyophilisation.

The aprotic, polar and water-miscible solvent acetonitrile (ACN) is a commonly used eluent in reversed phase chromatography [9], especially in ion-pair reversed phase chromatography. The decreasing demand for acrylonitrile results in a worldwide shortage of acetonitrile (ACN), since ACN is the by-product of acrylonitrile production. Furthermore, metabolic derivatives of ACN like hydrogen cyanide cause toxic effects. ACN is characterised by its sufficient elution strength for biomolecules paired with minor UV absorption (>190 nm) and low backpressure. Therefore, ACN has been the most suitable solvent for high resolution reversed

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phase separation and fractionation of dinucleoside polyphosphates, peptides and proteins [3,5,10–12]. Primary alcohols like methanol, ethanol or *n*-propanol are water-miscible, polar and protic solvents too. The polarity of these primary alcohols decreases with increasing number of methylene groups.

Therefore, the present study compares the chromatographic characteristics of ACN with methanol, ethanol and *n*-propanol as eluents in chromatographic separation of biomolecules, using both conventional C18 reversed phase columns as well as conventional C5 reversed phase columns.

2. Materials and methods

Acetonitrile, ethanol, methanol and tetrabutylammonium sulfate (TBA) were purchased from Merck (Germany); trypsin (modified sequencing grade) was purchased from Roche (Switzerland). *n*-Propanol and all other substances were purchased from Sigma–Aldrich (Germany). Amicon Ultra centrifugal filter devices (10 kDa) were purchased from Millipore (Germany). HPLC water was generated with a Seralpur Delta ultra filtration unit from ELGA LabWater (Germany). Reversed phase chromatographic separation was performed using conventional C18 monolithic reversed phase columns (Chromolith® Performance RP-18e (100 mm × 4.6 mm I.D., pore size 0.01–2 μm, Merck, Germany)) and conventional C5 reversed phase columns (Discovery® Bio Wide Pore C5-3 (100 mm × 4.6 mm I.D., particle size 3 μm, Sigma–Aldrich, Germany)).

2.1. Comparison of the eluents of interest in reversed phase chromatography in the presence of Ion-pair reagent triethylammonium acetate (TEAA)

Diadenosine polyphosphates (Ap_nA with n = 2–6; each 1 μg) were separated by gradient elution using water and the ion-pair reagent triethylammonium acetate (TEAA) (40 mmol/l (final concentration; pH 6.5; adjusted with triethylamine and formic acetic acid)) as eluent A, and the Chromolith® Performance RP-18e reversed phase column. Eluent and gradient conditions are shown in Table 1. Elution gradient of water–acetonitrile (eluent B_1), water–ethanol (eluent B_2), water–*n*-propanol (eluent B_3) was performed with 10% B_1, B_2, B_3, respectively, within 30 min. Elution gradient of methanol (eluent B_4) was performed with 20% B_4 within 30 min. The column temperature was ambient (22 ± 1 °C). The mobile phase was pumped at a constant flow rate of 1 ml/min by a high-pressure gradient pump system (Merck, Germany). The diadenosine polyphosphate mixture was dissolved in water and was mixed with eluent A. Injection volume was 110 μl. UV absorption was measured at 254 nm. The column eluate was monitored with a variable wavelength UV detector (759 A, Absorbance Detector, Applied Biosystems, Germany). Data were recorded and processed with the Chromeleon Lab System 6.60 (Dionex, Germany).

2.2. Comparison of the eluents of interest in Reversed phase Chromatography in the presence of Ion-pair reagent tetrabutylammonium hydrogen sulfate (TBA)

Diadenosine polyphosphates Ap_nA (with n = 2–6; each 1 μg) were separated by gradient elution on the Chromolith® Performance RP-18e reversed phase column in the presence of TBA (4 mmol/l) in a phosphate buffer (20 mmol/l K_2HPO_4, final concentration; pH 6.5 (adjusted with H_3PO_4 and KOH)) as eluent A. Eluent and gradient conditions are shown in Table 2. Additional experimental conditions were identical as described above.

2.3. Comparison of the eluents of interest in Reversed phase Chromatography in the presence of Ion-pair reagent Trifluoroacetic acid (TFA)

The peptide mixture (bradykinin, angiotensin II, angiotensin I (each 2 μg)) was separated by gradient elution on the Chromolith® Performance RP-18e reversed phase column in the presence of ion-pair reagent 0.1% trifluoroacetic acid (TFA) in water as eluent A. Eluent and gradient conditions are shown in Table 3 in detail.

Table 1
Conditions of chromatographic fractionation of diadenosine polyphosphates ($Ap_{2-6}A$) using a monolithic reversed phase C18 column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TEAA (40 mmol/l) (pH 6.5)	Water/TEAA (40 mmol/l) (pH 6.5)	Water/TEAA (40 mmol/l) (pH 6.5)	Water/TEAA (40 mmol/l) (pH 6.5)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Water–ethanol (50:50, v/v)	Water–*n*-propanol (50:50, v/v)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 10% B_1 at 30 min	0% B_2 at 0 min 10% B_2 at 30 min	0% B_3 at 0 min 10% B_3 at 30 min	0% B_4 at 0 min 20% B_4 at 30 min

Table 2
Conditions of chromatographic fractionation of diadenosine polyphosphates ($Ap_{2-6}A$) using a monolithic C18 reversed phase column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)	Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)	Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)	Water/TBA/K_2HPO_4 (4 mmol/l/20 mmol/l) (pH 6.5)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Ethanol (100%)	*n*-Propanol (100%)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 40% B_1 at 30 min	0% B_2 at 0 min 20% B_2 at 30 min	0% B_3 at 0 min 10% B_3 at 30 min	0% B_4 at 0 min 50% B_4 at 30 min

Table 3
Conditions of chromatographic fractionation of peptides (bradykinin, angiotensin I, angiotensin II) using a monolithic C18 reversed phase column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TFA (0.1%)	Water/TFA (0.1%)	Water/TFA (0.1%)	Water/TFA (0.1%)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Ethanol (100%)	*n*-Propanol (100%)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 40% B_1 at 30 min	0% B_2 at 0 min 40% B_2 at 30 min	0% B_3 at 0 min 40% B_3 at 30 min	0% B_4 at 0 min 50% B_4 at 30 min

Table 4
Conditions of chromatographic fractionation of proteins (cytochrome *C*, trypsin inhibitor type II-S, myoglobin) using a monolithic C5 reversed phase column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TFA (0.1%)	Water/TFA (0.1%)	Water/TFA (0.1%)	water/TFA (0.1%)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Ethanol (100%)	*n*-Propanol (100%)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 80% B_1 at 90 min	0% B_2 at 0 min 80% B_2 at 90 min	0% B_3 at 0 min 80% B_3 at 90 min	0% B_4 at 0 min 80% B_4 at 90 min

Protein mixture (cytochrome *C*, trypsin inhibitor type II-S, myoglobin (each 10 μg)) was separated by gradient elution on the Discovery® Bio Wide Pore C5-3 reversed phase column using the same conditions as described above. Eluent and gradient conditions are displayed in Table 4. UV absorption was measured at 220 nm. All additional experimental conditions were identical as described above.

Next, human serum albumin was digested with aqueous ammonium bicarbonate (50 mmol/l, pH 7.5; adjusted with NH_4OH and HCl) and 0.5% w/v trypsin (37 °C; 24 h). Tryptic peptides were filtered through 10 kDa Amicon Ultra centrifugal filter device.

Tryptic digested human serum albumin were separated by gradient elution on the Chromolith® Performance RP-18e reversed phase column in the presence of ion-pair reagent 0.1% trifluoroacetic acid (TFA) in water as eluent A. Eluent and gradient conditions are displayed in Table 5. UV absorption was measured at 280 nm.

The human serum albumin after tryptic digestion was separated by gradient elution on the Chromolith® Performance RP-18e reversed phase column in the presence of ion-pair reagent 0.1% trifluoroacetic acid (TFA) in water as eluent A. Eluent and gradient conditions are given in Table 5 in detail.

Table 5
Conditions of chromatographic fractionation of human serum albumin after typtic digestion using a monolithic C18 reversed phase column.

Eluent A	Eluent A	Eluent A	Eluent A
Water/TFA (0.1%)	Water/TFA (0.1%)	Water/TFA (0.1%)	Water/TFA (0.1%)
Eluent B_1	Eluent B_2	Eluent B_3	Eluent B_4
Water–acetonitrile (20:80, v/v)	Ethanol (100%)	Water–*n*-propanol (50:50, v/v)	Methanol (100%)
Gradient	Gradient	Gradient	Gradient
0% B_1 at 0 min 50% B_1 at 90 min	0% B_2 at 0 min 50% B_2 at 90 min	0% B_3 at 0 min 50% B_3 at 90 min	0% B_4 at 0 min 80% B_4 at 90 min

3. Results and discussion

This study is focused on the broad variation of mobile phase characteristics in order to replace acetonitrile in ion-pair reversed phase chromatography. Experimental chromatographic conditions, like eluents, ion-pair reagents and elution gradients were modified in large range and the resulting chromatographic separation was compared to chromatographic separation using the conventional eluent ACN.

Previous studies indicate that low molecular weight biomolecules like dinucleoside polyphosphates are quantifiable by ion-pair reversed phase chromatography in the presence of TBA as ion-pair reagent. Since TBA is not removable from dinucleoside polyphosphates, TEAA is used as ion-pair reagent in case of subsequent analysis of dinucleoside polyphosphates by mass-spectrometrical techniques [5]. For amphoteric molecules like peptides and proteins TFA is generally common in order to mask the negative and positive charges of amino acid residues (e.g., [7,8,13])

Fig. 1 shows characteristic reversed phase chromatograms of Ap_nA (with $n = 2–6$) using a conventional C18 monolithic silica reversed phase column in the presence of TEAA as ion-pair reagent using water–acetonitrile (20:80, v/v) (Fig. 1A), water–ethanol (50:50, v/v) (Fig. 1B), water–*n*-propanol (50:50, v/v) (Fig. 1C), and 100% methanol (Fig. 1D).

The peaks labelled in Fig. 1 represent the UV absorptions of Ap_nAs (with $n = 2–6$). Diadenosine polyphosphates were separated by using eluent water–acetonitrile (20:80, v/v) and a gradient of 10% water–acetonitrile within 30 min (Fig. 1A). The resolution is comparable to water–ethanol (50:50, v/v) using an identical gradient (Fig. 1B), but extended retention times were observed. Larger amount of the eluent water–ethanol caused minor separa-

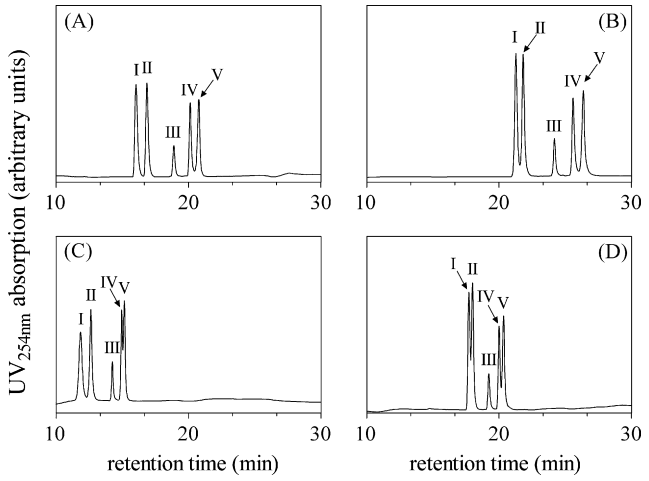

Fig. 1. Effect of different eluents on reversed phase chromatography of diadenosine polyphosphates (Ap_nA; with $n = 2–6$) on an analytical monolithic C18 reversed phase high performance liquid chromatographic column (Chromolith® Performance RP-18e (100 mm × 4.6 mm I.D., Merck, Germany)) in the presence of the ion-pair reagent triethylammonium acetate (TEAA) (40 mmol/l) in water (pH 6.5): (A) water–acetonitrile (20:80, v/v); gradient: 0 min: 0% B; 0–30 min: 0–10% B; 30–33 min: 10–100% B; 33–36 min: 100% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A. (B) Water–ethanol (50:50, v/v); gradient: 0 min: 0% B; 0–30 min: 0–10% B; 30–33 min: 10–100% B; 33–36 min: 100% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A. (C) Water–*n*-propanol (50:50, v/v); gradient: 0 min: 0% B; 0–30 min: 0–10% B; 30–33 min: 10–100% B; 33–36 min: 100% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A. (D) 100% methanol; gradient: 0 min: 0% B; 0–30 min: 0–20% B; 30–33 min: 20–100% B; 33–36 min: 100% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A.

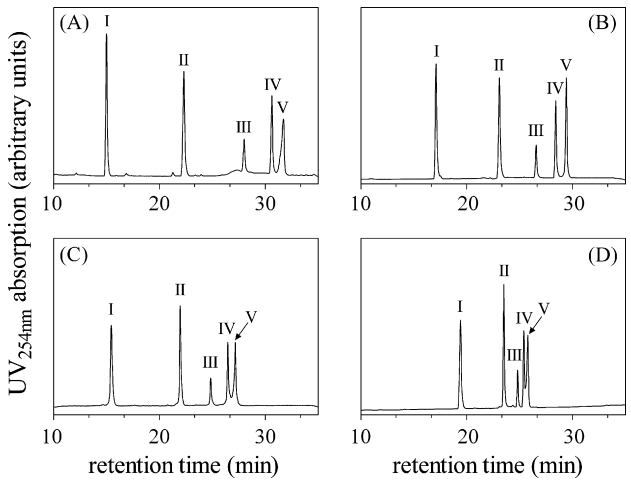

Fig. 2. Effect of different eluents on reversed phase chromatography of diadenosine polyphosphates (Ap_nA; with $n = 2–6$) on an analytical monolithic C18 reversed phase high performance liquid chromatographic column (Chromolith® Performance RP-18e (100 mm × 4.6 mm I.D., Merck, Germany)) in the presence of the ion-pair reagent tetrabutylammonium hydrogen sulfate (TBA) (4 mmol/l) and 20 mmol/l K_2HPO_4 in water (pH 6.5): (A) water–acetonitrile (20:80, v/v); gradient: 0 min: 0% B; 0–30 min: 0–40% B; 30–33 min: 40–100% B; 33–36 min: 100% B, 36–45 min: 0% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A. (B) 100% ethanol; gradient: 0 min: 0% B; 0–30 min: 0–20% B; 30–33 min: 20–100% B; 33–36 min: 100% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A. (C) 100% *n*-propanol; gradient: 0 min: 0% B; 0–30 min: 0–10% B; 30–33 min: 10–100% B; 33–36 min: 100% B. Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A. (D) 100% methanol; gradient: 0 min: 0% B; 0–30 min: 0–50% B; 30–33 min: 50–100% B; 33–36 min: 100% B. Labelled peaks are Labelled peaks are I: Ap_2A; II: Ap_3A; III: Ap_4A; IV: Ap_5A; V: Ap_6A.

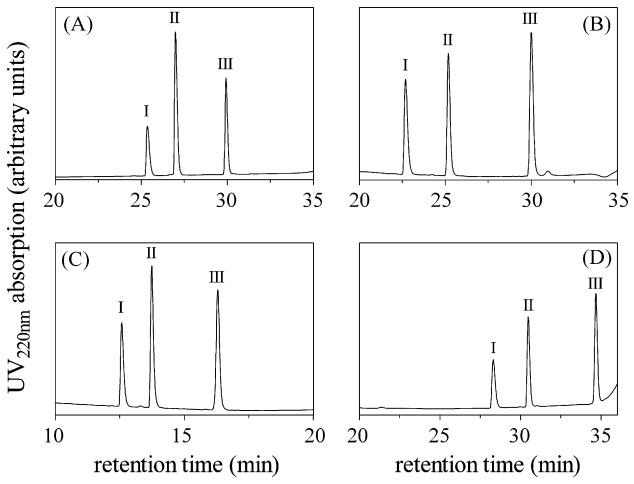

Fig. 3. Effect of different eluents on reversed phase chromatography of synthetic peptide mixture (bradykinin, angiotensin I, angiotensin II,) on an analytical monolithic C18 reversed phase high performance liquid chromatographic column (Chromolith® Performance RP-18e (100 mm × 4.6 mm I.D., Merck, Germany)) in the presence of the ion-pair reagent (0.1%) trifluoroacetic acid (TFA) in water: (A) water–acetonitrile (20:80, v/v); gradient: 0 min: 0% B; 0–30 min: 0–40% B; 30–33 min: 40–100% B; 33–36 min: 100% B. Labelled peaks are I: bradykinin; II: angiotensin II; III: angiotensin I. (B) 100% ethanol; gradient: 0 min: 0% B; 0–30 min: 0–40% B; 30–33 min: 40–100% B; 33–36 min: 100% B. Labelled peaks are I: bradykinin; II: angiotensin II; III: angiotensin I. (C) 100% *n*-propanol; gradient: 0 min: 0% B; 0–30 min: 0–40% B; 30–33 min: 40–100% B; 33–36 min: 100% B. Labelled peaks are I: bradykinin; II: angiotensin II; III: angiotensin I. (D) 100% methanol; gradient: 0 min: 0% B; 0–30 min: 0–50% B; 30–33 min: 50–100% B; 33–36 min: 100% B. Labelled peaks are I: bradykinin; II: angiotensin II; III: angiotensin I.

tion resolution and unlike water–acetonitrile abbreviated retention times resulted. These results indicate that water–ethanol (50:50, v/v) is adequate to replace acetonitrile in ion-pair reversed phase chromatography in the presence of TEAA. A gradient of 10% water–*n*-propanol (50:50, v/v) within 30 min displayed a high resolution of Ap_2A, Ap_3A and Ap_4A, but a lower resolution in the separation of Ap_5A and Ap_6A was observed (Fig. 1C). Thus, retention times were shortened and high backpressure occurred. These results indicate that water–*n*-propanol shows superior elution strength compared to water–acetonitrile but is not capable to substitute acetonitrile in ion-pair reversed phase chromatography.

In contrast, a gradient of 20% B_4 (100% methanol) within 30 min leads to a low resolution of Ap_2A and Ap_3A as well as Ap_5A and Ap_6A but delivered the highest resolution of separation of all examined elution gradient using methanol as eluent (Fig. 1D). A gradient of 5% B_4 in 30 min as well as 10% B_4 in 30 min does not increase resolution but enhance retention times of diadenosine polyphosphates (data not shown). Therefore, methanol is not appropriate to replace acetonitrile in ion-pair reversed phase chromatography.

In contrast, Fig. 2 shows reversed phase chromatograms of Ap_nA (with $n = 2–6$) in the presence of TBA as ion-pair reagent. Again, a C18 monolithic silica reversed phase column was used for separation using the eluent water–acetonitrile (20:80, v/v) (Fig. 2A). Chromatographic separation using the alternative eluents ethanol, *n*-propanol and methanol (each 100%) are shown in Fig. 2B–D. Diadenosine polyphosphates were baseline separated using water–acetonitrile (gradient: 40% B_1 within 30 min) (Fig. 2A) and ethanol (gradient: 20% B_2 within 30 min) (Fig. 2B) as eluent. Chromatography using ethanol as eluent led to an increased resolution of Ap_5A and Ap_6A, compared to chromatography using water–acetonitrile. Therefore, ethanol is a superior alternative for acetonitrile in reversed phase chromatography.

While a sufficient resolution of Ap_2A, Ap_3A and Ap_4A using *n*-propanol was achieved by decreasing the elution gradient to 10% B_3 within 30 min, the separation of Ap_5A and Ap_6A was insufficient (Fig. 2C). Thus, the retention times were shortened and high backpressure occurred. These results indicate that water–*n*-propanol shows superior elution strength compared to water–acetonitrile; however, it is not capable to replace acetonitrile in ion-pair reversed phase chromatography.

In order to achieve sufficient resolution in separation using methanol, the elution gradient had to be increased to 50% B_4 within 30 min; however, a non-sufficient resolution of Ap_5A and Ap_6A was observed (Fig. 2D). Higher amounts of methanol (between 60% B_4 within 30 min and 80% B_4 within 30 min) decreased the resolution and the retention times of diadenosine polyphosphates (data not shown). These results indicate that methanol is an inferior eluent compared to water–acetonitrile and is not adequate to replace acetonitrile in ion-pair reversed phase chromatography in the presence of TBA.

Fig. 3 shows a reversed phase separation of peptides using a C18 monolithic reversed phase column. Fig. 3A shows the elution of a peptide mixture of bradykinin, angiotensin II and angiotensin I using water–acetonitrile (20:80, v/v) and a gradient of 40% B_1 within 30 min. Fig. 3B shows the elution with 100% ethanol using identical gradient conditions. The peptides were baseline separated using both eluents water–acetonitrile and ethanol. Ethanol shows a similar elution strength and separation characteristics as water–acetonitrile.

Elution using *n*-propanol was performed using a gradient of 40% B_3 within 30 min (Fig. 3C) resulting in a sufficient resolution of the peptides. However, *n*-propanol is not an appropriate alternative for water–acetonitrile as an eluent, since the retention times decreased and high column backpressure was caused this eluent. To realize an ample fractionation of the peptide mixture using methanol as eluent, the elution gradient had to be increased to 50% B_4 within 30 min (Fig. 3D). However, methanol caused a strong baseline shift at larger amounts of methanol.

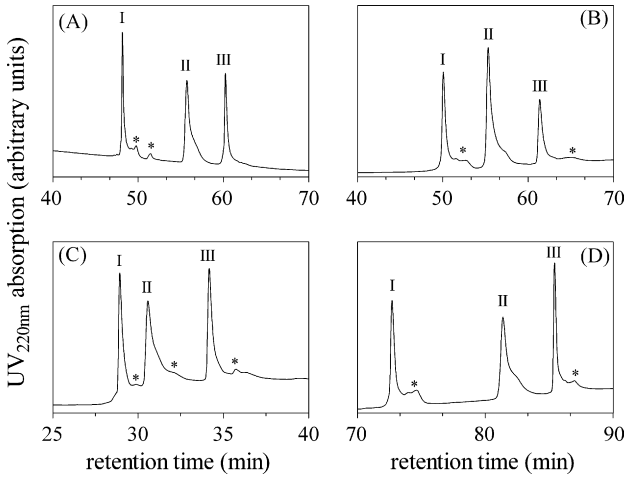

Fig. 4. Effect of different eluents on reversed phase chromatography of a protein mixture (cytochrome *C*, trypsin inhibitor type II-S, myoglobin,) on an analytical reversed phase high performance liquid chromatographic column (Discovery® Bio Wide Pore C5-3 (100 mm × 4.6 mm I.D., Sigma, Germany)) in the presence of the ion-pair reagent (0.1%) trifluoroacetic acid (TFA) in water: (A) water–acetonitrile (20:80, v/v); gradient: 0 min: 0% B; 0–90 min: 0–80% B; 90–93 min: 80–100% B; 93–96 min: 100% B. Labelled peaks are I: cytochrome *C*; II: trypsin inhibitor type II-S; III: myoglobin; *: contamination of the used proteins. (B) 100% ethanol; gradient: 0 min: 0% B; 0–90 min: 0–80% B; 90–93 min: 80–100% B; 93–96 min: 100% B. Labelled peaks are I: cytochrome *C*; II: trypsin inhibitor type II-S; III: myoglobin; *: contamination of the used proteins. (C) 100% *n*-propanol; gradient: 0 min: 0% B; 0–90 min: 0–80% B; 90–93 min: 80–100% B; 93–96 min: 100% B. Labelled peaks are I: cytochrome *C*; II: trypsin inhibitor type II-S; III: myoglobin; *: contamination of the used proteins. 100% methanol; gradient: 0 min: 0% B; 0–90 min: 0–80% B; 90–93 min: 80–100% B; 93–96 min: 100% B. Labelled peaks are I: cytochrome *C*; II: trypsin inhibitor type II-S; III: myoglobin; *: contamination of the used proteins.

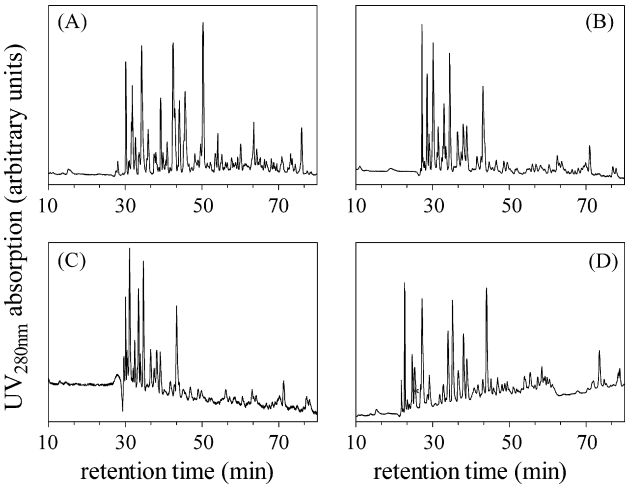

Fig. 5. Effect of different eluents on reversed phase chromatography of human serum albumin after typtic digestion on an analytical monolithic C18 reversed phase high performance liquid chromatographic column (Chromolith® Performance RP-18e (100 mm × 4.6 mm I.D., Merck, Germany)) in the presence of the ion-pair reagent (0.1%) trifluoroacetic acid (TFA) in water: (A) water–acetonitrile (20:80, v/v); gradient: 0 min: 0% B; 0–90 min: 0–50% B; 90–93 min: 50–100% B; 93–96 min: 100% B. (B) 100% ethanol; gradient: 0 min: 0% B; 0–90 min: 0–50% B; 90–93 min: 50–100% B; 93–96 min: 100% B. (C) Water–*n*-propanol (50:50, v/v); gradient: 0 min: 0% B; 0–50 min: 0–80% B; 90–93 min: 50–100% B; 93–96 min: 100% B. (D) 100% methanol; gradient: 0 min: 0% B; 0–90 min: 0–80% B; 90–93 min: 80–100% B; 93–96 min: 100% B.

Fig. 4 shows characteristic reversed phase chromatograms of a characteristic protein mixture composed of cytochrome *C*, trypsin inhibitor type II-S and myoglobin using trifluoroacetic acid (TFA) as ion-pair reagent and a C5 reversed phase column (Fig. 4). The proteins were baseline separated using water–acetonitrile (20:80, v/v) (Fig. 4A), and ethanol 100% (Fig. 4B), respectively. Comparison of the characteristic chromatogram shown in Fig. 4A and B demonstrates that also for proteins ethanol is obviously an adequate alternative for the replacement of ACN in ion-pair reversed phase chromatography. Chromatography using *n*-propanol as eluent results in lower resolution of cytochrome *C* and trypsin inhibitor type II-S and decreased retention times. Furthermore, *n*-propanol showed a strong interference at 220 nm, resulting in a baseline shift (Fig. 4C).

Using methanol as eluent and a gradient of 80% B_4 within 90 min led to a sufficient chromatographic resolution (Fig. 4D), but extended retention times were necessary to achieve this resolution, which caused an increased consumption of solvents. Because of the limited backpressure of the C5 reversed phase column an increased flow rate was realizable to reduce the time for the chromatographic separation.

Fig. 5 shows a reversed phase separation of tryptic digested human serum albumin as an example of complex peptide mixture using a C18 monolithic reversed phase column. Fig. 5A shows the elution of tryptic digested human serum albumin using water–acetonitrile (20:80, v/v) and a gradient of 50% B_1 in 90 min. Fig. 5B shows the elution using 50% B_2 (100% ethanol), using identical gradient conditions as in case of eluent water–acetonitrile. The complex peptide mixture was sufficiently separated using the eluents water–acetonitrile as well as ethanol. Again, ethanol shows large similarities in elution strength and separation quality compared to water–acetonitrile. Elution using *n*-propanol was performed by using a gradient of 50% B_3 (100% *n*-propanol) in 90 min (Fig. 5C) led to lower resolution separation of complex peptide mixture. In addition, reasonable by its higher elution strength abbreviated retention times are observed. Using methanol as eluent the elution gradient has to be increased to 80% B_4 (100% methanol) in 90 min to achieve a sufficient separation of the peptides (Fig. 5D); but enhanced retention times were caused and baseline shift is observed at higher amounts of methanol during the separation.

Ethanol obviously displays most comparable chromatographic characteristics with water–acetonitrile, both for separation of low molecular weight substances like dinucleoside polyphosphates in the presence of TEAA or TBA and for separation of peptides, proteins and complex peptide mixtures, like tryptic digested human serum albumin, in the presence of TFA. Ethanol displays rather the same or even superior characteristics in separation of biomolecules with ion-pair reversed phase chromatography compared to acetonitrile. Therefore, ethanol is the most suitable solvent to replace acetonitrile in reversed phase chromatography. Nevertheless, the elution gradients for the specific chromatography need to be adjusted. Representative chromatographic separation should be performed to optimise the elution gradient using ethanol for separation.

By the reason that the present study focuses on the replacement of acetonitrile as solvent in reversed phase chromatography of biomolecules by an appropriate alternative, we fractionated representative low, middle and high-molecular weight biomolecules and a complex mixture of biomolecules, using conventional linear gradients without optimising the separation and the run time in detail. This optimising procedure is recommended in case of a specific chromatographic problem, considering the less high viscosity of ethanol, which may limit the mass transfer at increased temperature. Since drastic increase of temperature is not essential in chromatography of biomolecules in general, the less high viscosity of ethanol is less important in most cases.

Isolation and quantification of low molecular, middle molecular as well as high-molecular weight biomolecules using ion-pair reversed phase chromatography with acetonitrile as eluent are replaceable by corresponding chromatographies using ethanol as eluent, resulting in a comparable or slightly increased separation

quality and resolution. In addition, the price ethanol (about € 25/l) [14] is much lower than the price for acetonitrile (about € 80/l) [15]. Furthermore, itself ethanol has a minor toxicity than acetonitrile.

Although the chromatographic characteristics of ethanol are comparable or superior to acetonitrile, and ethanol is causing less impact on the environment, than acetonitrile, acetonitrile is surprisingly most often used in biochemical applications, most like because of historical reasons. There was a strong difference in the price of acetonitrile and ethanol in the past, at least because of different tax rates. Because of the drastic increase of purchase price of acetonitrile recently, this advantage does not exist any longer.

In conclusion, we recommend the use of ethanol in comparison to acetonitrile in ion-pair reversed phase chromatography for the separation of low, middle and high-molecular weight substances because of both an increased resolution and decreased costs of ethanol compared to acetonitrile.

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