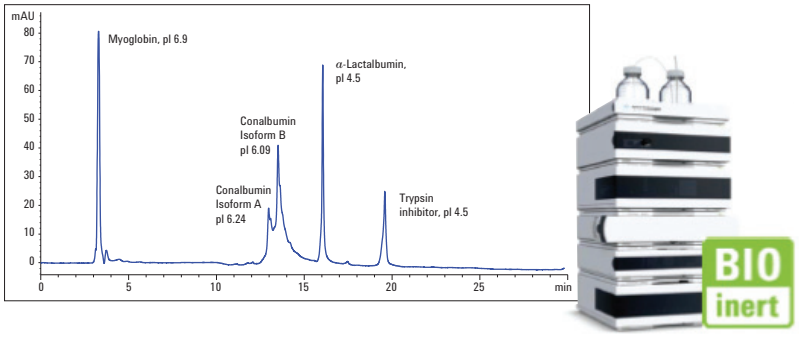
Proof of Performance

Analysis of proteins by anion exchange chromatography

Feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC System for applications using high-salt buffers

Application Note

Chemical and Pharmaceutical Analysis



Author

Sonja Schneider
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

In this Application Note, we show the separation of four proteins by anion exchange chromatography using four different high salt-containing elution buffers (up to 2 M) for linear and step gradients. Due to its iron/steel-free design, the Agilent 1260 Infinity Bio-inert Quaternary LC System was able to withstand the harsh conditions used in bio-analytic and bio-purification applications and thereby maintain its UHPLC performance. High precision of retention time and area was demonstrated using linear gradients for four salt types: sodium chloride (2 M), potassium chloride (1 M), sodium acetate (1 M) and tetramethylammonium chloride (1 M). In addition, the stability of retention time and resolution over 48 hours was proven using 2 M sodium chloride as eluting buffer.

Under these conditions, stainless steel-based LC-systems face problems such as salt-based corrosion and therefore require special care and tedious cleaning procedures. These procedures are no longer necessary with the Agilent 1260 Infinity Bio-inert Quaternary LC System thus increasing throughput and efficiency of bio-analytical or bio-purification application.

Agilent Technologies

Introduction

High salt-containing mobile phases, such as those used in ion exchange (IEX) or size exclusion chromatography (SEC), can be problematic for stainless steel-based LC systems. Due to corrosion effects after long-term usage of salt-containing buffers, these LC systems are at risk of being damaged. Tedious cleaning procedures are the consequences. It is therefore highly recommended to use an 'iron-free' system that is not affected by high salt concentrations.

The Agilent 1260 Infinity Bio-inert Quaternary LC System consists of metal-free components in the sample flow-path. All capillaries and fittings throughout the autosampler, column compartment and detectors are completely metal-free so that biomolecules interact only with ceramics or PEEK. This allows the user to deploy buffers containing high amounts of salt. Additionally, the system is stable in a wider pH range (1–13, short term 14) compared to the Agilent 1260 Infinity LC System[1].

In this Application Note, the feasibility of the Agilent 1260 Infinity Bio-inert Quaternary LC System for anion exchange chromatography (AEX), as an example for a high salt application, is demonstrated. A mix of four proteins was separated by anion exchange chromatography (AEX) by using four different eluting salts: sodium chloride (2 M NaCl), potassium chloride (1 M KCl), sodium acetate (1 M CH3COONa) and tetramethylammonium chloride (1 M [(CH3)4N]Cl). Precision of retention time and area was analyzed for linear and step gradients. In addition, long-time stability (48 h) of retention time and resolution using 2 M NaCl elution buffer was investigated.

Experimental

The Agilent 1260 Infinity Bio-inert Quaternary LC System consisted of the following modules:

- Agilent 1260 Infinity Bio-inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High Performance Bio-inert Autosampler (G5667A)
- Agilent 1260 Infinity DAD VL (G1315D) with bio-inert standard flow cell, 10 mm
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)

Column

Agilent Bio WAX, 5 µm, 4.6 × 250 mm, PK

Software

Agilent OpenLAB CDS, ChemStation Edition for LC & LC MS Systems, Rev. C.01.02 [14]

Solvents

Buffer A: 20 mM Tris, pH 7.6

Buffer B: 20 mM Tris, pH 7.6 +

- 2 M NaCl
- 1 M KCl
- 1 M CH_3COONa
- 1 M $[(CH_3)_4N]Cl$

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Tris was purchased from Fluka (Sigma-Aldrich, St. Louis, USA). NaCl was purchased from VWR, Radnor, PA, USA. $[(CH_3)_4N]Cl$ and KCl were purchased from Merck KGaA, Darmstadt, Germany. CH_3COONa was purchased from J.T. Baker (VWR, Radnor, PA, USA).

Chromatographic conditions

	Linear gradients	Step gradients
Gradient 1 M	5 min – 100% A	5 min – 100% A
	20 min – 70% B	5.01 min – 20% B
	25 min – 100% B	10 min – 20% B
		10.01 min – 40% B
		15 min – 40% B
		15.01 min – 60%B
		20 min – 60 %B
		20.01 min – 100%B
Gradient 2 M	5 min – 100% A	5 min – 100% A
	20 min – 35% B	5.01 min – 10% B
	25 min – 50% B	10 min – 10% B
	25.01 min – 100%B	10.01 min – 20% B
		15 min – 20% B
		15.01 min – 30%B
		20 min – 30 %B
		20.01 min – 100%B
Stop time	30 min	25 min
Post time	20 min	20 min
Temperature	25 °C	
Flow rate	0.5 mL/min	
Injection volume	5 µL	
DAD	280 nm	
Peak width	0.025 min (0.5 s response time) (10 Hz)	

Piston seal wash

- 100% ultrapure water
- Active for 0.3 min every 1.5 min

Proteins

- Myoglobin from equine skeletal muscle, 17,053 Da, pI 6.9 (1 mg/mL)
- Conalbumin from Chicken Egg White, 76,000 Da, pI 6.24 and 6.092 (2 mg/mL)
- α-Lactalbumin from bovine milk, 14,175 Da, pI 4.5 (1 mg/mL)
- Trypsin Inhibitor from Glycine max (soybean), 20,100 Da, pI 4.5 (1 mg/mL)

All proteins were purchased from Sigma-Aldrich, St. Louis, USA.

Results and discussion

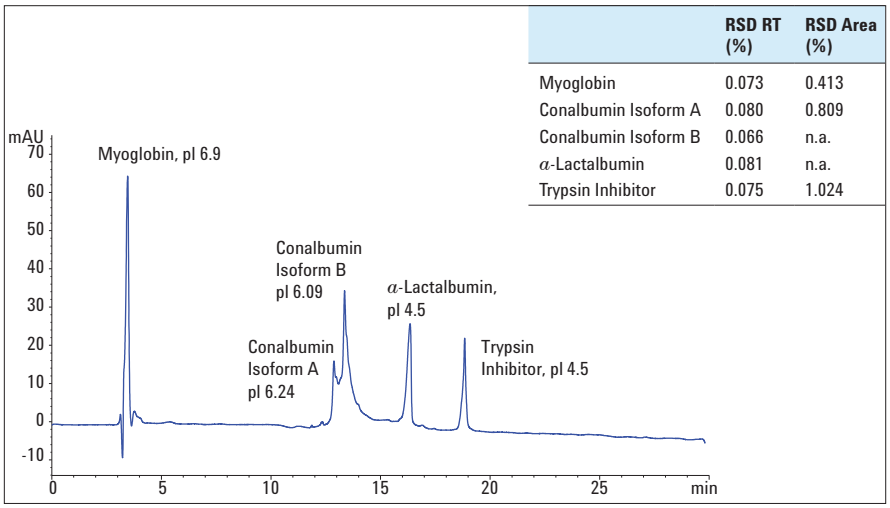
Linear gradients

Anion exchange chromatography using 2 M NaCl

A protein mixture of four proteins was separated via AEX with linear gradients using 2 M NaCl as eluting salt in buffer B. Figure 1 shows the chromatogram of the protein separation by a linear gradient. The proteins are mostly eluting according to their isoelectric point (pI). The separation of α-lactalbumin and trypsin inhibitor is possible due to charge changes on the surface of the protein, which are not necessarily identical with the total net charge. Precision was determined over seven runs as relative standard deviation of retention time and area. Conalbumin exists in different isoforms, depending on the amount of bound iron[2]. Therefore, five peaks were used for the evaluation of retention time precision. In contrast, the conalbumin peaks were not implemented into the calculation of area RSD due to the non-baseline separation of the isoforms. RSD of retention time was <0.09 % for all five peaks. RSD of area was <1.1 % for the evaluated proteins.

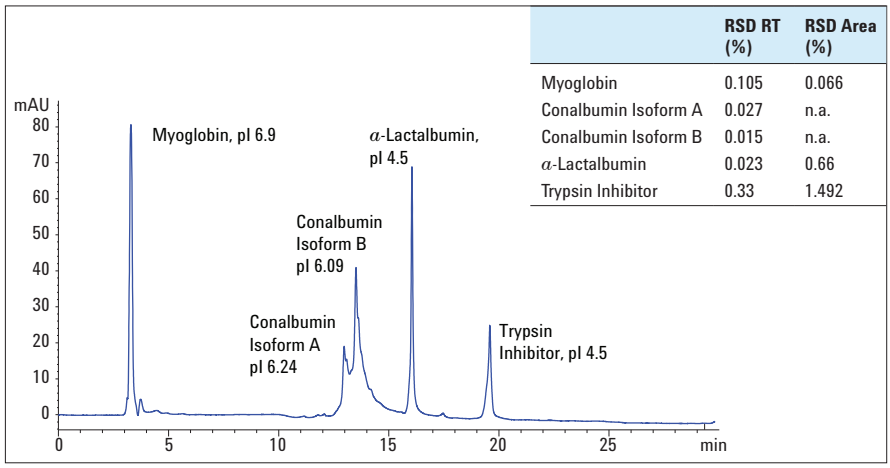
Anion exchange chromatography using different salt types

Resolution and selectivity can be influenced by a changing of the eluting ions, respectively salt types[3]. Depending on the protein to be analyzed, the salts might have different chromatographic effects on the elution of the protein. To find the most appropriate eluting ion for the individual application, various salts can be tested. If more than three salt types are to be tested in one sequence, a solvent selection valve is a good option for method development. In this Application Note, four salts were evaluated in the elution buffer. Figures 2, 3 and 4 show the protein separation using linear gradients with 1 M KCl (Figure 2), 1 M CH_3COONa (Figure 3) and 1 M $[(CH_3)_4N]Cl$ (Figure 4) as eluting salts. RSD of retention time and area was calculated with n = 7.



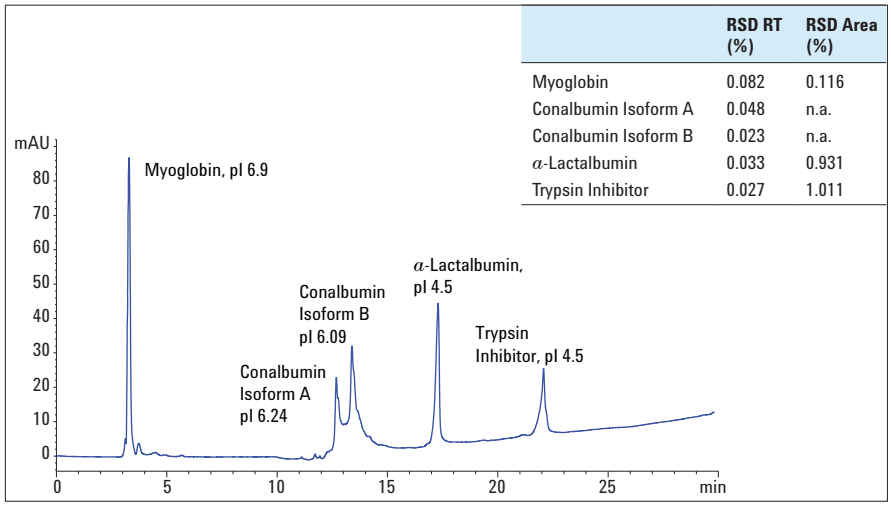
	RSD RT (%)	RSD Area (%)
Myoglobin	0.073	0.413
Conalbumin Isoform A	0.080	0.809
Conalbumin Isoform B	0.066	n.a.
α-Lactalbumin	0.081	n.a.
Trypsin Inhibitor	0.075	1.024

Figure 1
Protein separation by AEX by a linear gradient using 2 M NaCl as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.105	0.066
Conalbumin Isoform A	0.027	n.a.
Conalbumin Isoform B	0.015	n.a.
α-Lactalbumin	0.023	0.66
Trypsin Inhibitor	0.33	1.492

Figure 2
Protein separation by AEX by a linear gradient using 1 M KCl as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.082	0.116
Conalbumin Isoform A	0.048	n.a.
Conalbumin Isoform B	0.023	n.a.
α-Lactalbumin	0.033	0.931
Trypsin Inhibitor	0.027	1.011

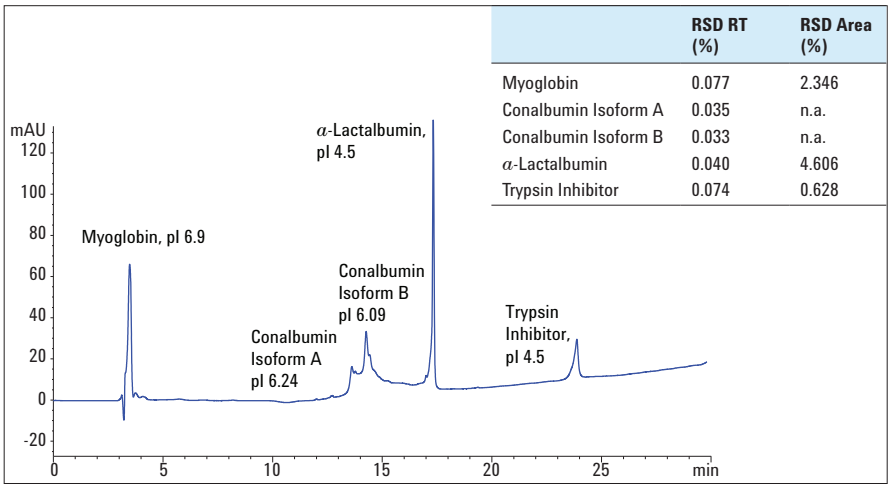
Figure 3
Protein separation by AEX by a linear gradient using 1 M CH_3COONa as eluting salt.

The experiments demonstrate changes in retention time, resolution and in addition changes in peak shape and intensity, depending on the eluting salt used. Especially, the resolution of conalbumin A and B differs between the salt types, whereas 1 M CH_3COONa showed the best resolution. Considering all factors mentioned above (best resolution, best peak shape and highest intensity) together with a flat baseline 1 M KCl was the optimal salt for the separation of the four proteins used.

Step gradients

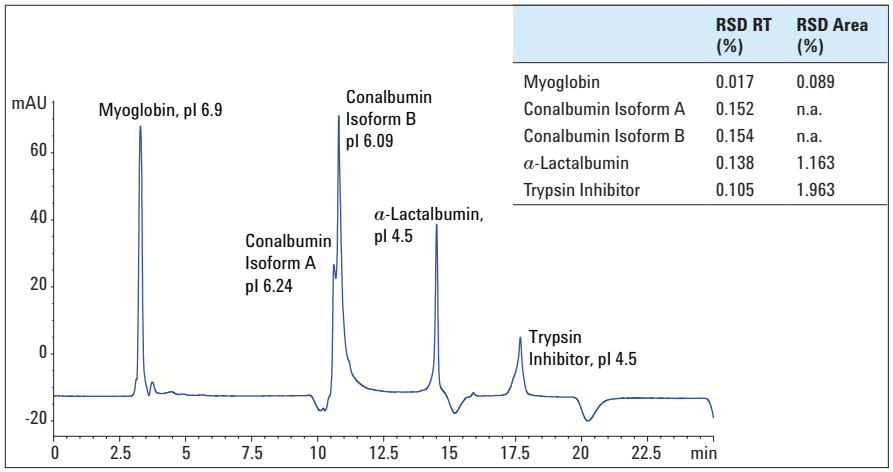
With the use of step gradients, especially if only one protein is to be separated, it is possible to accelerate separation time and thus reduce buffer consumption. Figures 5 and 6 show protein separations by step gradients using 1 M KCl (Figure 5) and 1 M CH_3COONa (Figure 6). RSD of retention time and area is calculated with n = 7.

Due to long equilibration times of the AEX column, precision of retention time and area is slightly inferior compared to the linear gradient. In addition, negative peaks could be observed at the time point of the gradients steps, which is likewise based on missing equilibration time.



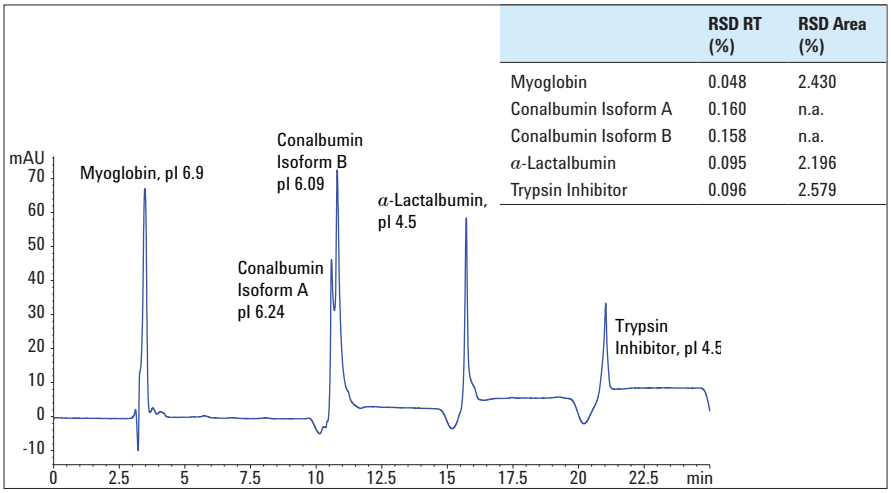
	RSD RT (%)	RSD Area (%)
Myoglobin	0.077	2.346
Conalbumin Isoform A	0.035	n.a.
Conalbumin Isoform B	0.033	n.a.
α-Lactalbumin	0.040	4.606
Trypsin Inhibitor	0.074	0.628

Figure 4
Protein separation by AEX by a linear gradient using 1 M $[(CH_3)_4N]Cl$) as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.017	0.089
Conalbumin Isoform A	0.152	n.a.
Conalbumin Isoform B	0.154	n.a.
α-Lactalbumin	0.138	1.163
Trypsin Inhibitor	0.105	1.963

Figure 5
Protein separation by AEX by a step gradient using 1 M KCl as eluting salt.



	RSD RT (%)	RSD Area (%)
Myoglobin	0.048	2.430
Conalbumin Isoform A	0.160	n.a.
Conalbumin Isoform B	0.158	n.a.
α-Lactalbumin	0.095	2.196
Trypsin Inhibitor	0.096	2.579

Figure 6
Protein separation by AEX by a step gradient using 1 M CH_3COONa as eluting salt.

To prove the long-term stability of retention time and resolution with high salt containing buffers, protein separation (linear gradient) using 2 M NaCl as eluting salt was monitored over 48 h, see Figure 7 and 8. The stability of retention time and resolution could be demonstrated over the whole time period. During all measurements, the piston seal wash (containing 100% water) was active in regular intervals for 0.3 min every 1.5 min to remove salt from the pump pistons.

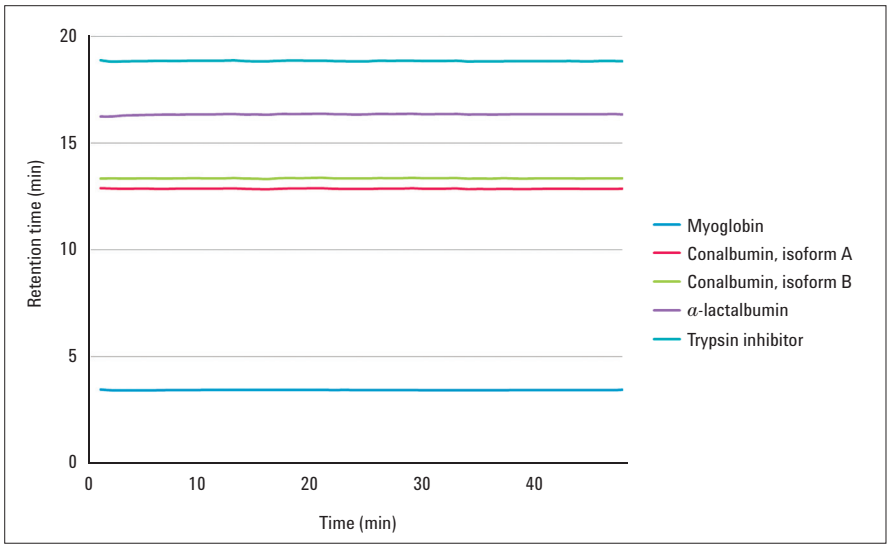


Figure 7
Stability of retention time over 48 h with 2 M NaCl.

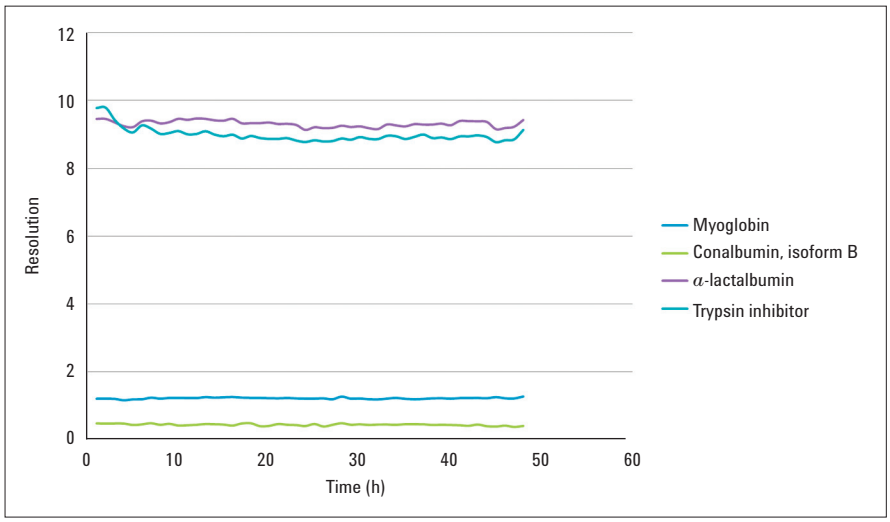


Figure 8
Stability of resolution over 48 h with 2 M NaCl.

Conclusions

This Application Note demonstrated that the Agilent 1260 Infinity Bio-inert Quaternary LC System is an ideal system for high salt applications. By employing anion exchange chromatography for the separation of four different proteins, high precision was achieved for linear gradients with four different eluting ions/salt types. Depending on the salt type used, the chromatograms varied with regards to retention time, resolution, peak shape and intensity. Hence, it is recommended to test different salt types to find the most adequate one for optimal sample separation. If more than three salt types are to be tested, a solvent selection valve is a good option for method development.

Retention time and resolution stability over a time period of 48 hours was confirmed for the analysis of the protein mix with 2 M NaCl as eluting salt.

With the use of step gradients, it is possible to speed up separation time and buffer consumption. However, long equilibration times of the columns have to be considered. Therefore, it is highly recommended to consider enough equilibration time between the single runs, both in step and linear gradients. It is also very important to activate the seal wash (containing 100% water) in regular intervals (for example, 0.3 min every 1.5 min) to remove salt from the pump pistons.

The Agilent 1260 Infinity Bio-inert Quaternary LC System as well as the Agilent Bio WAX, 5 µm, 4.6 x 250 mm, PK column show high stability concerning RT and resolution for high salt applications.

References

1.
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