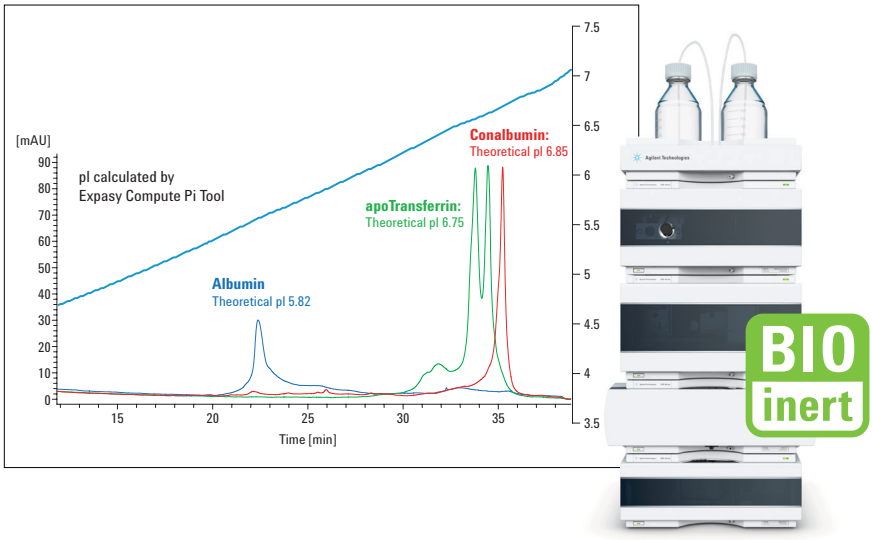
Protein Separation with pH Gradients Using Composite Buffer Systems Calculated by the Agilent Buffer Advisor Software

Technical Overview



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Abstract

This Technical Overview provides some tips and tricks on how to correctly apply the Agilent Buffer Advisor Software for the generation of pH gradients over a wide range using composite buffer systems. With the application of composite buffer systems, the Buffer Advisor Software is able to calculate and optimize linear pH gradients without compromising buffer capacity.

Verified for Agilent
1260 Infinity II LC
Bio-inert System

Agilent Technologies

Introduction

In ion-exchange chromatography, protein mixtures can be separated either by salt-gradient or pH-gradient elution. The elution principle in both modes is different. In salt-gradient mode, the protein is eluted by increasing the washing stringency after applying a NaCl gradient, then, the sodium ions compete with the protein for the charged binding sites on the stationary phase. In pH/gradient mode, the protein's net charge changes during a pH gradient due to protonation/deprotonation of functional groups on the protein. The protein is expected to elute at, or close to, its isoelectric point (pI). Using pH-gradient elution, the proteins are focused in narrower bands enabling higher resolution than usually achieved with salt-gradient elution[1].

Linear pH gradients are difficult to generate because changing ratios between acidic and basic components lead to a constant change of the ionic strength of the mobile phase, which itself has a significant impact on pH[2]. Simple mixing of single buffers with two different pH values in linear range volumes is not applicable due to limitations in buffering capacity depending on the pH to be mixed. With the use of a single buffer system (for example, MES, phosphate, and so on), it is possible to achieve relatively linear gradients over approximately 1.5 pH units. If wider pH ranges are needed, different buffer salts have to be mixed. To generate pH gradients over a wide pH range, Buffer Advisor Software offers so-called composite buffer calculations. Composite buffer systems are buffers consisting of multiple buffering components possessing different pKa values. The composite buffer covers a wide pH range without compromising the buffer capacity if the gradient is optimized by the software[3].

Figure 1 shows the principle of pH gradient calculations with Buffer Advisor Software using a cation exchange gradient mode (CEX) for a validated CEX composite mix with phosphate and citrate salts. For pH gradient generation, only solvent channels C and D are needed. Several additional gradient steps are inserted using the Optimize Gradient function to enable linearity over the complete pH gradient. For anion exchange chromatography (AEX), complementary buffer systems are available.

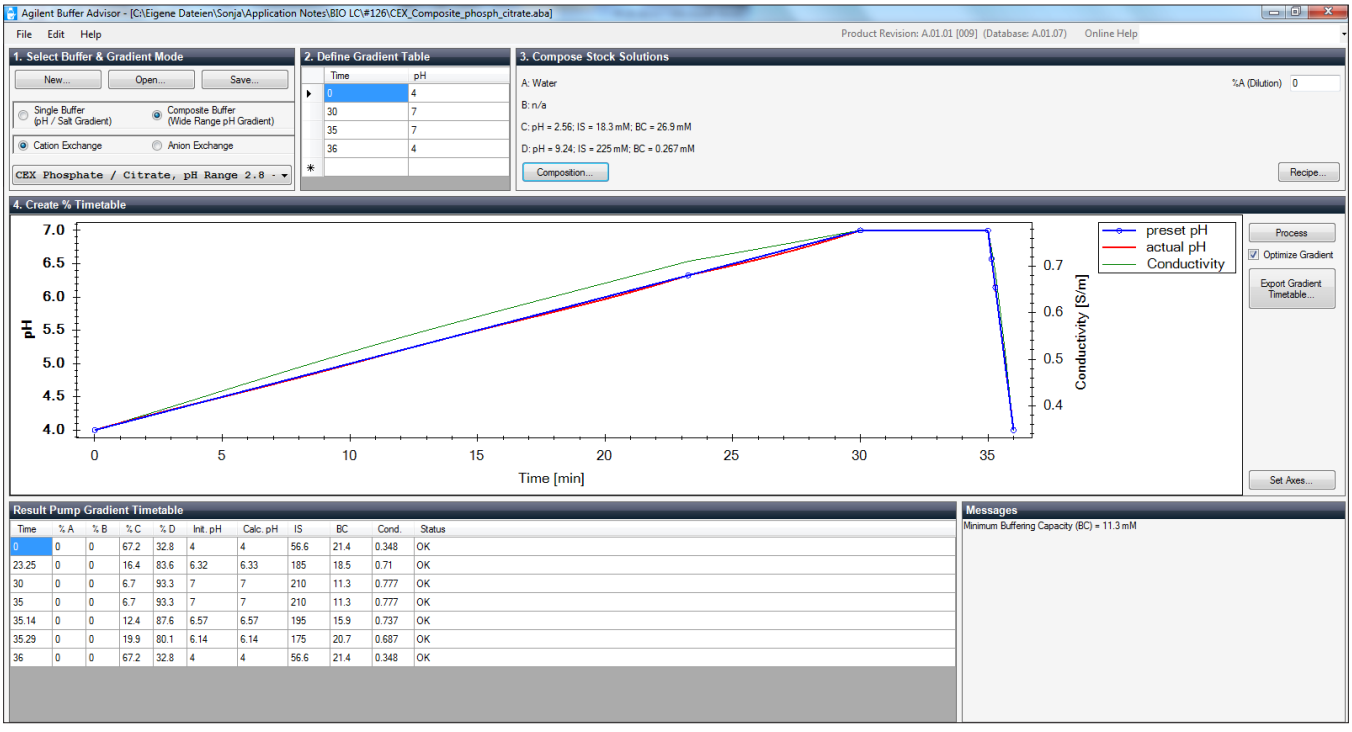

Figure 1
pH gradient calculation from Agilent Buffer Advisor Software.

Figure 2 shows the composition of the used stock solutions. By pressing the Recipe button, the amount of salt needed for 1 L buffer is displayed. For this validated buffer system, consisting of phosphate and citrate salts, the resulting pH values in bottle C is 2.56 and the pH values of bottle D 9.24, leading to a pH range from 2.8 to 7.1 with sufficient buffering capacity.

If a higher or different pH range is required, the user has the freedom to choose from several buffer components and to adjust the concentration of each compound according to his needs. This feature of Buffer Advisor Software offers maximum flexibility to create user-defined pH gradients for CEX or AEX chromatography.

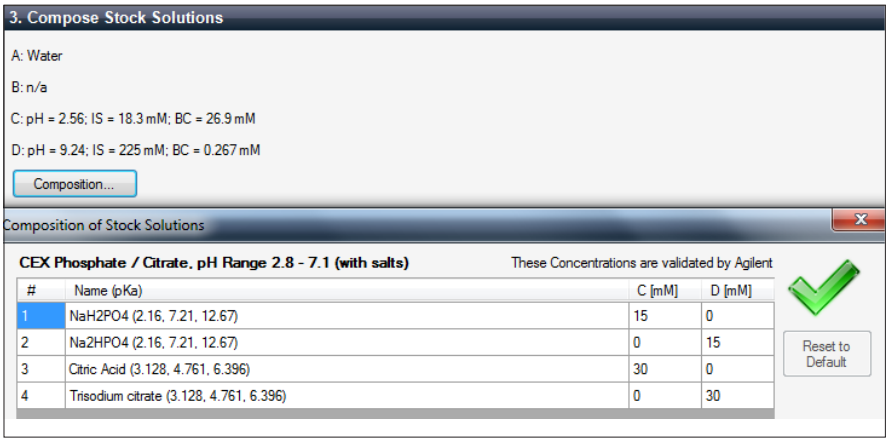

Figure 2
Composition of stock solution for CEX phosphate/citrate composite buffer.

Experimental

Instrumentation

Table 1 shows the configuration of the Agilent 1260 Infinity Bio-inert Quaternary LC System used for the experiments.

Description	Model number
Agilent 1260 Infinity Bio-inert Quaternary Pump	G5611A
Agilent 1260 Infinity Bio-inert High Performance Autosampler	G5667A
Agilent 1290 Infinity Thermostat (for sample cooling)	G1330B
Agilent 1260 Infinity Thermostatted Column Compartment with bio-inert solvent heat exchanger	G1316C
Agilent 1260 Infinity Diode Array Detector VL with 10-mm, bio-inert standard flow cell	G1315D

Table 1
Configuration of the Agilent 1260 Infinity Bio-inert Quaternary LC System.

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, revision C.01.04
- Agilent Buffer Advisor Software, revision A.01.01

Solvents and Samples

All solvents used were LC grade (Table 2). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Citric acid was purchased from Merck, Darmstadt, Germany.

The protein samples used were bovine serum albumin, conalbumin, lactoferrin, and apotransferrin. The proteins, ortho-phosphoric acid, disodium phosphate, trisocium citrate, BORAX (sodium tetraborate), and boric acid were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Chromatographic Conditions

Table 3 shows the chromatographic conditions. The pH gradients from pH 4–7 and from pH 5–9 in 30 minutes were calculated with the Buffer Advisor Software. All pH measurements were carried out online using a customized flow cell and sensor for this application.

	CEX validated mix pH 4 – 7		CEX user mix pH 5 – 9	
Buffer A	–		–	
Buffer B	–		–	
Buffer C	NaH_2PO_4 Citric acid	15 mM 30 mM	H_3PO_4 Citric acid H_3BO_3	20 mM 20 mM 40 mM
Buffer D	Na_2HPO_4 Trisodium citrate	15 mM 30 mM	Na_2HPO_4 Trisodium citrate Sodium tetraborate	20 mM 20 mM 40 mM

Table 2
Solvents.

	CEX validated mix pH 4 – 7	CEX user mix pH 5 – 9
Flow rate:	0.75 mL/min	0.75 mL/min
Column:	Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm (p/n 5190-2407)	Agilent Bio MAb PEEK, 4.6 × 250 mm, 5 µm (p/n 5190-2407)
Gradient:	pH 4 or 4.8–7 in 30 minutes	pH 5–9 in 30 minutes
Stop time:	45 minutes	45 minutes
Post time:	20 minutes	20 minutes
Injection volume:	15 µL	15 µL
Column temperature:	Room temperature	Room temperature
Thermostat autosampler:	6 °C	6 °C
Detection:	280 nm/4 nm Ref.: OFF Peak width > 0.05 minutes (1.0 second response time) (5 Hz)	280 nm/4 nm Ref.: OFF Peak width > 0.05 minutes (1.0 second response time) (5 Hz)

Table 3
Chromatographic conditions.

Results and Discussion

Figure 3 shows a chromatogram with corresponding pH gradient for the protein separation of albumin with a theoretical pI of 5.82, apotransferrin with a theoretical pI of 6.75, and conalbumin with a pI of 6.85. All pI values were calculated using the Expasy Compute Pi Tool based on their primary structure. The proteins were eluted from the weak cation exchange column roughly at their theoretical pI, enabling approximate pI estimation.

With the calculations from Buffer Advisor Software, it was possible to run a linear pH gradient with and without a column, resulting only in a time shift due to the column volume (Figure 4).

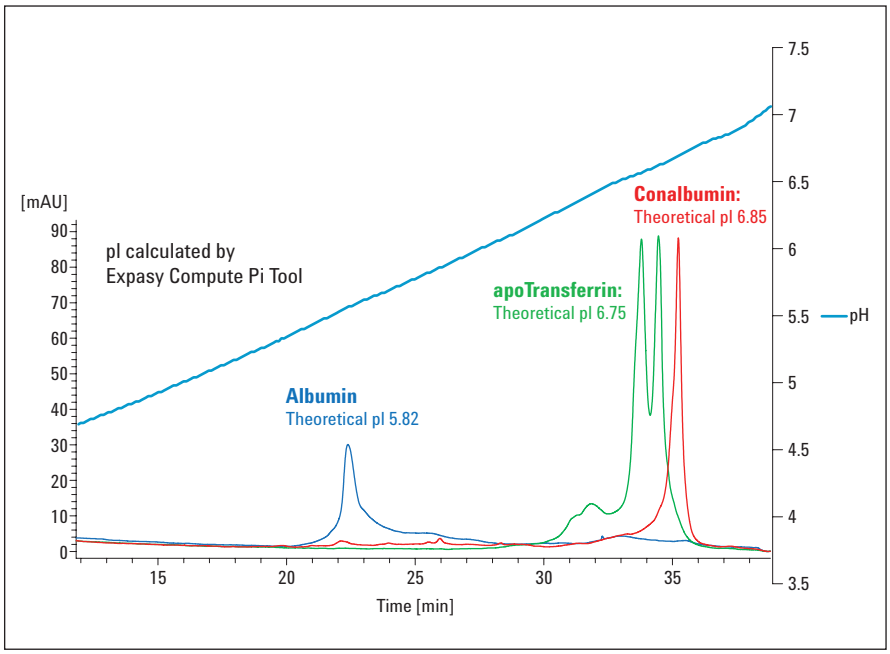

Figure 3
pH gradient using composite CEX validated mix buffer from pH 4–7.

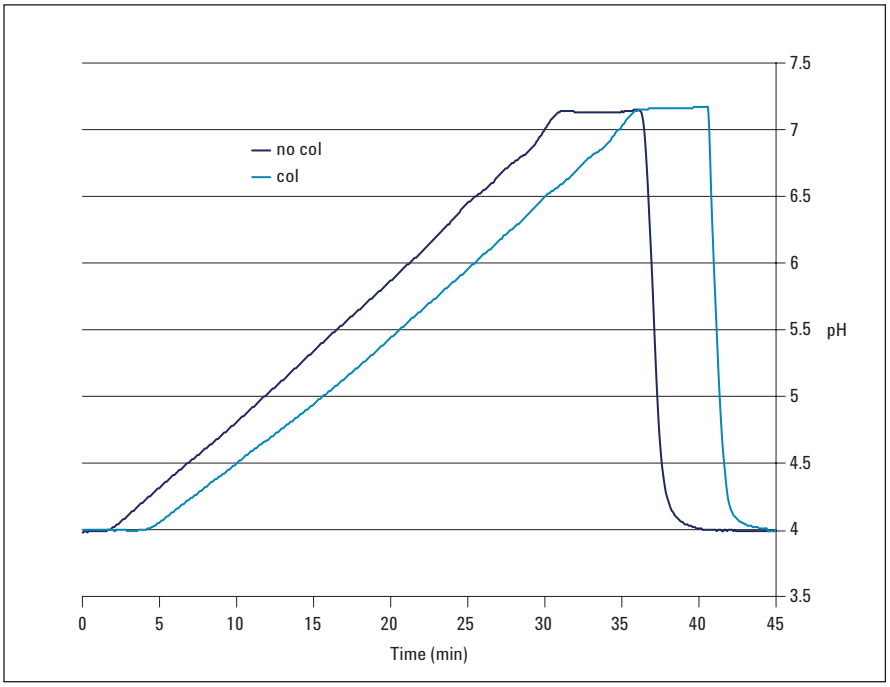

Figure 4
pH gradient with column (col) and without column (no col).

To enable a wider pH range for protein separation, a CEX User Mixture was created, composed of citrate, phosphate, and borate buffer components. With these components, a pH range from pH 5–9 was possible enabling the elution of more basic proteins like lactoferrin with a pI of 8.69 in addition to albumin and conalbumin (Figure 5).

Using the Optimize Function of the Buffer Advisor Software enables a nearly linear gradient from pH 5–9. Without optimizing, the pH gradient deviates a huge amount from the desired linear curve (Figure 6).

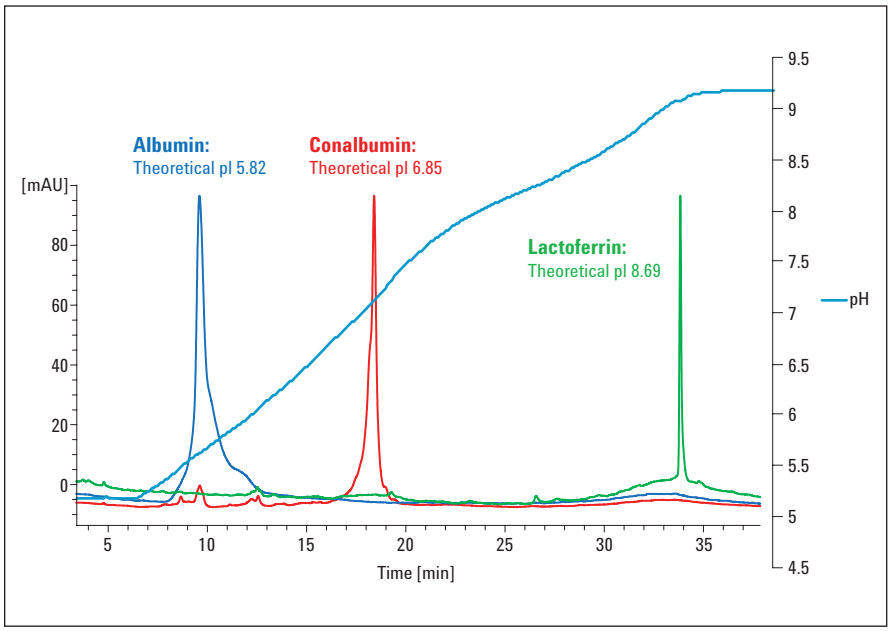

Figure 5
pH gradient using composite CEX User Mix buffer from pH 5–9.

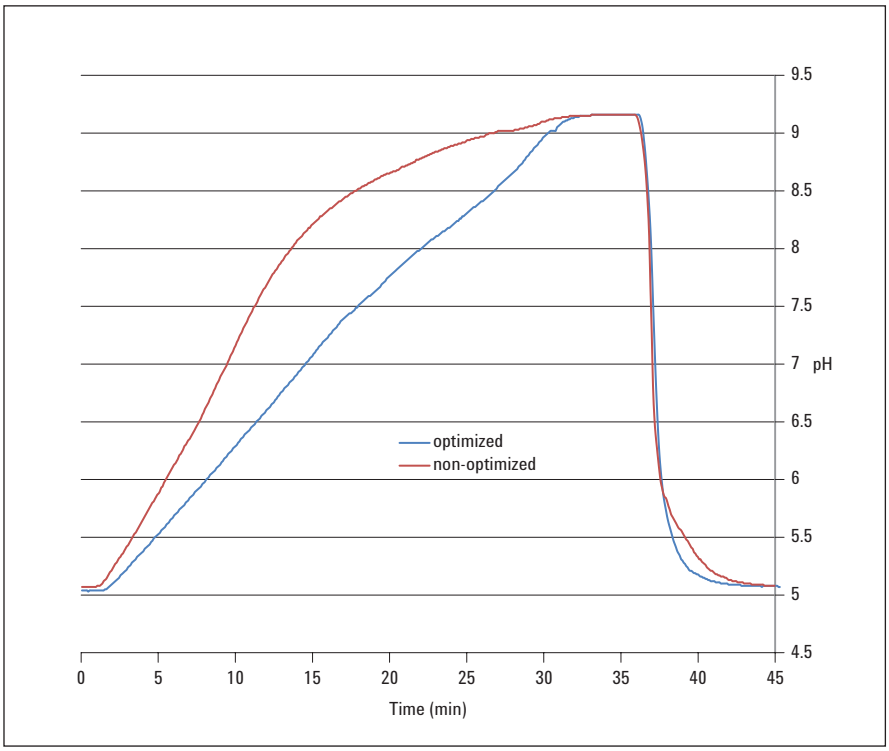

Figure 6
pH gradient from pH 5–9 with CEX User Mix, optimized by Agilent Buffer Advisor Software compared to non-optimized.

Conclusion

This Technical Overview shows protein separation with pH gradients over a wide range using composite buffer systems for cation exchange chromatography. The pH gradients were calculated and optimized with Agilent Buffer Advisor Software for linear pH gradients over a wide pH range. In addition to validated buffer systems for CEX and AEX chromatography, the user can also create customized User Mix composite buffers for an even wider or different pH range. With the Optimize Gradient function of the Agilent Buffer Advisor Software, linear gradients over a wide pH range are possible.

The Agilent Buffer Advisor Software together with the Agilent 1260 Infinity Bio-inert Quaternary LC system enables the generation of linear pH gradients over a wide pH range using composite buffer systems.

Literature

1.
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www.agilent.com/chem/bio-inert

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