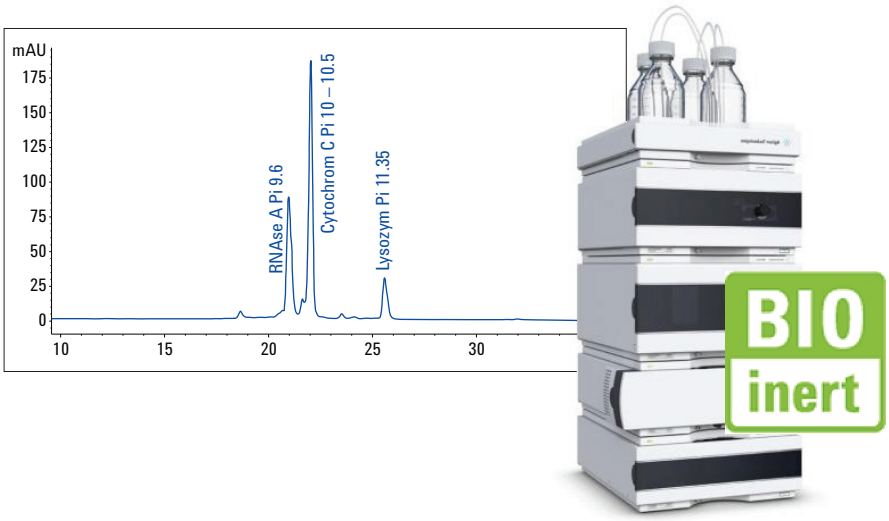
Optimizing Protein Separations with Cation Exchange Chromatography Using Agilent Buffer Advisor

Protein separation with the Agilent 1260 Infinity Bio-inert Quaternary LC System

Technical Overview

Suitable for Agilent
1260 Infinity III LC



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Abstract

This Technical Overview shows that the Agilent Buffer Advisor software in combination with the Agilent 1260 Infinity Bio-inert Quaternary LC System is an ideal solution for automated protein separation by ionic strength gradients. Usually, pH scouting using premixed two-component gradients is time-consuming and work-intensive. Dynamically mixed four-component gradients calculated by the Buffer Advisor software shorten and simplify the workflow for pH scouting. In addition, excellent retention time precision and pH consistency were gained using the gradients calculated by the Buffer Advisor software.

Verified for Agilent
1260 Infinity II LC
Bio-inert System

Agilent Technologies

Introduction

Proteins consist of many different amino acids comprising weak acidic (carboxylic) and basic (amine) groups. Therefore, proteins are amphoteric molecules that exist mostly as zwitterions in a certain pH range. The pH where the protein has no net charge and does not interact with a charged medium is the isoelectric point (pI). In ion exchange chromatography (IEX), the unique relationship between net surface and pH can be used for optimal protein separation. The pH defines the number of charges on the protein and also helps to stabilize the native structure of the protein in the buffer used during analysis.

To ensure optimal binding and elution characteristics of proteins of interest to the IEX column, pH and ionic strength of the deployed buffer are important factors. Even small changes in these two parameters can affect the separation. As a consequence, pH scouting is an important method to find the optimal separating conditions when working with ionic strength gradients. In contrast to pH-gradients, the pH is kept constant in ionic strength gradients. By increasing the ionic strength (salt concentration) of the mobile phase, the less strongly bound proteins are eluted earlier than the stronger bound proteins.

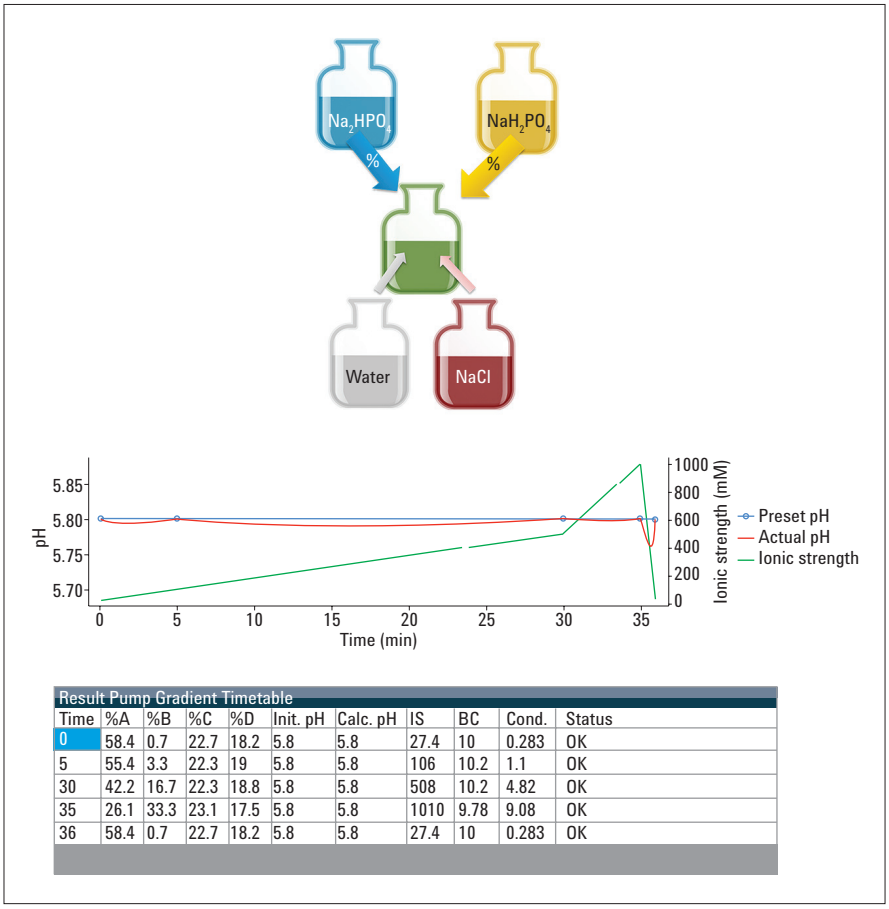
In general, a premixed two-component gradient is prepared for analysis with a starting buffer of low ionic strength and an elution buffer containing high ionic strength. This includes the following preparation steps:

- Dissolving the appropriate buffering compounds at defined concentration
- Titrating the pH with acid/base to the desired pH of the mobile phase
- Splitting the buffer and adding salt to one portion (elution buffer)
- Titrating the pH of the elution buffer with acid/base to the desired pH, if necessary

To perform pH scouting using premixed two-component gradients, prepare different bottles of buffer. To test, for example, six different pH values, it is necessary to prepare 12 bottles of premixed buffer. In contrast, with dynamically mixed four-component gradients, it is necessary to prepare only four bottles to generate various pH values. Further, dynamical mixing of a buffer eliminates the necessity to titrate the buffer solutions manually, which is typically time-consuming and errors prone. The application of dynamically mixed four-component gradients simplifies method development and reduces the time needed for buffer preparation to a large extent by just providing four bottles with stock solutions:

- Line A: Water
- Line B: Salt solution
- Line C: Acidic buffer component
- Line D: Basic buffer component

Using the four stock solutions, different buffers at different pH and salt concentration can be prepared. The Buffer Advisor software is a helpful tool to calculate the percentages of the stock solutions in order to achieve the desired pH, buffer concentration, and ionic strength (Figure 1).



Result Pump Gradient Timetable

Time	%A	%B	%C	%D	Init. pH	Calc. pH	IS	BC	Cond.	Status
0	58.4	0.7	22.7	18.2	5.8	5.8	27.4	10	0.283	OK
5	55.4	3.3	22.3	19	5.8	5.8	106	10.2	1.1	OK
30	42.2	16.7	22.3	18.8	5.8	5.8	508	10.2	4.82	OK
35	26.1	33.3	23.1	17.5	5.8	5.8	1010	9.78	9.08	OK
36	58.4	0.7	22.7	18.2	5.8	5.8	27.4	10	0.283	OK

Figure 1
Quaternary mixing to create a salt gradient with constant pH.

The Buffer Advisor software generates a timetable, which can be imported into the method of the 1260 Infinity Bio-inert Quaternary LC Pump using the *Import Solvent Blending File* function of the Agilent OpenLAB CDS ChemStation Edition software (Figure 2).

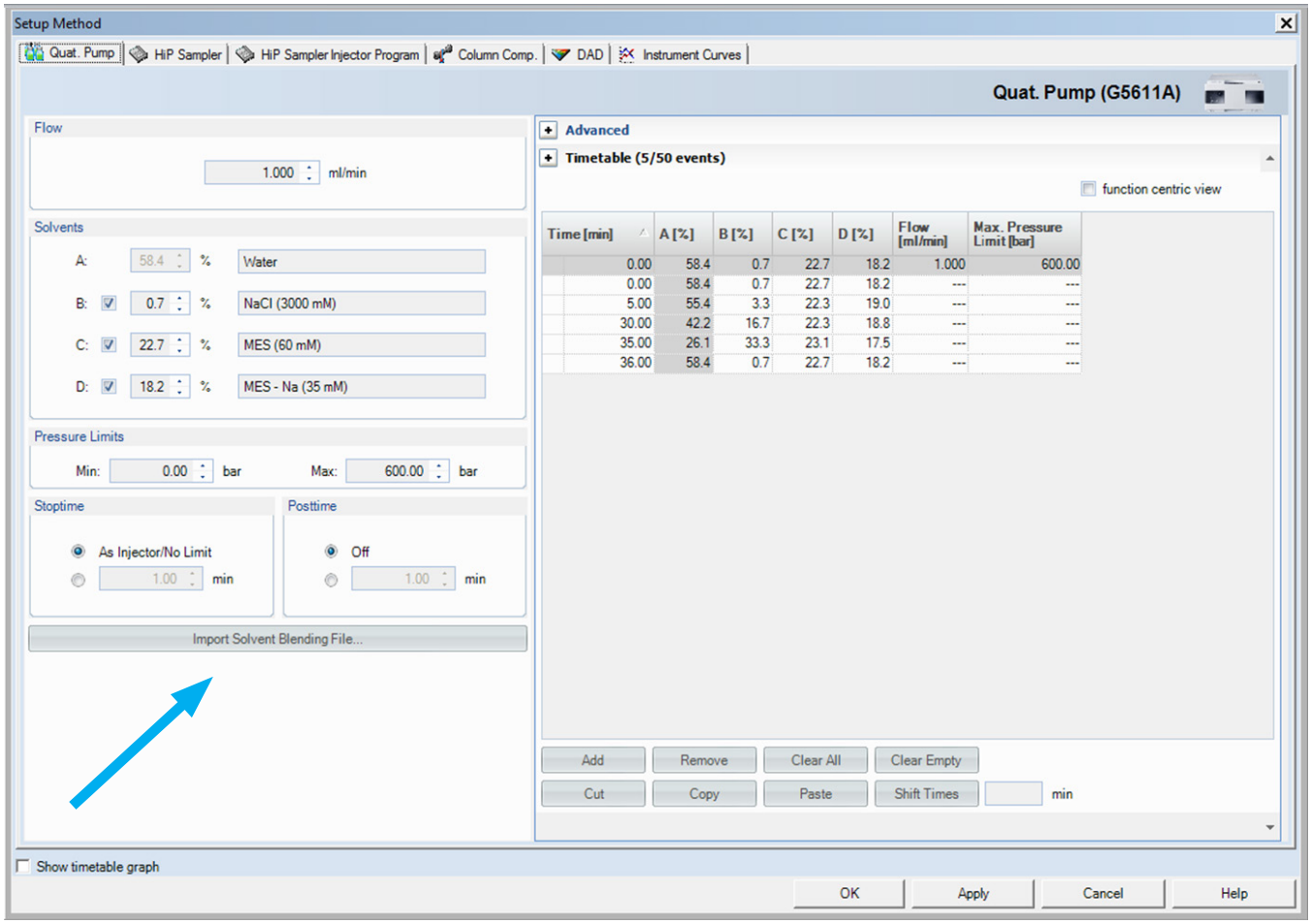


Figure 2
The generated Timetable can be imported into the method of the Agilent 1260 Infinity Bio-inert LC System through the Agilent OpenLAB CDS ChemStation Edition software.

The Buffer Advisor software can be applied for ionic strength or pH gradients in anion or cation exchange chromatography. The software provides a wide choice of different selectable buffers for single buffer (ionic strength gradients) or for composite buffer (pH gradients) applications. Depending on the proteins of interest and the used columns, the user can select buffers either for anion or cation exchange (Figure 3). To ensure optimal buffering capacity, recommended pH ranges and concentrations in which the buffers should be used are displayed.

A common issue in ion exchange chromatography with ionic strength gradients is the decrease in pH as an effect of added neutral salt like NaCl[1,2]. The Buffer Advisor software counteracts this issue by recalculation of the overall mobile phase composition considering the concentration of acidic and basic buffer (Line C and D) to maintain the desired constant pH. In addition, if the pH deviation gets too large, the Buffer Advisor software automatically inserts additional time points into the pump timetable.

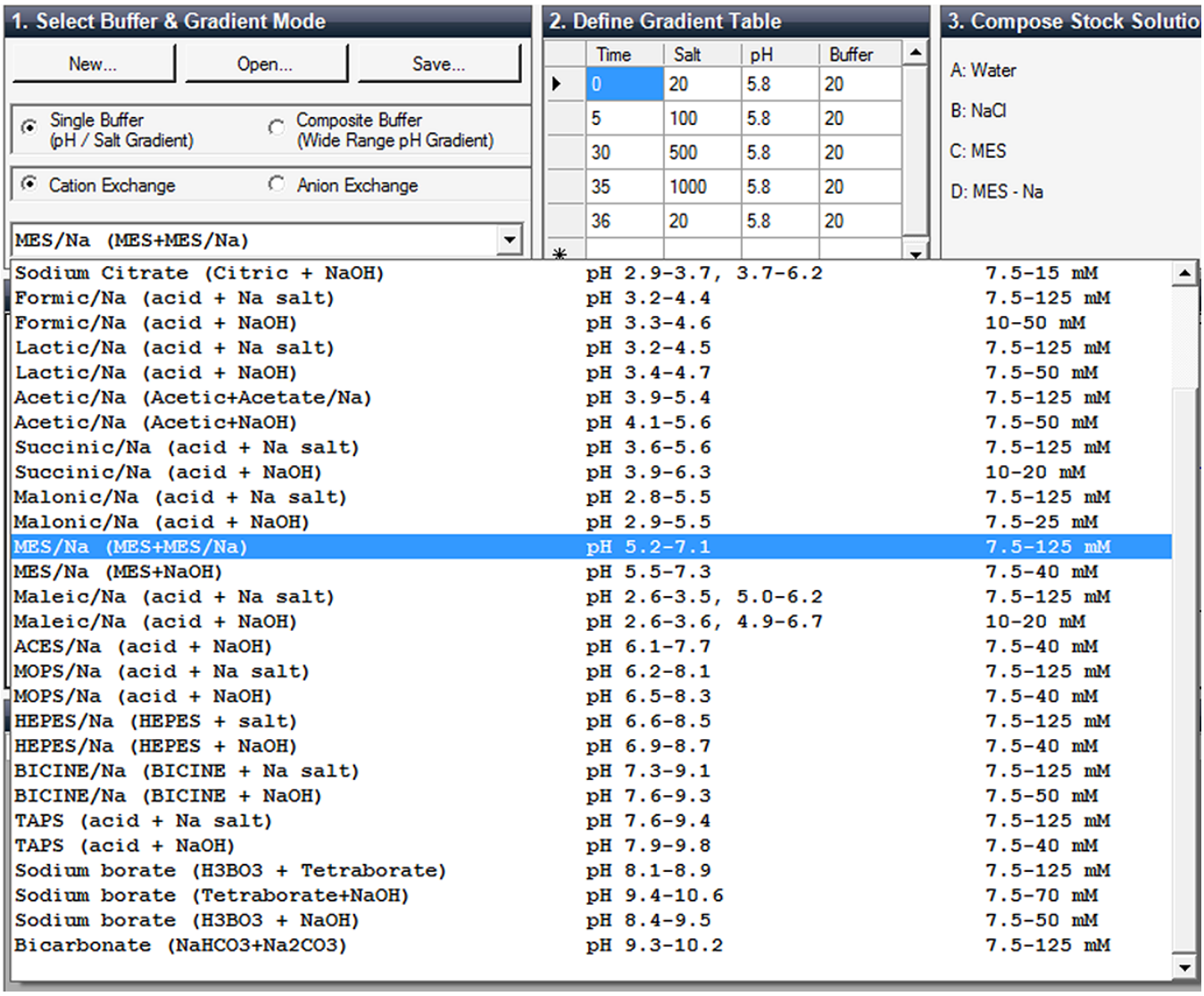


Figure 3
Buffer list for cation exchange chromatography, sorted by recommended pH range.

Experimental

Instrumentation

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 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C) with bio-inert solvent heat exchangers
- Agilent 1260 Infinity Diode Array Detector VL (G1315D with bio-inert standard flow cell, 10 mm)
- Agilent 1260 Infinity Bio-inert Analytical-scale Fraction Collector (G5664A)

Column

Agilent Bio MAb Column, PEEK, 4.6 × 250 mm, 5 µm

Software

- Agilent OpenLAB CDS ChemStation Edition for LC and LC/MS Systems, Rev. C.01.03 [32]
- Agilent Buffer Advisor, Rev. A.01.01

Solvents

Buffer A: H_2O

Buffer B: NaCl 3 M

Buffer C: MES (2-(N-morpholino) ethanesulfonic acid monohydrate) 60 mM

Buffer D: MES-Na (2-(N-morpholino) ethanesulfonic acid sodium salt) 35 mM

Sample

Mix of three proteins, solved in PBS (phosphate buffered saline), pH 7.4

Ribonuclease A:	13,700 Da	pI 9.6
Cytochrom C:	12,384 Da	pI 10–10.5
Lysozyme:	14,307 Da	pI 11.35

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). MES (2-(N-morpholino)ethanesulfonic acid monohydrate) and MES-Na (2(N-morpholino)ethanesulfonic acid sodium salt) were purchased from Merck, Darmstadt, Germany. NaCl was purchased from VWR, Radnor, PA, USA.

Chromatographic conditions

Flow rate:	1 mL/min
Gradient:	0 min – 20 mM NaCl 5 min – 20 mM NaCl 30 min – 500 mM NaCl 35 min – 1000 mM NaCl 36 min – 20 mM NaCl
Injection volume:	10 µL
Thermostat:	4 °C
Temperature TCC:	25 °C
DAD:	280 nm/4 nm Ref.: OFF
Peak width:	Peak width: > 0.05 min (1.0 s response time)(5 Hz)

Results and discussion

pH scouting was performed using calculations from the Agilent Buffer Advisor software for pH values from 5.8 to 6.8. A mix of three proteins (ribonuclease A, cytochrome C and lysozyme) was separated using a four-component salt gradient at six different pH values. Dynamically mixed four-component gradients were generated using the calculations from the software. The Buffer Advisor software simplifies the generation of different four-component gradients by calculating the percentage of the individual stock solutions in the mobile phase at defined time points (Figure 4).

1. After definition of the gradient parameters, such as time, maximum salt concentration, pH, and buffer concentration
2. The Buffer Advisor software calculates the needed stock concentrations.

   The *Recipe* button displays the absolute amount of needed chemicals for the preparation of the stock solutions (Figure 5). These proposed stock concentrations can be adjusted by the user.
3. Select the *Process* tab.
4. The Buffer Advisor software calculates the needed amount of each channel to maintain the correct pH during the complete chromatographic run. Furthermore, it calculates whether the pH, salt concentration and buffer concentration entered is suitable for the buffer system that was selected. The timetable displays also additional data, such as buffering capacity of the mobile phase.

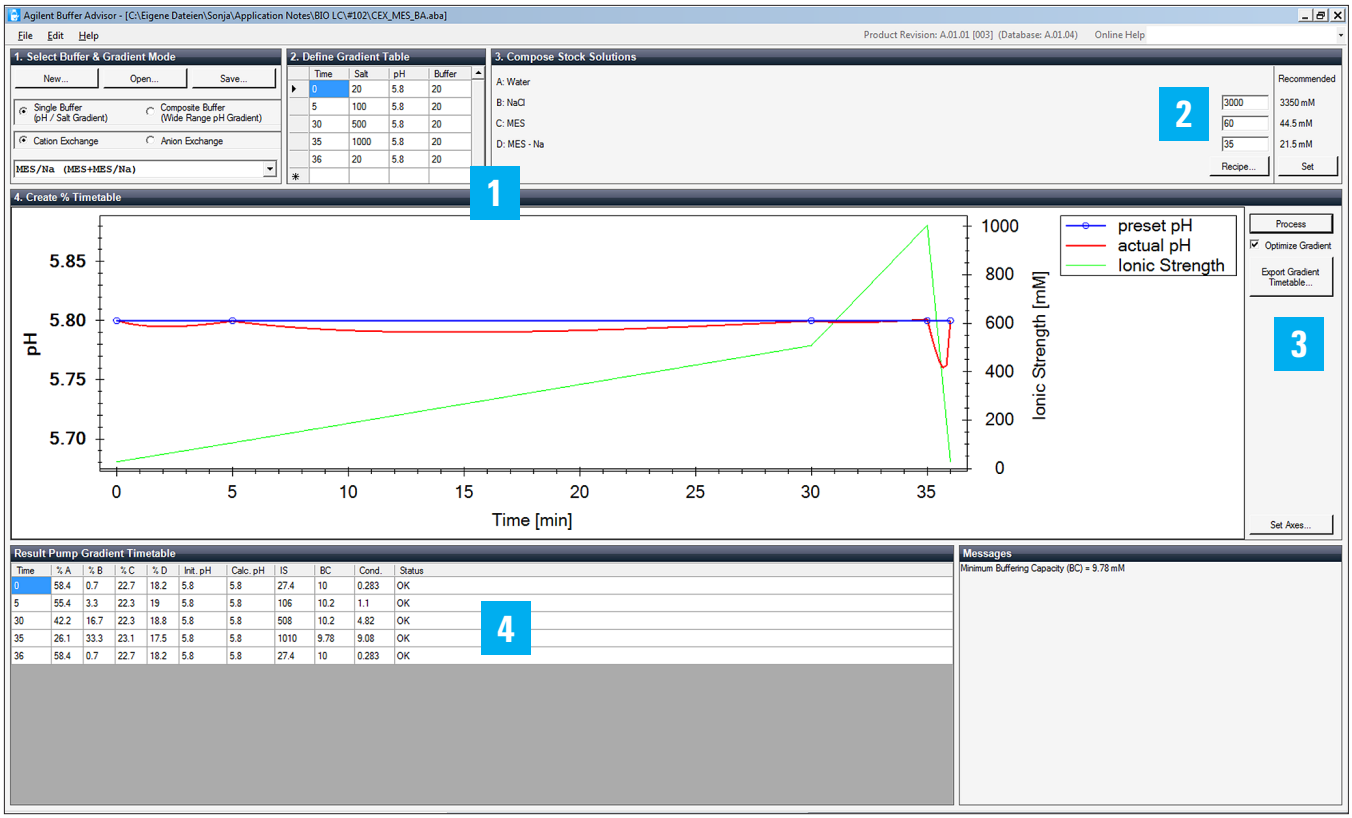


Figure 4
Agilent Buffer Advisor software, showing the steps described in "Results and discussion".

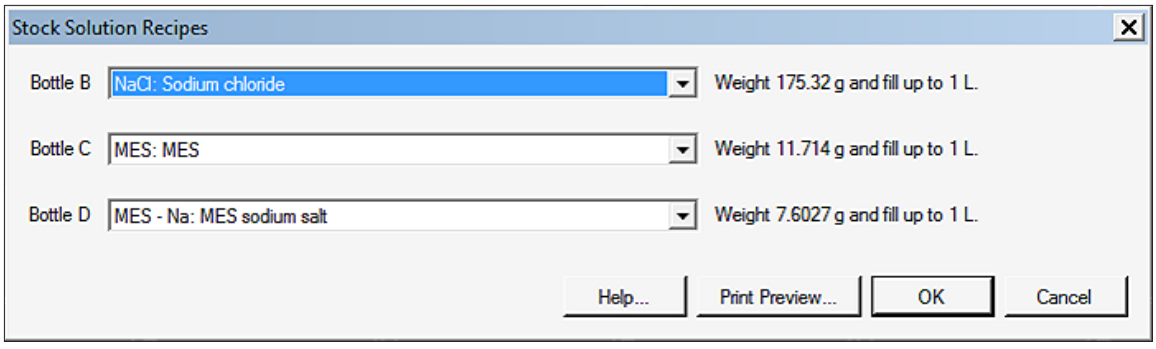


Figure 5
Stock solution recipes.

The pH scouting for the three-protein mix of ribonuclease A (A), cytochrome C (B) and lysozyme (C) demonstrates the benefits of the Agilent Buffer Advisor software (Figure 6). Even small pH changes of 0.2 have a strong influence on the retention of the proteins on the weak cation exchange (WCX) column. Changes in the elution order become obvious when the pH is changed from 5.8 to 6.8.

Manual preparation of corresponding buffers for premixed two-component gradients includes several steps. For each pH and for each prepared bottle (one with low and one with high ionic strength), a manual titration of the buffers is necessary. pH scouting for six different pH values in order to achieve the optimal resolution results in preparation of 12 solvent bottles (including weighing chemicals, pH adjustment). This is a very time-consuming procedure and highly prone to error and variation.

In contrast, the Buffer Advisor software is capable of automatically and reproducibly mixing all six separation conditions out of four stock solutions without any manual interference. The optimal resolution was achieved at pH 5.8 (Figure 6).

Based on the results, the user has various options on how to proceed:

1. Fine-tuning of the resulting pH values and gradients
2. Transfer of the dynamically mixed four-component gradient to other instruments through the OpenLAB CDS ChemStation timetable
3. Implementation of pH scouting results into two-component gradients using premixed buffers

In the last case, however, deviations from correct pH are expected, due to the pH optimization procedure of the Buffer Advisor software.

In these experiments, a pH of 5.8 was used for further analysis with respect to precision of retention times and pH consistency. Using dynamically mixed four-component gradients, the RSD of retention times was found to be < 0.11% for all three proteins. Figure 7 shows the pH values of a dynamically mixed four-component gradient without column, measured offline using collected fractions. The pH of 5.8 remains constant over the complete run.

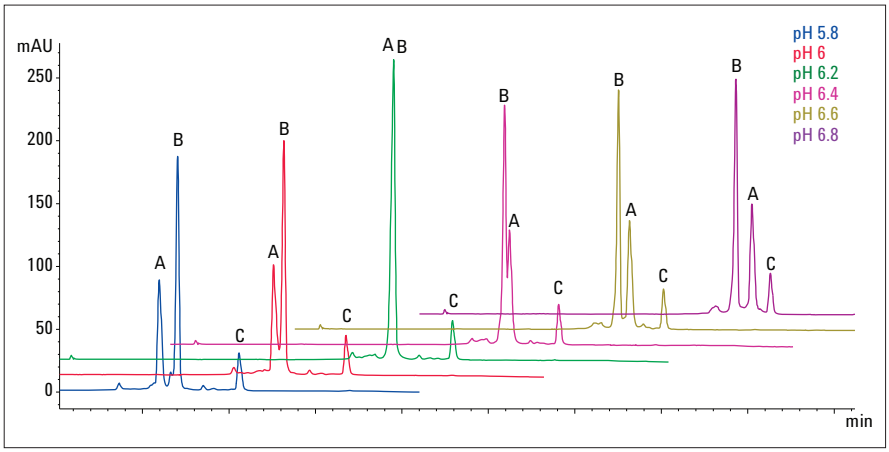


Figure 6
pH scouting for the separation of a three-protein mix using dynamically mixed quaternary gradients.

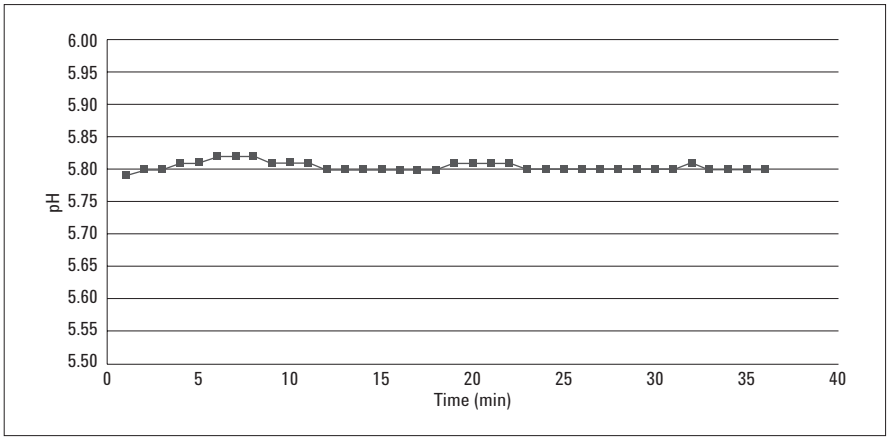


Figure 7
Offline pH measurement.

Conclusions

Using dynamically mixed four-component gradients, calculated by the Buffer Advisor software, shortens and simplifies the workflow for pH scouting. The employment of dynamically mixed gradients calculated with the Buffer Advisor software results in a significant decrease in buffer preparation time, particularly when compared to manual preparation of buffers for premixed two-component gradients. The Buffer Advisor software provides a wide range of prevalidated, user-selectable buffer systems for anion and cation exchange chromatography and delivers recipes for preparation of the most suitable stock solutions. Due to pH optimization of the software, resulting pH values are more accurate and precise than those resulting from premixed gradients formed out of manually prepared buffer solutions. The Buffer Advisor software counteracts this issue by the recalculation of the four-component gradient regarding the concentration of acidic and basic buffer to maintain the desired constant pH.

The Buffer Advisor software in combination with the Agilent 1260 Infinity Bio-inert Quaternary LC System is excellent for generating four-component gradients. The calculations of Buffer Advisor software lead to exact and reproducible protein analysis while providing an excellent tool for automated pH scouting and accurate ion exchange chromatography. The Buffer Advisor software is, therefore, an ideal tool for automatic development of analytical methods in ion-exchange chromatography, which can be seamlessly transferred to the corresponding QA/QC departments.

References

1.
R. J. C. Brown & M. J. T. Milton. Observation of a combined dilution and salting effect in buffers under conditions of high dilution and high ionic strength, *Accred Qual Assur* 8(11): 505-510, **2003**.

2.
A. E. Voinescu *et al*. Similarity of Salt Influences on the pH of Buffers, Polyelectrolytes, and Proteins, *J. Phys. Chem. B* 110: 8870-8876, **2006**.

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