Illustrating bioseparations with colorful proteins

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Abstract

Advances in biology are prompting new discoveries in the biotechnology, pharmaceutical, medical technology, and chemical industries. Developing commercial-scale processes based on these advances requires that new chemical engineers clearly understand the biochemical principles behind the technology, in addition to developing a firm grasp of chemical engineering principles.[1] This paper outlines the development of educational materials in biochromatography, one of the major techniques used to separate and purify biological molecules.

This project aims to determine operating conditions for displaying the separation of colorful proteins in a variety of course settings. Anion exchange chromatography demonstrations have been developed, showing that a mixture of flavodoxin (orange color) and green fluorescent protein (green color) can be selectively eluted at different salt concentrations, providing a powerful demonstration of the principles of protein binding and elution. These concepts have been expanded to full-scale experiments suitable for unit operations laboratories or upper-level biochemical engineering electives. This paper describes how these visually-appealing demonstrations and lab exercises centered on bioseparations can be incorporated into lecture- and lab-based chemical engineering courses.

Introduction

Advances in biology are prompting new discoveries in the biotechnology, pharmaceutical, medical technology, and chemical industries. Developing commercial-scale processes based on these advances requires that new chemical engineers clearly understand the biochemical principles behind the technology, in addition to developing a firm grasp of chemical engineering principles.[1] To successfully deliver this knowledge to students, engineering educators require additional resources to illustrate relevant biological concepts throughout the curriculum.

This paper outlines the development of educational materials in biochromatography, one of the major techniques used to separate and purify biological molecules. In a typical bioprocess, the majority of the costs are associated with isolating and purifying the desired biological compound.[2] In many of the later stages of purification, over 50% use some type of chromatography.[3] Exposing students to biochromatography provides an introduction to bioseparations and the underlying biochemistry concepts. As separation processes are based on the physical and chemical properties of the product and chief impurities, a wide range of concepts can be included, such as overall cell composition, protein biochemistry, recombinant protein production techniques, and bioprocess optimization.

This paper explains how these concepts can be introduced by improving undergraduate courses and laboratories through the development of exciting, visually-appealing experiments. The use of visually-appealing materials has been shown to motivate and captivate students in biology and chemical engineering settings.[4-9] Additionally, some elements of bioseparation (adsorption, ion-exchange, and chromatography) are difficult to teach in a lecture-based format, as these are rate-based, time-dependent processes.[10] These experiments will improve instruction in this difficult area by employing a range of colorful proteins with different biophysical properties.

To facilitate vertical integration in a variety of chemical engineering courses, two types of exercises have been developed. Demonstrations allow for these concepts to be introduced into lecture-based courses, and can be expanded to short hands-on exercises in small courses. These demonstrations will be piloted during Spring 2005 at Rowan University in the junior-level Separation Processes course. Full-scale experiments allow for these concepts to be studied in more detail through unit operations and elective courses, where students can explore the effect of process parameters on separation efficiency. These experiments will be piloted at Rowan University in Fall 2005 in the senior-level Biochemical Engineering elective. Both types of exercises are described in this paper.

Materials

A set of four colorful proteins with a range of physical properties were selected. The use of colorful proteins removes the need for UV detectors in a demonstration setting, and allows for each protein to be monitored at a distinct visible wavelength in full-scale experiments. In order to illustrate the two main driving forces for biological separations, proteins with different sizes and ionic properties were chosen. Table 1 describes physical properties of the four proteins.

Table 1. Physical properties of the colorful proteins

Protein	Color (λ_{max})	Molecular Weight	Isoelectric Point[11]
Cytochrome c	Red (410 nm)	12 kDa	9.6
DsRed2	Pink (561 nm)	103 kDa[12]	6.3
EGFP	Green (488 nm)	27 kDa[13]	5.6
Flavodoxin	Orange (464 nm)	19 kDa	4.2

To clarify the source of these proteins, cytochrome c is from horse heart, DsRed2 is from a *Discosoma* genus of coral, EGFP is a modified form of the green fluorescent protein from *Aequorea victoria* jellyfish, and flavodoxin is from *Cyanobacterium anabaena*.[14-17] At Rowan University, these proteins have been produced by students in Junior and Senior Clinic through recombinant protein expression in bacteria. These proteins are also available from commercial sources (e.g. horse heart cytochrome c from Sigma, catalogue# C7752, $382 for 1 g; DsRed2 from BD Biosciences, catalogue# 632436, $265 for 100 µg; EGFP from BD Biosciences, catalogue# 632439, $265 for 100 µg).

Anion exchange chromatography resin, gel filtration chromatography resin, and chromatography columns are available from a variety of sources. In this paper, DEAE Sepharose Fast Flow resin (Amersham Biosciences, catalogue# 17-0709-10, $42 for 25 g) and 24 mL low-pressure Kontes columns (Fisher, catalogue# K420401-1030, $16.36 per column) were used. Chromatography

resin was prepared and packed into a column using the directions supplied with the resin. A variety of fluid delivery systems can be used, including pipette and gravity-fed flow (results in Figure 1), peristaltic pumps, and complete chromatography systems such as the Akta Basic from Amersham Biosciences (results in Figure 3). Additional information on the theory of ion exchange chromatography and equipment needs can be found in bioseparation or biochemical engineering textbooks.[18-20]

Demonstrations

Protein purification and physical biochemistry concepts can be incorporated into lecture-based courses through the use of biochromatography demonstrations. This type of demonstration is suitable for separation processes, unit operations, mass transfer operations, and introductory-level biotechnology courses. In anion exchange chromatography, the resin is positively-charged. A protein will bind to the resin if it is negatively-charged, in a solution with a pH above its isoelectric point. A simple demonstration of this principle would be to place two proteins from Table 1 in a buffer with a pH between the protein's pIs, load the mixture onto anion exchange resin at the same pH, and watch as the low-pI protein is retained. For instance, mixing cytochrome c and EGFP at a pH of 8.0 would allow for EGFP to be retained on the column, while cytochrome c would flow through.

A more interesting demonstration can be created by using conditions that allow all proteins to bind the resin. Following binding, the proteins can be selectively eluted by changing the pH or salt concentration. Figure 1 illustrates this concept with time-lapse photographs for a mixture of EGFP and flavodoxin in 10 mM sodium phosphate at pH 6.5. The first panel demonstrates that both EGFP (green) and flavodoxin (orange) have bound to the resin. The second and third panels illustrate the movement of EGFP down the column after 200 mM sodium chloride is added to the top of the column. In the fourth panel, flavodoxin was recovered by washing the column with 2 M sodium chloride.

These demonstrations could also be expanded into short, hands-on exercises during lectures. The example in Figure 1 was presented in a developmental learning sequence: the students were presented with the material in stages, with protein binding followed by elution of each individual protein. Students could also perform this short exercise in small groups during a lecture period. With a slight change, the material could be presented in an inductive learning framework. Students could be presented with a given mixture of proteins at three different pH values. After loading each mixture onto a column filled with anion exchange resin, students could monitor the results. In an attempt to explain the variety of binding behavior, students would deduce that each protein has a unique "charge change point" (equivalent to isoelectric point), and that modifying the solution pH and salt concentration are important determinants of anion exchange chromatography performance.

The four overall objectives of this demonstration are to show students that:

1. ion exchange chromatography is based on ionic interactions
2. the ionic character of the resin is fixed (anion exchange resin is positively charged)
3. the ionic character of a protein is determined by the solution pH and the pI of the protein
4. by adjusting the solution pH or salt concentration, proteins with different pI values can be separated through ion exchange chromatography

Student mastery of these objectives can be measured through exam or homework problems. For instance, students could display their knowledge by designing a chromatographic separation for a mixture of three proteins with given isoelectric points. If students have mastered the material, they should be able to select a resin, select a buffer pH and salt concentration that will allow the protein of interest to bind to the resin, and design a pH or salt gradient that will selectively elute the proteins.

Full-scale experiments

The underlying biological concepts can also be covered in full-scale experiments, where the emphasis is on bioseparation process optimization. This style of experiment is suitable for unit operation laboratories and upper-level elective courses with laboratory components. To illustrate the importance of process parameters on bioseparation performance, a more difficult protein separation was developed.

EGFP and DsRed2 were mixed in 25 mM Tris at pH 8.5 or 8.0. Three different chromatography methods were evaluated: linear salt gradient at 25 mM Tris, pH = 8.5; linear salt gradient at 25 mM Tris, pH =8.0; and step salt gradient at 25 mM Tris, pH 8.0. Figure 2 outlines a general gradient-based chromatography method. These experiments were performed on an Amersham Biosciences Akta Basic chromatography unit, equipped with a UV detector capable of monitoring three individual wavelengths. EGFP was monitored at 488 nm and DsRed2 was monitored at 561 nm. Alternatively, the process could be monitored off-line by collecting small fractions and measuring the absorbance on a visible spectrophotometer.

The quality of protein separation was quantified by a resolution calculation. This is illustrated in Equation 1 and Figure 3.[18]

$$\text{resolution} = \frac{V_{\max,b} - V_{\max,a}}{0.5(w_{b,a} + w_{b,b})} \quad (1)$$

Figure 3 shows what the peaks would look like for perfect protein separation (resolution = 1.0). If the peaks overlap then the resolution will fall between 0.0 and 1.0.

Figure 4 illustrates the separation created between EGFP and DsRed2 with method 3 using time-lapse photography. The resolution between EGFP and DsRed2 improved as two process parameters changed. When the pH was brought closer to the isoelectric points of the proteins by

changing from 8.5 to 8.0, the resolution improved. Also, when the salt gradient was made more shallow through the introduction of steps, the resolution improved. These results are summarized in Table 2. Further steps in these two directions could lead to improved resolution.

Table 2. Resolution between EGFP and DsRed2 using anion exchange chromatography

Method	pH	Salt Gradient	Resolution
1	8.5	Linear from 20 to 300 mM NaCl	0.12
2	8.0	Linear from 20 to 300 mM NaCl	0.28
3	8.0	Steps at 20, 50, 110, 140 mM NaCl	0.40
4	7.5	Linear from 20 to 300 mM NaCl	0.34
5	7.5	Steps at 20, 50, 65, 80 mM NaCl	0.48

The objectives of the full-scale experiments are the same as the demonstration. The main difference is that the full-scale experiments provide an opportunity for students to explore the fourth objective in a laboratory setting. Depending on the available laboratory time, students can perform multiple separations at different solution conditions, with the ultimate goal of creating a separation protocol that produces the best resolution.

This experiment could be expanded to include additional colorful proteins or operating parameters to create experimental variety. With any combination of proteins, this experiment enhances student understanding of bioseparations, specifically the importance of solution pH, salt concentration, and protein physical parameters in anion exchange chromatography.

Summary

Demonstrations and full-scale experiments in anion exchange chromatography using a set of colorful proteins have been described. These educational materials will allow instructors to introduce important biochemical engineering and physical biochemistry principles into the chemical engineering curriculum. The visual appeal and low cost of supplies will make the demonstrations an effective teaching tool in core courses focused on separation processes. The variety of possible behavior will make the full-scale experiments a robust addition to unit operations laboratories or biochemical engineering electives. Further developments will make the modules available for dissemination to other universities.

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Biographical Information

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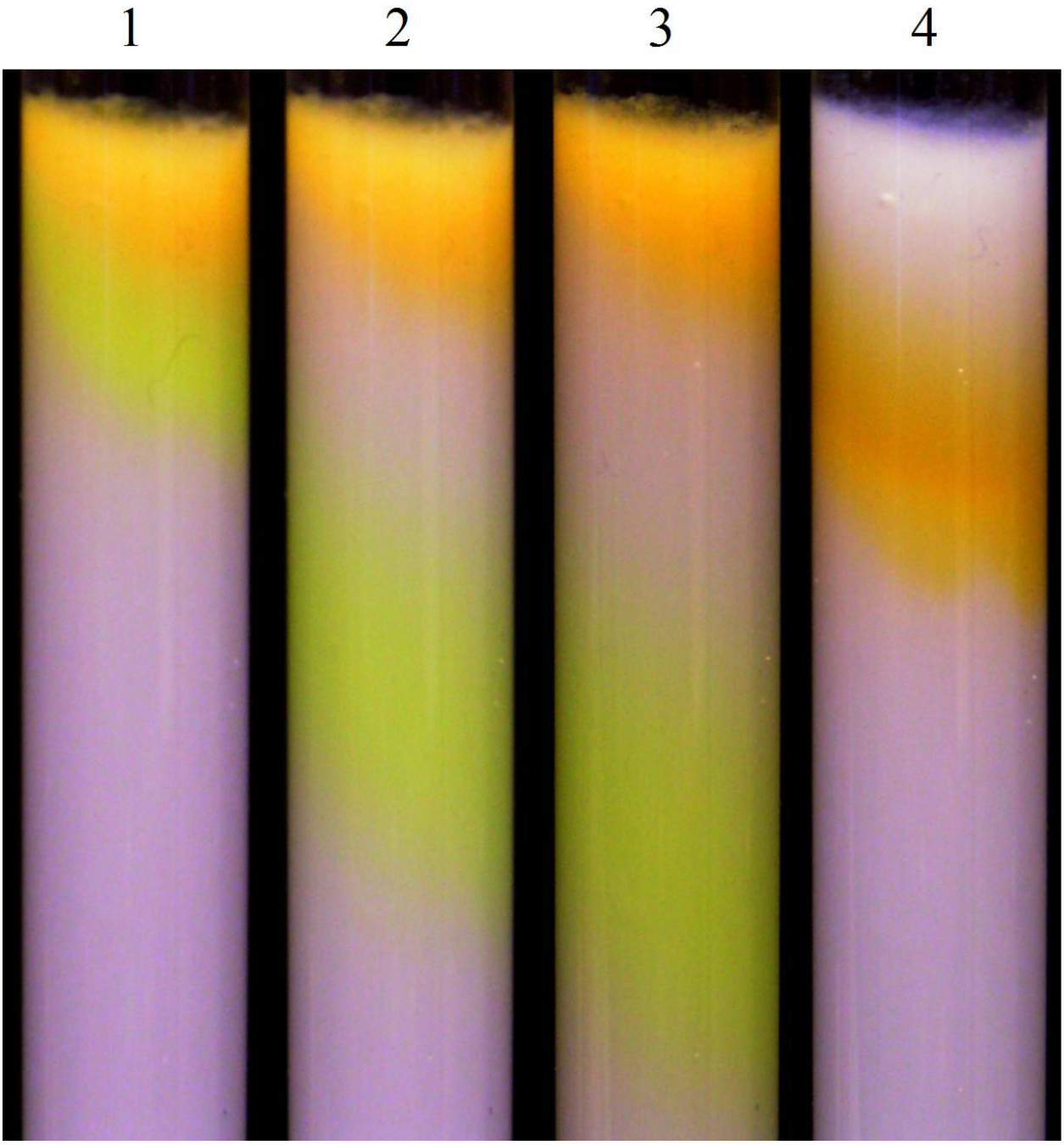

Figure 1. Anion exchange of a mixture of EGFP and flavodoxin.

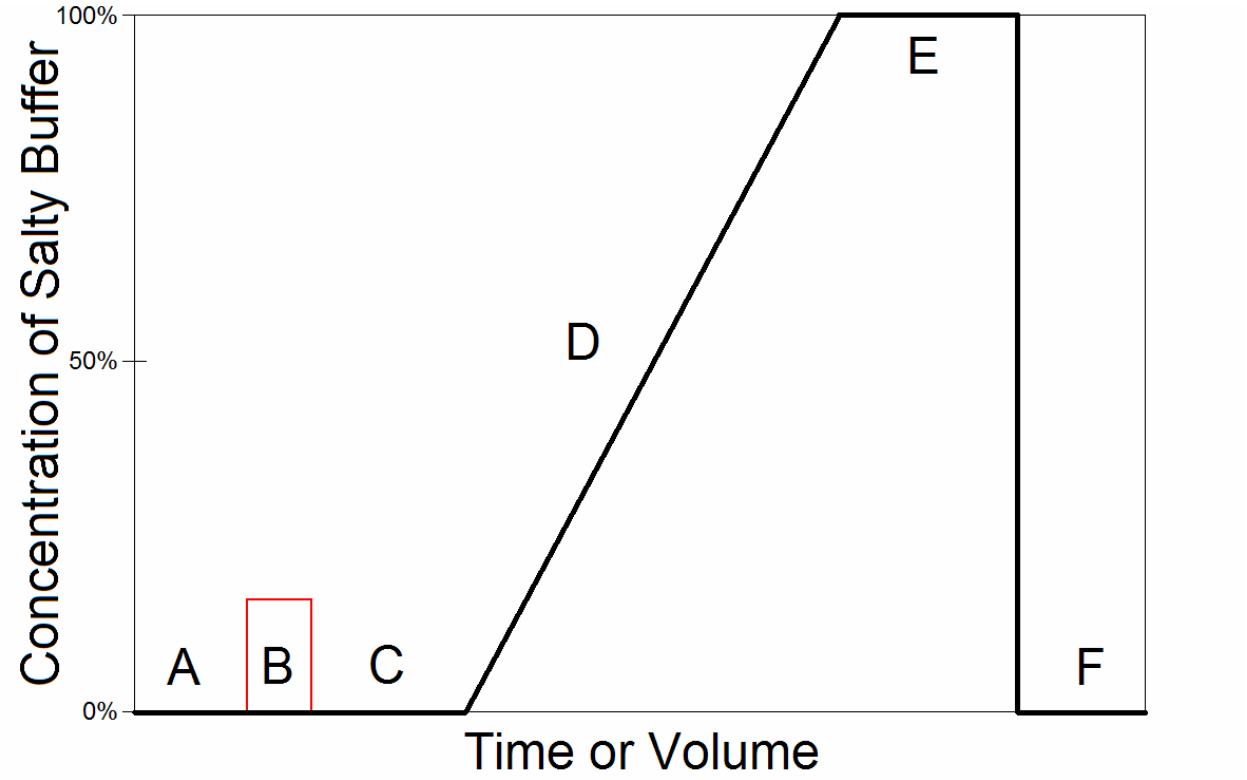

Figure 2. Outline of general gradient-based chromatography method. Broad guidelines for the duration of each phase are reported in parentheses in terms of column volumes, defined as the volume of resin in the column. During period "A" low-salt buffer is delivered to the column to equilibrate the resin (3-5 column volumes). During period "B" the sample is applied to the column (sample volume). During period "C" additional low-salt buffer is delivered to the column to wash away any unbound protein (1-2 column volumes). During period "D" the concentration of salt in the buffer is slowly incremented to selectively elute the proteins (3-5 column volumes). During period "E" additional high-salat buffer is delivered to remove tightly-bound protein (1-2 column volumes). During period "F" the column is re-equilibrated with low-salt buffer (1-2 column volumes). A pH gradient may be used in place of a salt gradient in anion exchange chromatography. Shaped gradients or a series of steps may be substituted for a linear gradient in period "D."

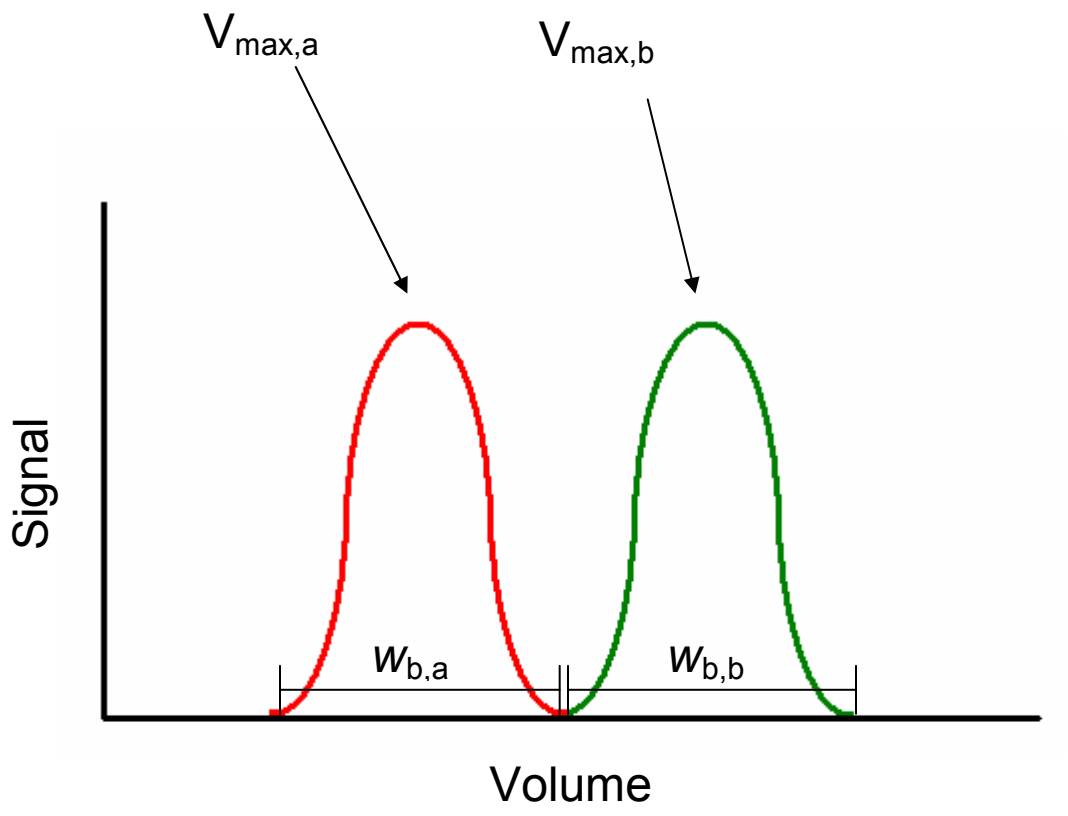

Figure 3. Chromatogram with illustration of resolution parameters.

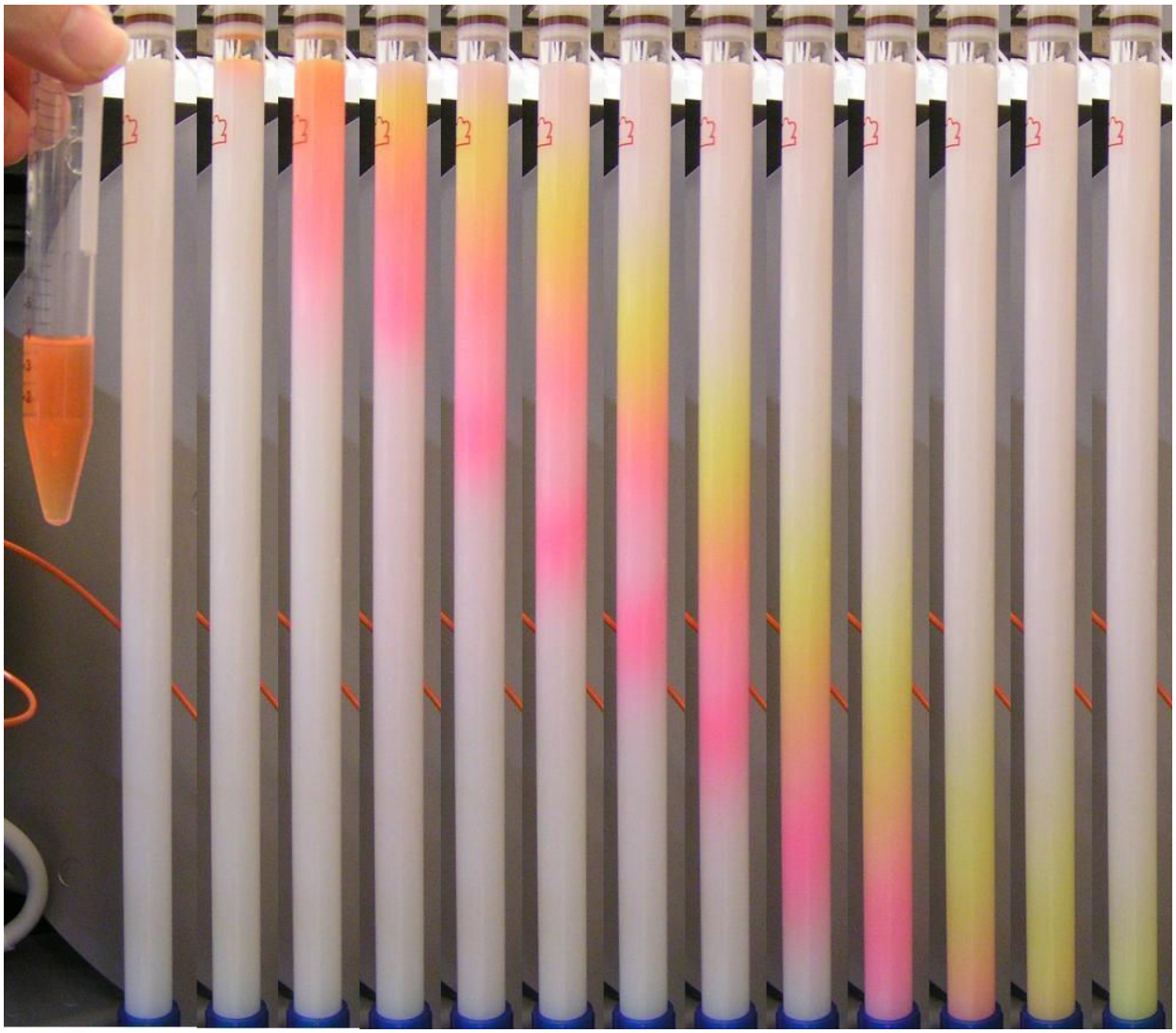

Figure 4. Anion exchange of a mixture of EGFP and DsRed2 using method 3 (see Table 2).