Chromatofocusing

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Chromatofocusing is a form of gradient elution chromatography performed using an ionexchange column packing and an internally generated pH gradient that travels through the column as a retained pH front.



Introduction

The technique of chromatofocusing is used to separate amphoteric substances, most commonly proteins, and was originally developed by Sluyterman and his colleagues (Sluyterman and Elgersma, 1978; Sluyterman and Wijdeness, 1978). In contrast to the related technique of isoelectric focusing, chromatofocusing does not utilize an electric field. Instead, a pH gradient is made to propagate inside an ion-exchange chromatography column as a retained front owing to the adsorption behaviour of the buffering species in the elution buffer. The separation achieved is based on charge differences between proteins. Proteins elute from a chromatofocusing column at a pH, generally termed the apparent isoelectric point, that is often close to the actual isoelectric point. Chromatofocusing is used both as an analytical and as a preparative method, and is capable of high resolution, with separations reported between protein isoforms differing by a single amino acid residue and by less than 0.05 pH units in apparent isoelectric points.

Outline and Applications of Method

The method as most commonly practised employs a weakbase, ion-exchange column packing and a polyampholyte elution buffer containing a mixture of polymeric buffering species that buffers a broad pH range. To perform the method, the column is first equilibrated at an initial pH using a starting buffer typically containing a single, weakbase buffering species. After the sample containing the proteins to be separated is injected into the column, the polyampholyte elution buffer, which has been titrated to a pH lower than the initial pH, is introduced into the column as a step change at the column entrance. The interaction of the column packing with the elution buffer produces a gradual, decreasing pH gradient that travels through the column as a retained front. The effect of the pH gradient on the adsorption behaviour of the proteins in the sample causes the analyte proteins to separate in the column effluent. Although early studies of the method employed column packings derivatized with diethylaminoethyl groups to provide the ion-exchange functionality, it was soon recognized that linear pH gradients could be produced more easily with polylethylenimine derivatized column packings. Currently, several commercial suppliers produce a variety of weak-base ion-exchange column packings and polyampholyte buffers with properties optimized for use in chromatofocusing. Illustrative applications of the method using these common procedures and materials are summarized in Table 1.

 Table 1
 Illustrative applications of chromatofocusing

Application	Reference
Separation of cortisol-bovine serum albumin conjugates Separation of two isoforms of the protein β_2 -microglobulin that differ by a single amino acid residue	Giraudi G and Baggiani C (1990) <i>Analyst</i> 115 : 1531–1534 Odani H, Oyama R, Titani K, Ogawa H and Saito A (1990) <i>Biochemical and Biophysical Research Communications</i> 168 (3): 1223–1229
Preparative-scale separation and purification of the peptides thymosin β_4 and thymosin β_9 from bovine tissue Purification and concentration of proteins produced by <i>Haemophilus influenzae</i> for use in proteome analysis	 Roboti A, Livaniou E, Evangelatos GP et al. (1994) Journal of Chromatography A 662: 27–34 Fountoulakis M, Langen H, Gray C and Takacs B (1998) Journal of Chromatography A 806: 279–291

Position and Width of Protein Bands in the Column Effluent

Figure 1a shows qualitatively the pH profile, and the concentration profiles of proteins in the column effluent, for the case where a weak-base, ion-exchange column packing and a descending pH gradient are used to perform chromatofocusing. **Figure 1b** shows the corresponding velocities of protein bands, and the velocities of pH values on the pH profile, both as functions of the liquid-phase pH. As illustrated, if any of the protein under consideration is located downstream from the centre of the protein band, it will tend to have a velocity less than the average velocity of the band, while the reverse is true for any of the protein located upstream from the centre of the band, so that all of the protein tends to accumulate into a narrow region at the point where the band velocity curve intersects the pH profile, as indicated by arrows in **Figure 1b**.

The width of a protein band during chromatofocusing is determined by the shape of the pH gradient, the degree to which the charge of the protein varies with pH, and other properties of the system. More specifically, Sluyterman and Elgersma (1978) have shown that the band width at the column outlet in terms of the pH change across the band follows eqn [1], where ϕ is the log of the ratio of hydrogen



Figure 1 (a) pH profile and concentration profiles of proteins focused into bands in the effluent from a chromatofocusing column. (b) Corresponding relation between the apparent isoelectric points, the curves giving the velocities of protein bands as functions of the liquid-phase pH, and the curves giving the velocities of pH values as a function of those values inside the chromatofocusing column.

ion concentrations in the fluid and adsorbed phases, which is equivalent to the dimensionless Donnan potential described by Sluyterman and Elgersma, d(pH)/dV is the slope of the pH gradient measured at the column outlet in units of pH per volume of mobile phase that has passed through the column, and dz/d(pH) is the rate of change of the characteristic binding charge of the protein with pH.

$$\Delta(\mathrm{pH}) \propto \sqrt{\frac{1}{\phi} \frac{\mathrm{d}(\mathrm{pH})/\mathrm{d}V}{\mathrm{d}z/\mathrm{d}(\mathrm{pH})}} \qquad [1]$$

Variations of Method

Soon after the introduction of the original method, efforts were made by several workers to replace the polyampholyte elution buffer with a chemically well-defined mixture containing a large number of low-molecular-weight buffering species (Hearn and Lyttle, 1981; Hutchens *et al.*, 1986). Under these conditions, the effluent pH profile changes from the smooth profile shown in **Figure 1** to a profile consisting of a sequence of discrete pH steps, with the number of steps corresponding to the number of buffering species in the elution buffer that produce ions that adsorb onto the column packing. Nevertheless, if an adequate number of these buffering species is present, the pH profile becomes sufficiently close to linear in shape that the method operates in essentially the same way as when a polyampholyte elution buffer is employed.

An approach to simplifying the elution buffer in chromatofocusing suitable for preparative chromatography has been described by Frey (1996) and Strong and Frey (1997) in which either a weak-base or strong-base ionexchange column packing is used together with a limited number of low-molecular-weight buffering species in the elution buffer. These workers demonstrated that when the starting and elution buffer compositions are properly optimized, the protein of interest can be selectively focused on a single, retained stepwise pH front in the column effluent while the impurities elute from the column in other regions of the pH profile. It was also observed by these workers that a retained, stepwise pH front formed using a small number of buffering species in the elution buffer can accomplish the displacement development of proteins to yield a form of displacement chromatography that eliminates the need for a traditional displacer component (Narahari et al., 1998). Other efforts to extend the method include studies of how to select the composition of the elution buffer to form a gradual pH gradient having a desired shape when a limited number of low-molecularweight buffering species are employed in the elution buffer (Bates and Frey, 1998; Logan et al., 1999), and studies involving the external mixing of two different elution buffers in varying proportions to modify the shape of a retained pH gradient (Liu and Anderson, 1997).

Conclusions

Since its introduction in the late 1970s, chromatofocusing has found widespread use as a high-resolution chromatographic procedure for separating proteins according to their apparent isoelectric points. Recent development efforts aimed at reducing its reliance on polyampholyte buffers and specialized column packings are likely to expand the future range of applications for the method, especially as a preparative separation technique.

References

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Further Reading

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