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Diafiltration: A Fast, Efficient Method for Desalting, or Buffer Exchange of Biological Samples

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Diafiltration is a technique that uses ultrafiltration membranes to completely remove, replace, or lower the concentration of salts or solvents from solutions containing proteins, peptides, nucleic acids, and other biomolecules. The process selectively utilizes permeable (porous) membrane filters to separate the components of solutions and suspensions based on their molecular size. An ultrafiltration membrane retains molecules that are larger than the pores of the membrane while smaller molecules such as salts, solvents and water, which are 100% permeable, freely pass through the membrane.

This article will cover the concepts of protein concentration and diafiltration. It will compare different ways of performing diafiltration and their impact on process time, volume, product stability, and recovery.

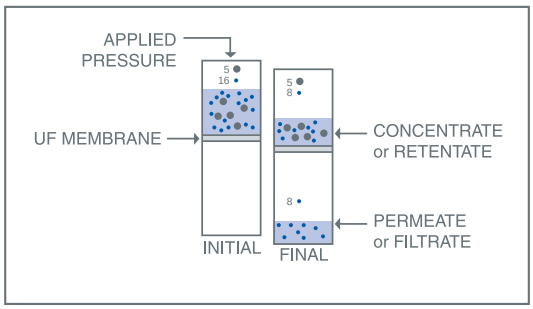
CONCENTRATION

The solution retained by the membrane is known as the concentrate or retentate. The solution that passes through the membrane is known as the filtrate or permeate.

A membrane for concentration is selected based on its rejection characteristics for the sample to be concentrated. As a general rule, the molecular weight cut-off (MWCO) of the membrane should be 1/3rd to 1/6th the molecular weight of the molecule to be retained (3-6X Rule). This is to assure complete retention. The closer the MWCO is to that of the sample, the greater the risk for some small product loss during concentration. The risk increases if diafiltration will also be used since the relative loss depends on the total volume of filtrate that will be generated. Membrane flux rate (filtrate flow rate per unit area of membrane) is related to pore size. The smaller the pores, the lower the flux rate for the same applied pressure. Therefore, when selecting a membrane for concentration / diafiltration, one must consider the time factor versus product recovery. In most biological applications, recovery outweighs the time consideration. The process time can always be reduced by increasing the amount of membrane area used.

Figure 1 below provides an example of concentration. The sample is placed in a device containing a suitable ultrafiltration membrane that will retain the large molecules. Pressure is applied until half the volume has passed through the membrane. The large molecules are retained in half the original volume (concentrate), which also contains half of the salt molecules. The filtrate contains the other half of the salt molecules but none of the large molecules. Therefore, the large molecules are concentrated as liquid and salt are removed. The salt molecule to volume ratio in the concentrate remains constant so the ionic strength of the concentrated solution remains relatively constant.

Figure 1.
2X Concentration of Sample by Ultrafiltration



- Large molecules – bigger than pores in membrane
- Small molecules – salts or solvent

The ionic strength of the concentrate (retentate) solution can subsequently be reduced by "washing" the remaining salt out with water, a process called diafiltration. This is essentially a dilution process and is performed in conjunction with a concentration process. Water is added while filtrate is removed. If the washing solution is another buffer instead of water, the new buffer salt will replace the initial salt in the sample.

For simplicity, the above and subsequent examples use a direct flow filtration device such as a centrifugal concentrator. The same principles apply to cross flow filtration devices such as cassettes and hollow fibers where the retentate is recirculated.

BENEFITS OF DIAFILTRATION

Conventional techniques used for salt removal or buffer exchange such as membrane dialysis and column-based gel filtration can be effective but have limitations. Dialysis procedures can take up to several days, require large volumes of water for equilibration and risk product loss through manual manipulation of the dialysis bags. Gel filtration results in a dilution of the sample and often requires an additional ultrafiltration step to concentrate it back. Adding steps to a process can risk sample loss or possible contamination. With diafiltration, salt or solvent removal as well as buffer exchange can be performed quickly and conveniently. Another big advantage of diafiltration is that the sample is concentrated on the same system, minimizing the risk of sample loss or contamination.

There are several ways to perform diafiltration. While the end result may be the same, the time and volume required to complete the process may vary considerably. It is important to understand the differences in the methods used and when to choose one over the other.

CONTINUOUS DIAFILTRATION

The technique of continuous diafiltration (also referred to as constant volume diafiltration) involves washing out the original buffer salts (or other low molecular weight species) in the retentate (sample) by adding water or a new buffer to the retentate at the same rate as filtrate is being generated. As a result, the retentate volume and product concentration does not change during the diafiltration process. If water is used for diafiltering, the salts will be washed out and the conductivity lowered. If a buffer is used for diafiltering, the new buffer salt concentration will increase at a rate inversely proportional to that of the species being removed.

The amount of salt removed is related to the filtrate volume generated, relative to the retentate volume. The filtrate volume generated is usually referred to in terms of “diafiltration volumes”. A single diafiltration volume (DV) is the volume of retentate when diafiltration is started. For continuous diafiltration, liquid is added at the same rate as filtrate is generated. When the volume of filtrate collected equals the starting retentate volume, 1 DV has been processed.

Using continuous diafiltration, greater than 99.5% of a 100% permeable solute can be removed by washing through 6 retentate volumes (6DV) with the buffer of choice.

Molecules that are larger than salts and solvents, but which are still smaller than the pores in the membrane, can also be washed out. The permeability of these molecules, however, may be less than 100%. In such cases, it will take more liquid, i.e. more DV’s, to completely wash a partially permeable molecule through the membrane, compared to a 100% permeable molecule. Typically, the larger the molecule, the lower the permeability and the greater the wash volume required.

The permeability of a molecule through a specific membrane can be determined by measuring the concentration of the molecule in the filtrate compared to the concentration in the retentate under specified conditions.

% permeability = $(\text{Conc.}_{\text{FILTRATE}} / \text{Conc.}_{\text{RETENTATE}}) \times 100$

Permeability is often described in terms of “Rejection Coefficient” of the membrane, i.e. the membrane’s ability to hold back or reject a given molecule from passing through.

Rejection Coefficient = $1 - (\text{Conc.}_{\text{FILTRATE}} / \text{Conc.}_{\text{RETENTATE}})$.

A rejection coefficient of 1 equals 0% permeability

A rejection coefficient of 0 equals 100% permeability

Permeability will be affected by such factors as transmembrane pressure (TMP), crossflow rate, retentate concentration, pH, and ionic strength, and gel layer formation (concentration polarization). Therefore, the permeability may change during the process.

Table 1 shows the relationship between permeability through a membrane and the number of diafiltration volumes required for removal of permeating species. As noted earlier, a greater volume of buffer is required to remove a molecule that is partially retained. To remove 99.9% of a molecule that is 25% permeable to the membrane requires 9 DV’s, while for a 100% permeable species, only 7 DV’s are required.

Table 1:
Continuous (Constant Volume) Diafiltration

Diafiltration Volumes	Permeability 100% Rejection Coefficient = 0	Permeability 75% Rejection Coefficient = 0.25
1	63%	53%
2	86%	77%
3	95%	89%
4	98.2%	95%
5	99.3%	97.6%
6	99.7%	98.9%
7	99.9%	99.4%
8		99.7%
9		99.9%

0% - Salts, solvents, buffers, etc.

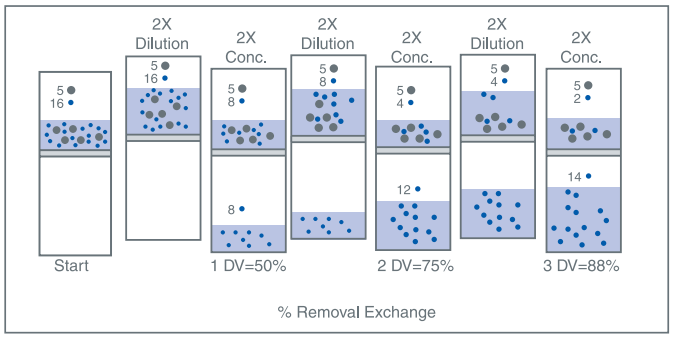
25%- Molecules lower in MW than MWCO of membrane but bigger than salts

DISCONTINUOUS DIAFILTRATION – Sequential Dilution

Discontinuous diafiltration by sequential dilution involves first diluting the sample with water or replacement buffer to a predetermined volume. The diluted sample is then concentrated back to its original volume by ultrafiltration. This process is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent dilution removes more of the small molecules.

As shown in Figure 2, the sample is generally diluted with an equal volume of buffer (1DV). Alternatively, multiple volumes can be added at once, provided the process tank is large enough to hold the entire volume. Diluting the sample usually lowers the viscosity, which may increase the filtrate flux rate.

Figure 2.
Discontinuous Diafiltration – Sequential Dilution

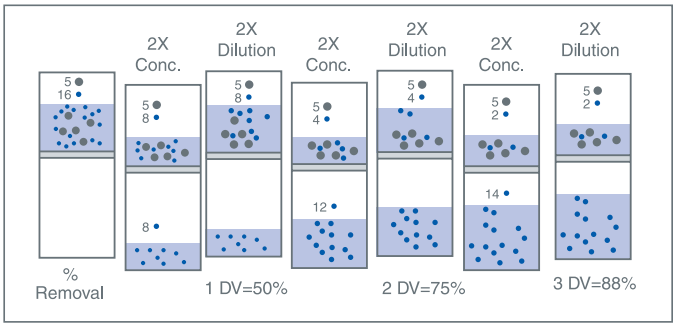


- Large molecules – bigger than pores in membrane
- Small molecules – salts or solvent

DISCONTINUOUS DIAFILTRATION – Volume Reduction

Discontinuous diafiltration by volume reduction reverses this procedure. The sample is first concentrated to a predetermined volume, and then diluted back to its original volume with water or replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent concentration and dilution removes more of the small molecule (Figure 3).

Figure 3.
Discontinuous Diafiltration with Volume Reduction



- Large molecules – bigger than pores in membrane
- Small molecules – salts or solvent

After the last buffer addition to complete diafiltration, the sample may be concentrated before analysis or the next purification step is performed.

The final product, after diafiltration by either method (discontinuous 2X volume reduction or sequential dilution) is at the same volume and concentration as when diafiltration started. The salt concentration has been equally reduced in both examples. However, the volume of diafiltration buffer used by the volume reduction method was half that used in sequential dilution. This is because the initial concentration step reduced the volume in half. A diafiltration volume is equal to the volume where dilution occurs. Therefore, half the volume was required.

This being the case, it would seem that concentrating before diafiltration, by either discontinuous sequential dilution or constant volume diafiltration, should reduce the required diafiltration buffer volume and save time. And in most cases this is true. The factor we have not accounted for is filtrate flux rate, which equates to process time. As the product becomes concentrated, viscosity increases and the filtrate flux rate decreases. The filtrate flux rate varies inversely as the log of the concentration factor.

$$J = k \ln(C_G / C_B)$$

Where

J = Filtrate Flux Rate

k = constant

C_G = gel layer concentration

C_B = retentate (bulk flow) concentration

This becomes very significant as the product concentration (C_B) increases above a few percent and is dependent on the characteristics of the specific molecules that make up the sample. So, although it might take significantly less volume to diafilter a concentrated sample, it could take considerably more time compared to a less concentrated sample. Simple protocols are available to find optimum conditions to maximize productivity.

Table 2.
Salt Reduction from Sample using Volume Reduction or Constant Volume Diafiltration

Diafiltration Volumes	2X Volume Reduction		Continuous Diafiltration (Constant Volume)	
	100% Permeable 0% Retention*	75% Permeable 25% Retention*	100% Permeable 0% Retention*	75% Permeable 25% Retention*
1	50%	41%	63%	53%
2	75%	65.%	86%	77%
3	88%	79%	95%	89%
4	94%	88%	98.2%	95%
5	96.9%	93%	99.3%	97.6%
6	98.4%	95.6%	99.7%	98.9%
7	99.2%	97.4%	99.9%	99.4%
8	99.6%	98.4%		99.7%
9	99.8%	99.0%		99.9%
10	99.9%	99.4%		

*Retention of smaller molecules

0% - Salts, solvents, buffers, etc.

25%- Molecules lower in MW than MWCO of membrane but bigger than salts

CONTINUOUS OR DISCONTINUOUS DIAFILTRATION - WHICH TECHNIQUE SHOULD BE USED?

When deciding which technique to use and where in the process to perform diafiltration, consider the following factors:

1) Initial sample volume, concentration and viscosity

2) Required final sample concentration

3) Stability of sample at various concentrations

4) Volume of buffer required for diafiltration

5) Total processing time

6) Reservoir size available

7) Economics

The choice of which method to use must be based on several criteria. Scale is an important consideration. What we will do at laboratory scale may be very different than at process scale, especially if the process is automated. At lab scale discontinuous diafiltration is often used for simplicity. Continuous diafiltration requires a pump or equipment to add the diafiltration solution at a constant rate. Both techniques can be automated for process applications. If we eliminate the equipment issue and focus on the process, we can compare the differences.

The ionic strength, buffer composition and stabilizer concentration can affect stability of the sample. Diafiltration may remove salts or stabilizing molecules, resulting in protein product denaturation and aggregation. The process of concentrating and diluting a protein solution can also affect molecular interactions resulting in denaturation or aggregation as well as subsequent precipitation and product loss. It is necessary to evaluate the effect of concentration on the product to determine where diafiltration is best performed relative to concentration effects.

Continuous diafiltration offers an advantage over discontinuous diafiltration in that the retentate concentration remains constant. It is often seen as a more gentle process relative to the stability of the product.

WHEN TO PERFORM DIAFILTRATION - BEFORE OR AFTER CONCENTRATION?

We have already seen that concentrating a sample first can significantly reduce the volume of diafiltration solution required. We have also seen that continuous diafiltration takes less volume than discontinuous diafiltration with sequential dilution. Therefore, if the sample is first concentrated to the final concentration required and then continuous diafiltration performed, acceptable results should be obtained.

However, above a certain concentration, filtrate flux rates may become prohibitively slow. It may actually takes longer to diafilter the concentrated sample than it would if the sample were first diluted to reduce the concentration. In this situation, even though continuous diafiltration of the diluted sample requires a greater diafiltration volume, the total processing time would be less due to the faster filtrate flux rate. (Process Time = Filtrate flow rate x Volume)

In general, the optimum retentate concentration for performing (continuous) diafiltration is at:

$\ln (C_G/C_R) = 1$ or $C_{R\ (optimum)} = C_G/e = 0.37C_G$ *

Where

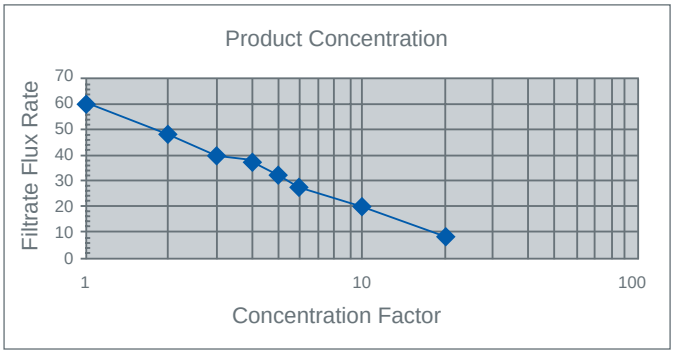
C_G = gel layer concentration

C_R = retentate concentration.

$C_{R(Optimum)}$ = highest retentate concentration where diafiltration should be performed

The C_G value for a sample can be determined from experimentation by concentrating a sample on a membrane and recording and plotting data for filtrate flux rate vs. log concentration (or concentration factor). The curve can then be extrapolated to filtrate flux rate = "0". The C_G value will be the same for this product regardless of the starting concentration or filtrate flux rate.

Figure 4.
Determination of the C_G Value for a product



In this example (Figure 4) the C_G value is a concentration factor of approximately 33X. Therefore the optimal concentration to perform diafiltration would be $0.37C_G$ = 12.2X. If the starting product concentration is 5mg/mL, then diafiltration should be performed when the concentration reaches 61mg/mL. If the final concentration will be less than 61mg/mL, then diafiltration should be performed after concentration, unless it is necessary to remove a specific molecule prior to concentration.

The ultrafiltration product selected may dictate choice of continuous or discontinuous diafiltration. Stirred cells and centrifugal devices are best suited for discontinuous diafiltration because of their mode of operation. Tangential flow devices have the advantage of being useful for either diafiltration technique.

Summary

Diafiltration is a fast and effective technique for desalting or buffer exchange of solutions. It can be performed in a continuous or discontinuous mode. Continuous diafiltration usually takes less volume to achieve the same degree of salt reduction as discontinuous diafiltration with sequential dilution and can be easier to perform.

Continuous diafiltration is also perceived as a kinder and gentler process on active biomolecules. On the other hand, discontinuous diafiltration with volume reduction takes less volume than continuous diafiltration. Concentrating the sample before diafiltration usually reduces the required filtrate volume and saves time. However if the sample viscosity becomes too great, the filtrate flux rate decreases and the process time can increase substantially. Determining the C_G for the sample can help answer the question - At what concentration should I perform diafiltration?

Initial volumes from a few milliliters up to thousasnds of liters can be processed using Pall's Tangential Flow Filtration (TFF) products. Pall's tangential flow systems allow easy addition of membrane surface area, providing the flexibility to reduce processing time or scale up the process. This range of devices and systems provides scalability as well as the flexibility to work with almost any sample volume. Pall's highly selective OMEGA™ polyethersulfone (PES) membranes with narrow pore size distribution and high flow rates provide fast processing time and efficient separation for diafiltration.

* Industrial Ultrafiltration Design and Application of Diafiltration Processes, Beaton & Klinkowski, J. Separ. Proc. Technol., 4(2) 1-10 (1983)

Glossary

Diafiltration: Diafiltration is a technique that uses ultrafiltration membranes to completely remove or lower the concentration of salt or solvent, or to replace buffer salts from solutions containing proteins and other large molecules,

Diafiltration Volume: One diafiltration volume equals the initial volume in which the molecule of interest is suspended. The number of diafiltration volumes required depends on whether the permeating species is freely passing (salts, buffers, solvents) or partially retained.

Continuous Diafiltration: The technique of continuous diafiltration (also referred to as constant volume diafiltration) involves washing out the original buffer salts (or other low molecular weight species) in the retentate (sample) by adding water or a new buffer to the retentate at the same rate as filtrate is being generated

Discontinuous Diafiltration-Sequential Dilution: Discontinuous diafiltration by sequential dilution involves first diluting the sample to a predetermined volume, then concentrating the sample back to its original volume with water or replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent dilution removes more of the small molecules

Discontinuous Diafiltration-Volume Reduction: Discontinuous diafiltration by volume reduction involves first concentrating the sample to a predetermined volume, then diluting the sample back to its original volume with water or replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent concentration and dilution removes more of the small molecule.

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