Developing a Vacuum Concentration Protocol

Introduction

Efficient removal of solvents by vacuum concentration is a routine step for many analytical and isolation protocols. For the most part, protocols simply involve spinning the sample while applying a vacuum. A vacuum concentrator reduces the atmospheric pressure which lowers the boiling point of the solution. The centrifugal force keeps the sample from boiling out of the tube, which is a generally referred to as *bumping*. However, some solutes are negatively affected by both temperature and solute concentration changes that occur during processing. For optimum yield and preservation of biological activity, these parameters should be considered for each new solute and solvent combination.

Solute Properties and Guidelines for Concentration

The ease by which a solute can be concentrated is dependent upon the characteristics of the solute and solvent. A resilient molecule, such as DNA, is easily concentrated when in an aqueous solution. It can be heated, concentrated to dryness and will still perform properly when introduced into a cell. Characteristics of the solvent being evaporated have a major impact on the process as well. Volatile solvents are much easier to remove. The ease by which solvent is removed from a sample is secondary when compared to the resulting quality of the concentrated solute product.

Not all solutes have the resiliancy of DNA. Many molecules can be denatured if heated or cooled and may become insoluble if dried. Therefore it is important to know the limitations of your solute. The following general guidelines can be applied:

- Small DNA molecules are resistant to moderate heating and drying, but extreme heating will lead to denaturation. Strands may become nicked but typically still function in vivo.
- Genomic DNA will also survive moderate heat, but may be difficult to dissolve in aqueous buffers after concentration.
- RNA is sensitive to basic conditions, and concentration should not produce a high pH. RNA can also be difficult to re-dissolve if thoroughly dried.
- Proteins are possibly the most difficult solute to concentrate intact. The diversified nature of each protein species, including primary sequence, secondary structures and post-translational modifications, make broad generalizations about handling proteins difficult. Temperature, freezing conditions, salts and protein concentration can all affect product integrity. Use caution when handling proteins.
- Carbohydrates, especially monomers, tolerate handling well. A potential problem may lie in the drying of polymers, such as starch, which may be difficult to re-hydrate. High temperatures can also damage carbohydrates.

When first working with a new molecule, it is difficult to know which parameters affect the solute being concentrated. Proceed with caution until you determine how much abuse your sample can tolerate. This is especially true for proteins.

SmaI

As previously noted, DNA is a resilient molecule easily concentrated to dryness. Unfortunately, many other molecules aren't as forgiving. For instance, the restriction enzyme SmaI is very sensitive to changes in temperature. It is inactivated by both heat (> 65°C) and extreme cold.

SmaI was diluted to a concentration of 1 U/Ìl in water and concentrated with a Labconco CentriVap Vacuum Concentrator using three methods. The enzyme was then rehydrated in 1X restriction enzyme buffer and tested for activity by cleaving Ï DNA. Concentrating the enzyme at room temperature using a low vacuum to the point of dryness has little effect on the SmaI activity. Using high vacuum conditions causes a solution of the enzyme to concentrate by freeze drying, which also has little effect on enzyme activity. However, concentrating the enzyme using high vacuum but without freezing the solution results in loss of activity. Results are summarized in Figure 1.

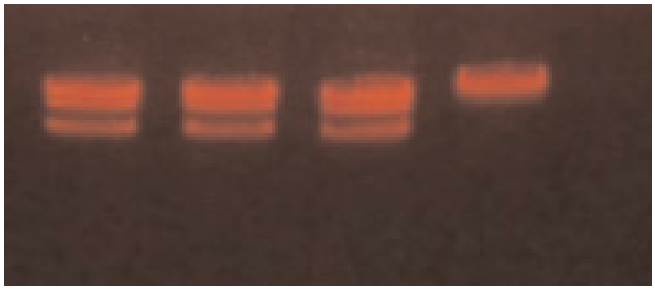

Figure 1. The fate of SmaI is unexpected when vacuum concentrated. Lane 1 is a SmaI control used to digest Ï DNA at 25°C. Lane 2 represents enzyme dried under low vacuum, and lane 3 was freeze dried under high vacuum with sufficient heat removal to freeze the sample. The SmaI of lane 4 was concentrated under low vacuum at 4°C. All lanes exhibit activity except lane 4.

Effect of High Salt Concentration

Other proteins are tolerant of high and low temperatures, but may become inactive or insoluble in high salt concentrations. The dehydration and inactivation of proteins during freeze drying can be caused by local accumulation of salt ions. Buffer exchange is one method used to reduce the concentration of salt ions prior to solute concentration. Gel filtration with G10 or G15 Sephadex (GE Healthcare, Piscataway, NJ) can be used to exchange buffers on peptides, proteins and other high molecular weight solutes. Generally samples become more dilute after gel filtration, but the water will soon be removed during concentration.

Result Between Surface Area, Temperature, and Pressure

The parameters that affect solutes during vacuum concentration are not limited to temperature. The interrelationship between surface area, temperature, and pressure also affects sample integrity. When a vacuum is applied, the sample temperature will decrease as solvent molecules vaporize. In a process referred to as adiabatic expansion, internal energy of the sample is transferred as solute moves between phases. As the vacuum removes the solvent vapors, the temperature of the liquid decreases and the rate of vaporization or sublimation if the sample is frozen, slows. With room temperature water (500 microliters in a microcentrigure tube), the sample temperature can decrease to -25°C in just a few minutes after applying a high efficiency vacuum (Figure 2). Methanol, which is more volatile than water, drops to below -40°C under the same conditions. It is also important to note that methanol boils at 64.7°C. Figure 3 demonstrates these differences.

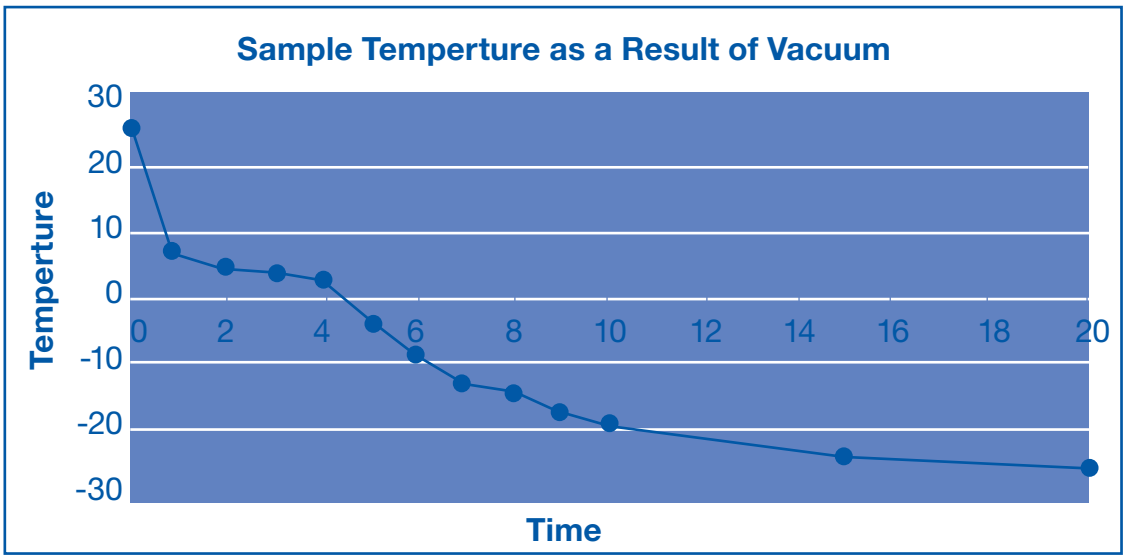

Figure 2. The temperature of water decreases rapidly when a deep vacuum is applied. As water molecules vaporize, heat is removed from the system which eventually leads to freezing. The rate of water loss then slows as the heat required for sublimation is greater than vaporization.

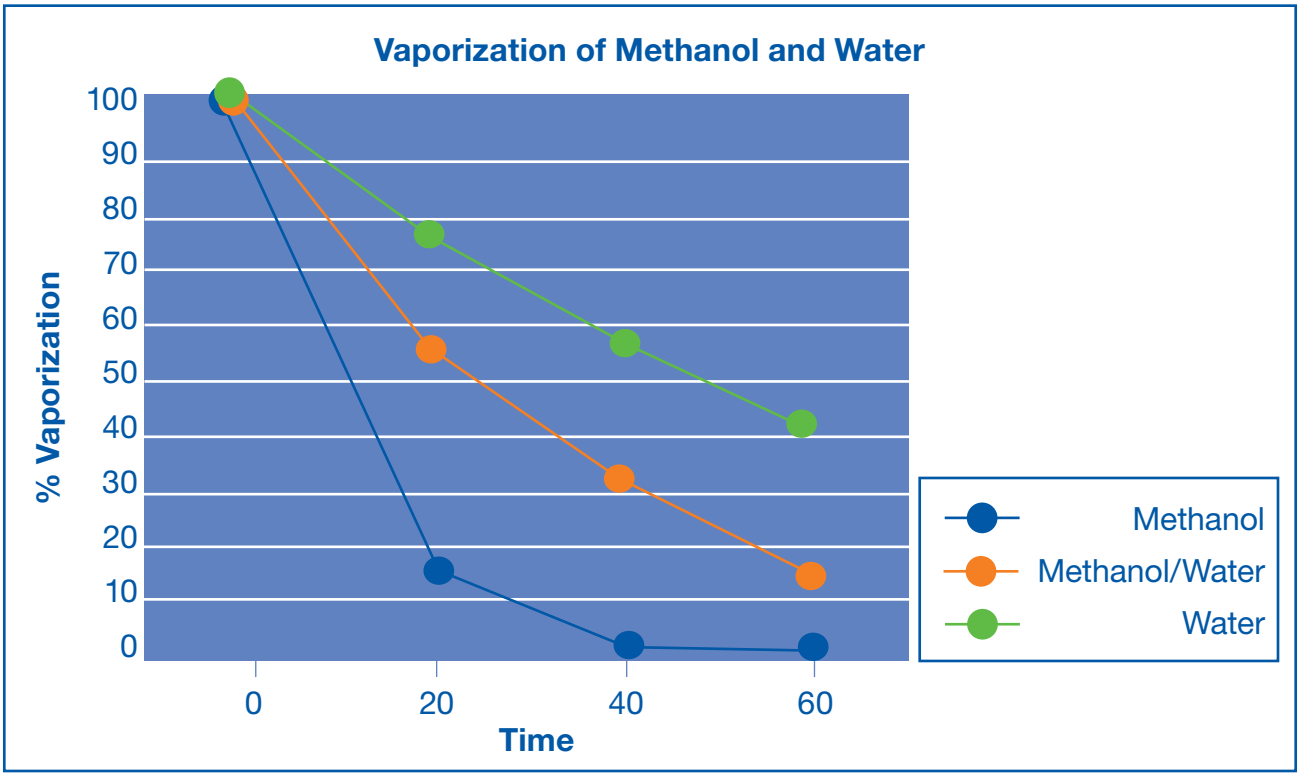

Figure 3. Vaporization of methanol and water demonstrates the nature of solvent volatility on concentration. Methanol was completely vaporized in approximately half the time of water. The methanol/water mixture shows a hybrid vaporization pattern.

Impact of Solvent Properties on the Concentration Process

The difference in vaporization between methanol and water illustrates the importance of solvent properties on the concentration process (Table 1). In comparing these solvents, the key property that affects concentration is boiling point. As vacuum is applied to the solvents and the boiling point decreases, molecules readily vaporize. Consequently, the order of solvent evaporation rate during sample concentration is methanol > ethanol > water (Figure 4).

Table 1. Physical Properties of Common Solvents

Solvent	Melting/Freezing Point	Boiling Point
Water	0,0	100.0
Ethanol	-114.1	78.5
Methanol	-97.7	64.7

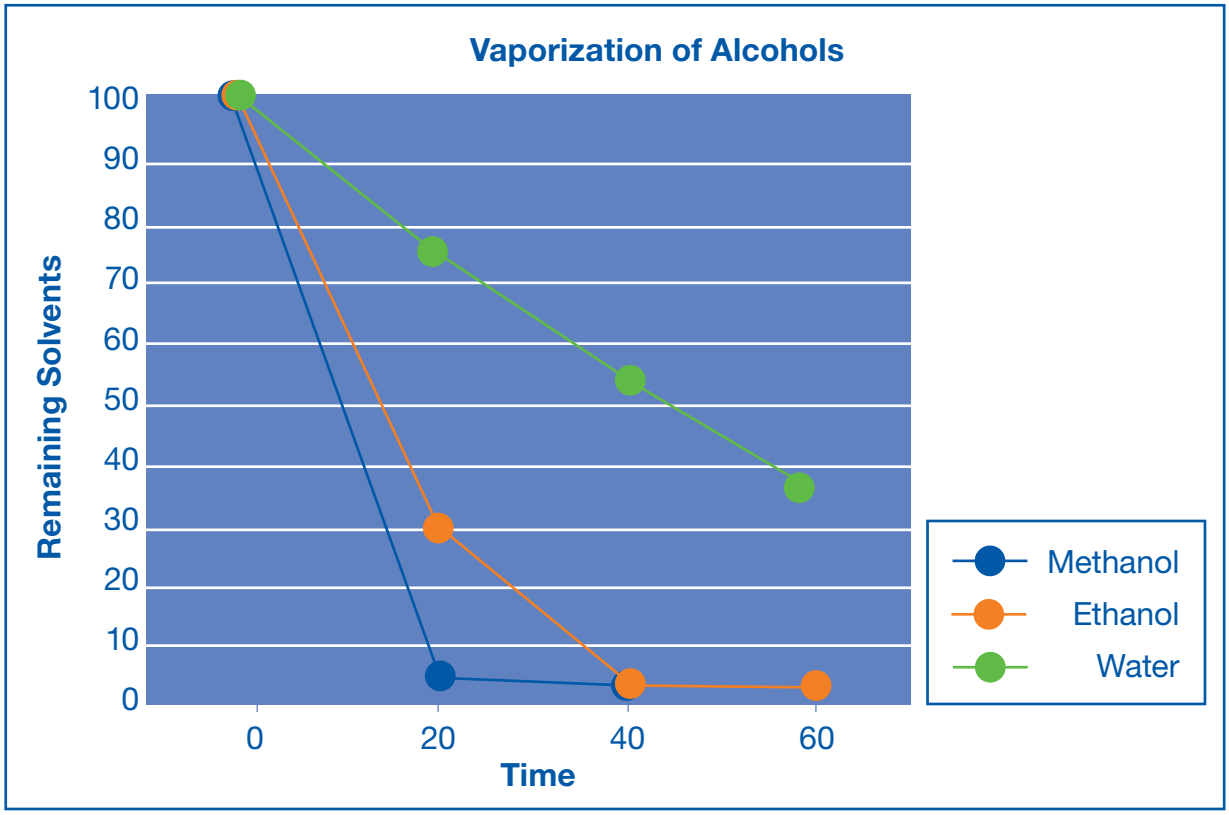

Figure 4. The graph illustrates the concurrent evaporation of methanol, ethanol and water samples in a Labconco CentriVap Centrifugal Concentrator. It demonstrates that solvents with lower boiling points vaporize faster. This process represents 500 microliters (ul) of solvent evaporated with a high efficiency vacuum pump for one hour.

Effect of Freezing Point on Concentration

The solvent freezing point also affects the concentration process. Under high efficiency vacuum conditions,

water vaporizes until it freezes. At that point, water loss is through sublimation, which requires greater heat energy than vaporization. A comparison of the rate of vaporization from water and phosphate buffered saline PBS demonstrates this point in (Figure 5). Using a CentriVap Centrifugal Concentrator with a high efficiency vacuum pump, water freezes more rapidly than the PBS due to the presence of salt in the latter. Though the PBS)does eventually freeze the small time difference results in greater water loss from the PBS samples.

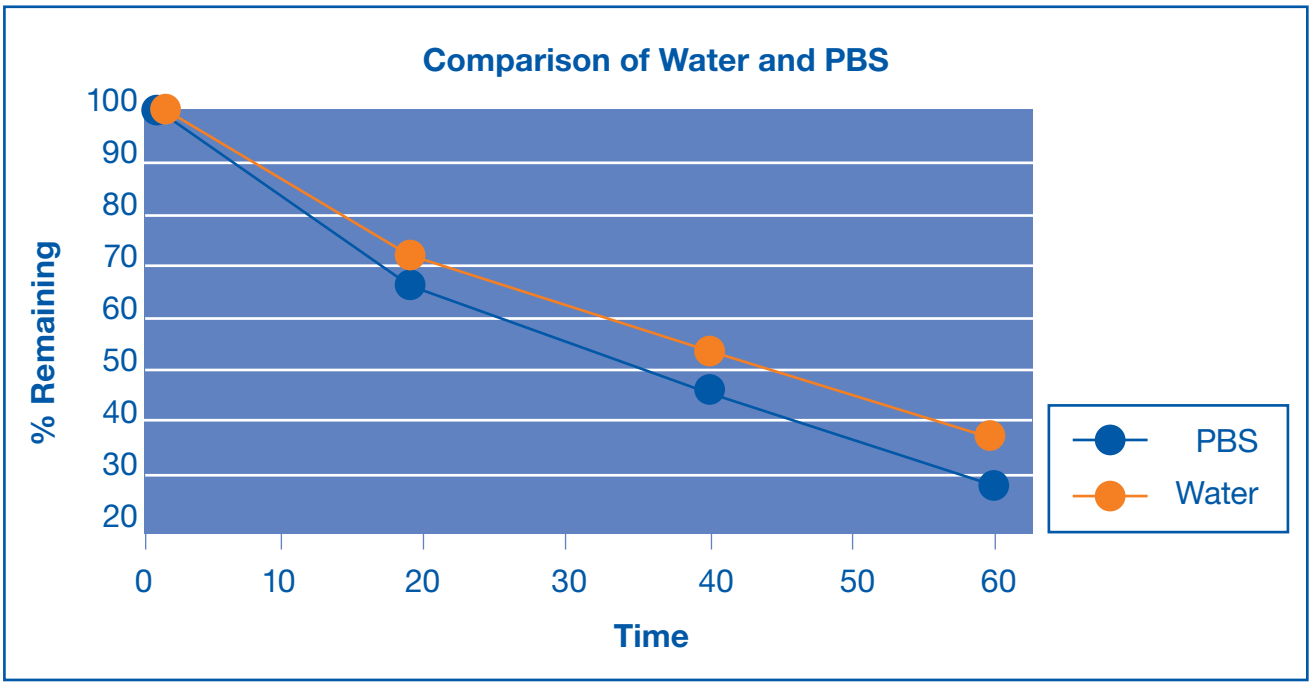

Figure 5. Vaporization of water and phosphate buffered saline (PBS) demonstrates that liquid samples vaporize at a rate faster than frozen samples. More energy is required to change in phase from solid to gas than from liquid to gas.

Introduction of Heat

Many vacuum concentrators introduce heat into samples to enhance evaporation. Although this can be effective, it is dependent upon the type of system employed. Centrifugal concentrators, such as the CentriVap, can provide temperature control to the chamber. A Refrigerated CentriVap is also available with cooling from -4°C and heating up to 100°C. Temperature control is useful for temperature sensitive proteins and analytes. However, an adjustment of atmospheric pressure may be necessary for heated chambers to be effective at accelerating evaporation.

Introducing heat into samples during the concentration process will not necessarily increase the temperature of the sample. If the atmosphere in the concentrator is under a near full vacuum, then heat introduced through the chamber will not affect the sample. Convection works by increasing the temperature (molecular motion) of the gas molecules in the local atmosphere. If there is little or no atmospheric pressure, which corresponds to a reduced number of atmospheric gas molecules, then heat transfer is ineffective. Using a bleed valve or lower

efficiency vacuum pump will increase the level of atmosphere pressure in the concentrator so that chamber heat is transferred to the sample. At high pressure, aqueous samples are less likely to freeze. This is especially important when concentrating bioactive compounds such a protein.

Phase Effect on Concentration

As shown in Figure 6 below, a comparison of sample water loss using CentriVap under high vacuum, high vacuum with infrared light and low vacuum demonstrates the phase effect on the concentration process. The high efficiency vacuum is most effective at evaporating water when the sample is liquid and the starting temperature is high. However, as the sample cools and freezes, the rate of water loss slows. Introducing heat by infrared radiation further enhances evaporation, but as the sample cools and eventually freezes, the rate declines again. Although low vacuum pressure is initially slow, the results are similar to those using a high efficiency vacuum. The rate of water loss eventually becomes greater since the sample does not freeze.

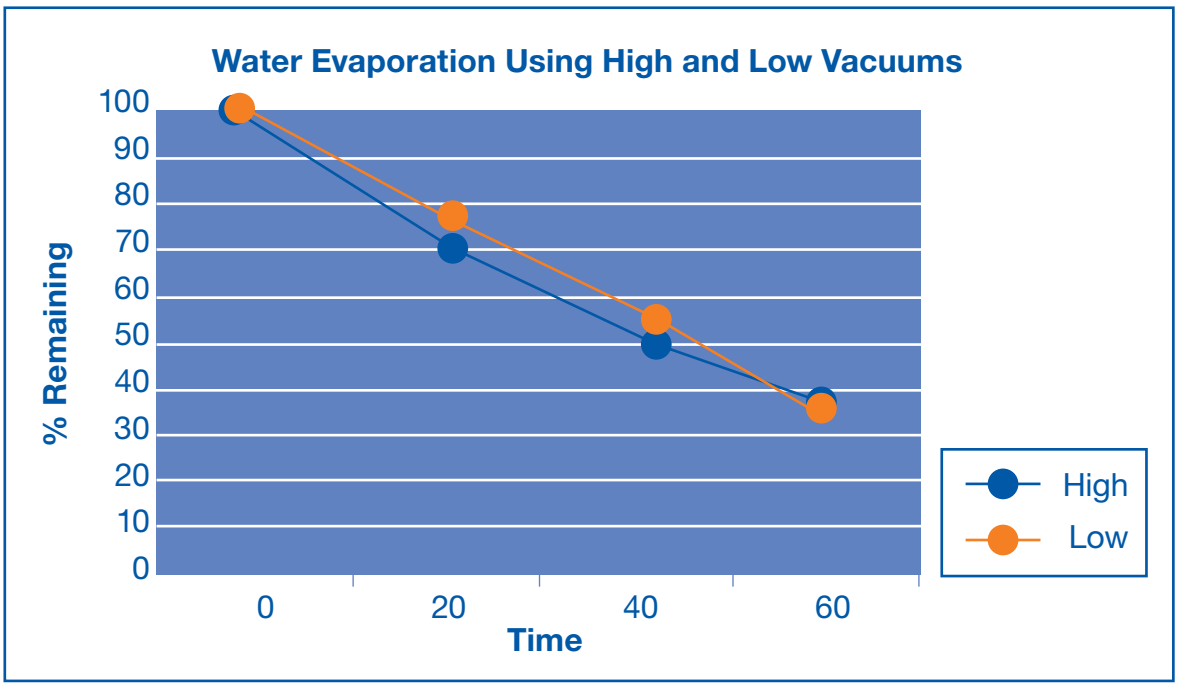

Figure 6. This graph demonstrates the comparison of water loss using high vacuum and low vacuum. Low vacuum concentration, though initially lagging, surpassed high vacuum concentration since the sample remained liquid.

Effect of Surface Area on Concentration

The surface area of the liquid also affects the rate of solvent loss. By increasing the surface area, solvent more readily escapes from solution. This implies that solvent contained in long, thin tubes or vessels will evaporate slower than with shorter containers with wider openings. To improve concentration, try placing tubes in a vacuum concentrator rotor at an angle to have a slanted surface area while spinning.

Summary

In order to effectively concentrate a solute, several important factors must be considered. First, the parameters that allow for solute stability must be identified and built into the process. High salt content, over drying and extreme temperatures can all negatively affect solute integrity. The concentration protocol must maintain parameters suitable to maintain stability for the solute being concentrated. If a choice of solvent is possible, using the solvent with the lower boiling point will result in faster evaporation. Make sure to use a vacuum level that is appropriate for the solute being concentrated. High efficiency vacuum may speed concentration, but loss of heat may cause the solvent to freeze and slow the process. Finally, use a large surface area to speed the removal of solvent from the sample.

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