

BASIC DESIGN OF LYOPHILIZATION PROTOCOLS FOR HUMAN BONE TISSUES

M. ITURRIA †, C. RODRÍGUEZ EMMENEGGER †, R. VIEGAS †, J. ZEBALLOS ‡,
O. WODOWOZ ‡, I. ÁLVAREZ ‡ and A. BOLOGNA ALLES †*

† *Departamento de Ingeniería de Materiales, Instituto de Ingeniería Química, Facultad de Ingeniería, Universidad de la República.*

‡ *Instituto Nacional de Donación y Transplante de Células, Tejidos, y Órganos, Hospital de Clínicas, Facultad de Medicina, Universidad de la República.*

**To whom all correspondence should be addressed. Universidad de la República, Julio Herrera y Reissig 565, 11300 Montevideo, Uruguay. E-mail: aballes@fing.edu.uy*

Abstract — For lyophilization protocols, the shelf temperature, chamber total pressure, and process time must be selected to maximize equipment throughput while maintaining highly reliable product characteristics. A general method to estimate these operation parameters was devised and applied to the production protocols of human cancellous chips, cortical struts, and morselized bone to be used as osteoconductive allografts. The sample freezing point was estimated utilizing thermodynamic principles and the shelf temperature was set just below this value. The total chamber pressure was adjusted according to the water vapor pressure above ice, and the process time during main and final drying were estimated considering both the amount of water to be removed from each sample and the rate of heat transferred by contact and radiation from the surroundings. The initial pressure-shelf temperature surfaces projected for all the drying stages were tuned experimentally to yield optimized freeze-drying protocols for each type of sample.

Keywords — Freeze drying, bone allograft, heat transfer.

I. INTRODUCTION

Cryodesiccation is the preferred method for drying thermally sensitive materials, because the low temperatures involved minimize unwanted reactions that usually occur in other drying processes, since frozen samples are dried under vacuum by sublimation of the ice present (Geankoplis, 1982). In order to maintain suitable drying rates, heat must be supplied to the frozen sample fast enough to prevent the sample temperature from dropping excessively resulting in exceedingly long processing times. Depending on the sample chemistry and morphology, a final drying period must be added to the normal main-drying stage to remove intimately combined water. Final drying is carried out at comparatively higher temperatures using the highest vacuum attainable by the equipment, in order to remove the last fraction of specimen humidity and meet the water activity required to allow the storage of the products at room temperature.

Despite lyophilization has been currently used in a wide variety of applications, little information is avail-

able for a rational approach to the initial estimation of freeze-drying start-up parameter values.

II. FREEZE DRYING CURVE ESTIMATION

The parameters needed to set a production protocol are the shelf temperature (T_S), the total chamber pressure (P_T) and the span of the main and final drying stages (t_M and t_F), if the latter is required.

The main drying shelf temperature selected must be low enough to assure that all the water present in the sample is under the form of ice before drying begins. Therefore, the freezing point depression of the water contained in the specimen must be estimated or measured, and a reasonable safety margin must be added to allow for local fluctuations in the sample characteristics. However, the highest possible safe shelf temperature should be selected to minimize processing time, since the drying process tends to decrease considerably the sample temperature and consequently the water mass transfer.

In general, two phenomena cause the depression of the freezing point of a pure fluid by decreasing its equilibrium vapor pressure, i.e., the presence of solutes, as well as the confinement of the liquid within a solid with surfaces akin to the fluid. Both contributions to the freezing point depression add up, and these can be measured, estimated from empirical equations, or calculated from thermodynamic principles. The latter approach was used in the past to derive Eq. 1, for the case of the presence of solutes in water (Atkins, 1980), and Eq. 2 for the capillary cryoscopic descent of water confined in solids with hydrophilic surfaces (Kurz and Fisher, 1984; Thomson, 1871; Woodruff, 1973):

$$T - T_0 = i K_f m \quad (1)$$

$$T - T_0 = -\frac{V_S \sigma_{LS} T_0}{\Delta H_{fusion}} \kappa \quad (2)$$

where T is the freezing point (K), T_0 is the normal freezing point (K), i is the Moles of ions per mole of solute, K_f is the cryoscopic constant ($\text{K}\cdot\text{kg}\cdot\text{mol}^{-1}$), m is the solution molality ($\text{mol}\cdot\text{kg}^{-1}$), σ_{LS} is the water-ice interface excess energy (J/m^2), V_S is the specific volume of the solid phase (m^3/kg), κ is the interface average curvature radius (m^{-1}) and ΔH_{fusion} is the fusion latent heat (J/kg)

The cryoscopic descent estimated for a solution five times more concentrated than an isotonic NaCl solution,

i.e., 45 g/L, neighbors 2.5 K, so the effect of solutes for most human tissue fluids appears to be moderate.

To make significant the confinement descent, water must be enclosed in capillaries with radii less than about 100 nm. As the minimum spacing between collagen fibers found in the bone matrix can be placed to be in the order of 5 nm (Fawcett, 1982), the use of Eq. 2 results in a 12 K cryoscopic depression for water confined in a capillary of 5 nm of radius, where σ_{LS} was taken to be 32 mJ/m² (Liu *et al.*, 2003). Adding both contributions, an average 15 K freezing point depression is estimated for water present in bone tissue, and a process shelf temperature of -25°C can be set to dry safely this material. A 10 K safety margin was chosen. When more sample lots were dried and reliable production information was collected, this safety margin could be eventually lowered to decrease processing time if necessary.

The total pressure within the chamber during main drying should be set according to the ice vapor pressure equilibrium curve. The sample when drying will be always below the shelf temperature. Thus, a total chamber pressure equal to the ice vapor pressure for a temperature of at least 5 K below the shelf temperature should be set in order to obtain adequate drying rates.

Process time depends on the amount of water to be removed from the sample and on the heat transfer rate in each drying stage. The prevalent heat exchange mechanisms operating in the chamber are radiation and contact between the sample and the heated shelf. Provided that most specimens processed can be considered to be thin, because lyophilization is a fairly slow process and thus temperature gradients within the sample should be negligible for most practical purposes, the drying process can be adequately described by:

$$Q_{total} = m_{water} \Delta h_{sub} + m_{sample} C_{P_{sample}} (T_{sample} - T_0) \quad (3)$$

$$Q_{total} = \left(h_C A_C \int_{t_1}^{t_2} (T_{shelf} - T_{sample}) dt \right) + f_{view} \varepsilon \sigma A_R \left(\int_{t_1}^{t_2} (T_{surroundings}^4 - T_{sample}^4) dt \right) \quad (4)$$

Where Q_{total} is the total heat transferred to the sample (J), m_{water} is the mass of water to be removed (kg), Δh_{sub} is the latent heat of sublimation (J/kg), m_{sample} is the sample dry mass (kg), $C_{P_{sample}}$ is the sample constant pressure specific heat (J·K⁻¹·kg⁻¹), T_{sample} is the sample temperature (K), T_0 is the reference temperature (K), f_{view} is the radiation view factor, ε is the sample surface emissivity, σ is the Stefan–Boltzmann constant (J m⁻² K⁻⁴ s⁻¹), A_R is the sample radiation area (m²), $T_{surroundings}$ is the average surroundings temperature (K), T_{shelf} is the shelf temperature (K), t_1 is the starting drying time (s), t_2 is the ending drying time (s), h_C is the contact heat transfer coefficient (J·m⁻²·K⁻¹·s⁻¹) and A_C is the sample contact area (m²).

By solving Eq. 3 and Eq. 4, the process time in each stage can be estimated beforehand, although proper values for the different variables involved must be found in the literature, measured, or estimated.

Most solids, unless they reflect electromagnetic radiation, behave as gray bodies with emissivities around

0.9. Nevertheless, bone has been reported to have an emissivity of nearly 1 (Stumme *et al.*, 2003).

The heat transfer area for cortical struts can readily be measured, however, this variable can be rather difficult to estimate in other samples such as cancellous chips or morselized bone. Since in both cases the surface of the tray used to put the samples onto the shelves was fully occupied, A_C was approximated by the tray area, while A_R was taken to be twice this value to account for the contribution of the lower and upper shelves radiating to the samples.

The average surroundings temperature prevailing in the process should be found near the value set for the shelf temperature. Although this, the true value depends on the particular geometry of the lyophilization chamber, primarily on the position of the fairly cold ice condenser relative to the shelves. Since experimental information was not available, the average surroundings temperature was taken to be the shelf temperature as a reasonable first approximation for the main drying stage.

The values for the f_{view} and h_C could not be found in the literature or estimated beforehand as above, thus, experimental measurements were carried out to record the information needed to calculate both parameters using Eq. 3 and Eq. 4. Once these parameters were estimated, new lyophilization protocols for different samples could be projected saving valuable experimental time.

The view factor in Eq. 4 is an adjustment parameter that depends on the geometry of the heat sources radiating to the sample and their temperatures, since only one average temperature value must be used in Eq. 4. A view factor close to a value of 1 will indicate that other parameters in Eq. 4 have been chosen properly enough, specially the average surroundings temperature.

For the final drying period, a shelf temperature in the vicinity of room temperature and the lowest possible total chamber pressure attainable by the equipment should be selected, and the needed time span can also be estimated by means of Eq. 3 and Eq. 4 as well.

The objective of this work is therefore to offer a basic calculation procedure to estimate the values of the initial parameters needed in setting up cryodesiccation protocols. The experimental values found in this work are clearly particular to the samples and equipment used, and these should only be used, if anything at all, as a guideline when different equipment or samples are involved. Although this, the calculation procedure described should be applicable to cryodesiccation in general regardless of the experimental conditions.

III. EXPERIMENTAL PROCEDURE

As-received human frozen samples from the Multi-Tissue Bank (INDT) (Álvarez *et al.*, 2004) of cancellous chips, cortical struts, and morselized bone from two donors, were processed in a Martin Christ Beta 1-16 freeze dryer. Samples of 30 g of cancellous chips (10 x 10 x 5 mm), about 50 g cortical struts from tibia and femur (100 x 30 x 5 mm), and 30 g samples of morselized

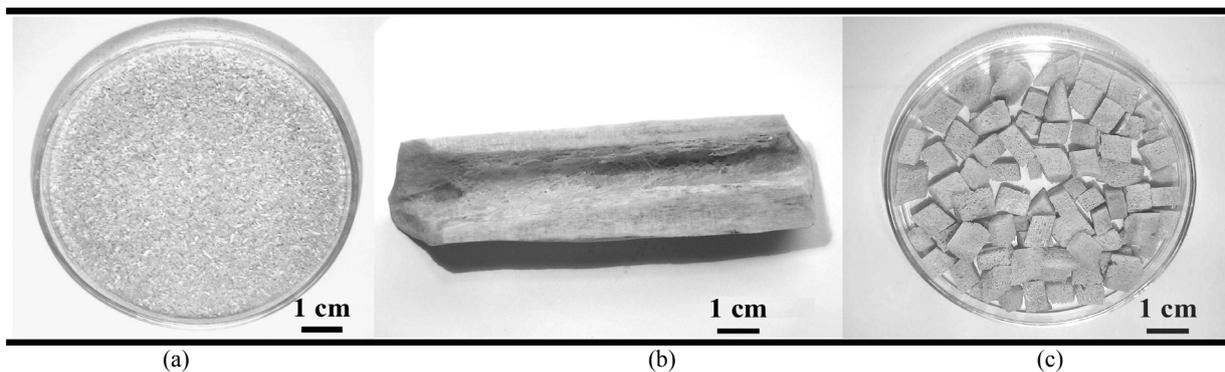


Figure 1. Bone tissue samples: (a) Morselized tissue. (b) Cortical strut. (c) Cancellous chips.

bone with ground sizes ranging between 6 and 50 mesh (U.S. sieves) were employed in each run (see Fig. 1).

Samples, placed on thin stainless steel trays- 15 cm in diameter- were centered on one of the 3 electrically heated shelves. RTD-Pt 100 sensors were affixed to the shelf and the sample to record their temperatures. The shelf temperature was set to reach -25°C in 3 hours at atmospheric pressure. This temperature was held for 30 minutes while the equipment made ready to rapidly evacuate the chamber and reach 22 Pa of total pressure in the main drying stage (see Fig. 2). When a final drying stage was incorporated, the shelf temperature was increased to 20°C in six hours under constant vacuum, and finally the highest achievable vacuum of 2 Pa was applied.

Various main and final drying times were tested. Abbreviated runs, incorporating a main drying stage only, were carried out followed by a one-hour ramp from -25°C to room temperature to measure the humidity content of various samples at that point.

To assess the contact heat transfer contribution, runs were carried out under the same conditions of normal operation, but the samples were separated 2 mm from the shelf by small holders made of a black stainless steel mesh.

The samples initial and residual water contents were determined by weight loss after heating in an oven at 105°C for 24 hours as the normal procedure. Longer water contents test runs were carried out at 50°C to rule out the unlikely presence of volatile species other than water. The values of these runs resulted in identical water contents than the 105°C procedure in average samples within experimental error.

IV. RESULTS AND DISCUSSION

A. Freeze Drying Curves

In the pre-freezing stage, cancellous chips obtained from the storage freezer began to equalize their temperature to the descending shelf value (see Fig. 2a). During this stage, at -6°C a sharp increase in the sample temperature to -3°C was observed (see Fig. 2a) indicating that part of the unbound water in the sample had undercooled before crystallizing. The value -3°C is close to the freezing point of an unconfined aqueous solution estimated above. Towards the end of this stage, despite the shelf temperature being -25°C , the specimens stabilized at -27°C due to the cold chamber environment (see Fig. 2a). Once vacuum was applied, a substantial drying rate was established, and the sample temperature decreased abruptly to -42°C (see Fig. 2 a). The large temperature difference between the sample and its surroundings allowed a relatively high heat transfer rate which provided the heat flow needed to sustain the high sublimation rate observed. Once the drying rate began to taper off, the sample temperature began to increase and stabilized once more at -27°C after only 5 hours of the 9 hours programmed for main drying (see Fig. 2 a). For all practical purposes, the drying process at this point had ceased to move onwards.

The behavior of morselized bone obtained from the same donor was fairly similar to that of the cancellous chips described above (see Fig. 2b). However, the cortical struts obtained from the same donor behaved markedly different, since main drying conditions resulted in relatively low drying that ceased even more prematurely

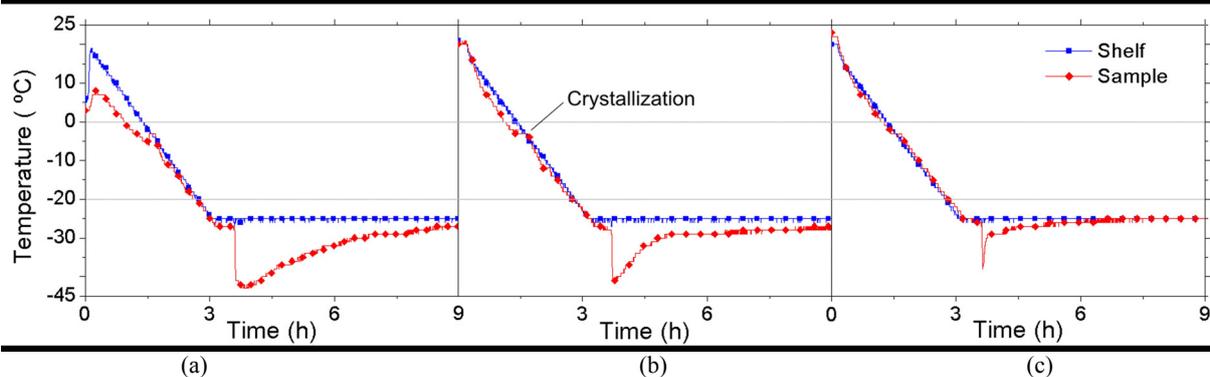


Figure 2. Main drying of bone tissue from Donor 1. (a) Cancellous chips. (b) Morselized bone. (c) Cortical strut.

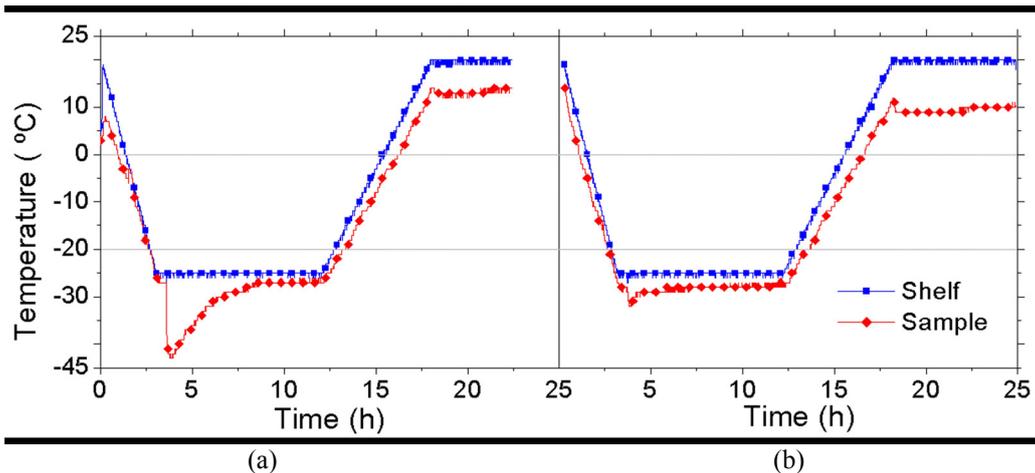


Figure 3. Freeze drying of cancellous chips. (a) Donor 1. (b) Donor 2.

Table 1. Donor 1 sample humidity (percent dry basis).

Sample	Initial content	After main drying	After final drying
Morselized bone	35	4,9	3,6
Cancellous chips	28	3,4	3,9
Cortical struts	14	10,4	4,9

(see Fig. 2c). This suggests that, in spite of the strut lower initial water content (see Table 1), most of the water present was bound water. In agreement with this, a marked 5°C decrease in sample temperature (not shown) was usually recorded for struts once the final 2 Pa chamber pressure was applied in the final drying stage.

The residual humidity contents offered in Table 1 indicate that, in principle, a final drying stage would only be required for cortical struts. It must be noted that two samples from the same lot, i.e. similar starting conditions, were dried for each type of sample, one was interrupted after main drying, and the second one was allowed to run the full cycle that included final drying. For cancellous chips, this procedure resulted in slightly higher humidity for the full cycle sample due to minor differences in the initial sample. However, porous cancellous chips from Donor 2 behaved quite differently than those of Donor 1 (see Fig. 3), indicating that differences in extraction, cleaning and handling procedures can strongly contribute to the inherent differences between donors (Biyikli *et al.*, 1986).

Some cryodesiccation equipment has the capability of altering programmed drying times on-the-fly by tracking the sample temperature. However, this mode of operation can be risky since proper sensor attachment to certain samples may be difficult, as well as just one sample location only is monitored. Thus, the variability of the starting material must be taken into account by implementing reasonable safety margins in the processing times of both drying stages to accommodate sample differences.

B. View Factor and Contact Heat

Numerical integration of Eqs. 3 and 4 during main drying was performed to estimate the radiation view factors

and the contact heat transfer coefficients displayed in Table 2. View factors were calculated from the runs without sample-shelf contact, and the values obtained were utilized to estimate the contribution of the heat transfer by contact in normal runs.

The sample-shelf distance used to eliminate contact heat transfer was kept to a minimum, since care must be exercised not to alter significantly the surroundings temperature prevailing in both types of runs. The assumption that the shelf temperature can be representative of the surroundings temperature is questionable for relatively large separation distances. Moreover, if the separation is not minimal, the sample will stabilize at a temperature substantially lower than that of the shelf, reflecting the presence in the chamber of the fairly cold ice condenser.

The value of the view factors obtained under the assumptions made above are reasonably close to the expected value of 1 (see Table 2), indicating that the calculation procedure yielded acceptable results. The relatively lower view factors calculated for morselized bone and cancellous chips can suggest a variety of things, from lower emissivities due to the presence of reflecting free ice, to differences on the value of the heat transfer area selected, to a lower true surroundings temperature than the value assumed. Strikingly, if the stabilization temperature of -27°C (see Fig. 2) for these samples is employed as the surroundings temperature, instead of -25°C the shelf set point, a view factor of 1.03 is calculated in both cases, suggesting that this temperature discrepancy probably played an important role in the low view factor values calculated. In spite of this, since the shelf temperature was taken to be the average surrounding temperature, the use of the view factor values listed in Table 2 will give the correct results when using Eq. 4.

Table 2. Calculated parameters for main drying.

Sample	f_{view}	h_c ($\text{J}\cdot\text{m}^{-2}\cdot\text{K}^{-1}\cdot\text{s}^{-1}$)	Contact heat transferred (%)
Morselized bone	0.77	3.7	44
Cancellous chips	0.71	3.5	43
Cortical struts	1.02	3.5	30

The use of the heat transfer coefficients calculated thus far, as well as the method depicted in estimating other parameters needed to project new cryodesiccation protocols, markedly narrowed down the experimental time required to set up optimized freeze-drying production protocols.

V. CONCLUSIONS

A method to set the parameters of a freeze drying process was developed and tested successfully with human bone tissues. Total drying times of about 21 hours were employed, with the shelves held at -25°C in main drying for about 6 hours and a total chamber pressure of 22 Pa, followed by a five-hour final drying stage at room temperature and 2 Pa. A final drying stage ought to be included in most cases in order to allow for sample variability. Radiation accounted for the majority of the heat transferred, chiefly for cortical struts, though contact heat transfer was quite relevant in all three cases tested and should be considered in projecting efficient T-t-P freeze drying surfaces.

REFERENCES

- Álvarez, I., M. Saldias, O. Wodowoz, H. Perez Campos, D. Machin, W. Silva, P. Sueta, N. Perez and M. Acosta, "Progress of National Multi-tissue Bank in Uruguay in the International Atomic Energy Agency (IAEA) Tissue Banking Programme," *Cells and Tissue Banking*, **4**, 173 – 179 (2004).
- Atkins, P.W., *Fisicoquímica*, 6ta ed., Editorial Omega, p. 179 (1980).
- Biyikli, S., M.F. Modest and R. Tarr, "Measurement of thermal properties for human femora," *J. Biomed. Mater. Res.*, **20**, 1335-1345 (1986).
- Fawcett, D.W., *Tratado de Histología*, 11va ed. Bloom-Fawcett, 207-208 (1982).
- Geankoplis, C., *Procesos de Transporte y Operaciones Unitarias*, C.E.C.S.A., 482-486 (1982).
- Kurz, W. and D.J. Fisher, *Fundamentals of Solidification*, Trans Tech Publications, Uetikon-Zürich, Switzerland, 13 (1984).
- Liu, Z., K. Mudrew, R. Wan and J. Elliot, "Measurement of freezing point depression of water in glass capillaries and the associated ice front shape," *Physical Review E*, **67**, 061602 (2003).
- Stumme, L.D., T.H. Baldini, E. Jonassen and J.M. Bach, "Emissivity of Bone," *Summer Bioengineering Conference*, Sonesta Beach Resort in Key Biscayne, Florida, June 25-29, 1013-1014 (2003).
- Thomson, W., *Philos. Mag.*, **42**, 448 (1871).
- Woodruff, D.P., *The Solid-Liquid Interface*, Cambridge University Press, London, 25 (1973).

Received: February 3, 2008.

Accepted: November 21, 2008.

Recommended by Subject Editor Orlando Alfano.