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Freeze-drying of pharmaceuticals in vials nested in a rack system / Artusio, Fiora; Adami, Marco; Barresi, Antonello A.; Fissore, Davide; Chiara Frare, Maria; Udrescu, Claudia I.; Zanetti, Francesco; Zunino, Gabriele; Pisano, Roberto. - ELETTRONICO. - (2023), pp. 27-34. (Intervento presentato al convegno 8th European Drying Conference (EuroDrying 2023) tenutosi a Lodz (POL) nel 4-7 July 2023).

Availability:
This version is available at: 11583/2982181 since: 2023-11-02T11:04:37Z

Publisher:
Lodz University of Technology

Published
DOI:

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12 October 2026

FREEZE-DRYING OF PHARMACEUTICALS IN VIALS NESTED IN A RACK SYSTEM

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Abstract: *The distribution of biopharmaceuticals often requires lyophilisation. The drug product is first frozen and potentially exposed to stress conditions that can be detrimental to its quality. These stresses are also encountered when a drug product has to be distributed under ultra-cold conditions. Adjusting the formulation and/or freezing conditions allows for limiting the impact of these stresses on the final product. This paper investigates two loading configurations, vials directly resting on the shelf and nested in a rack system, and their impact on the freezing and drying behaviour of a sucrose-based formulation. First, two key freezing parameters, i.e., ice nucleation temperature and cooling rate, were studied as they can affect the product behaviour during drying. The product freezing rate and the ice nucleation temperature distribution were affected by the loading configuration, resulting in larger ice crystals in the case of vials nested in a rack system. The analysis was also extended to the drying phase, showing that the loading configuration impacted the product temperature during drying and the overall heat transfer coefficient between the equipment and the product.*

Keywords*: freeze-drying; rack system; proteins; cryopreservation; heat transfer.*

1. Introduction

The administration of safe biopharmaceutical products and vaccines often relies on cold, i.e., 2-8°C, or ultra-cold, i.e., -20°C or -70°C, distribution conditions. Failures in the cold chain can lead to risks for the patients, inter batch thermal variability, and lack of compliance with regulatory standards. Given the high added value of biopharmaceuticals, also an economic aspect must be considered as a failed cold chain would lead to batch withdrawal.

Lyophilisation represents a reliable route to allow the distribution and storage of biopharmaceuticals under mild refrigeration or even ambient conditions. However, the liquid formulation must be frozen before entering the drying, i.e., ice sublimation, phase. Freezing is a critical step since it can be detrimental for the stability of therapeutic proteins (Privalov, 1990; Bhatnagar et al., 2007). During freezing, many processes are taking place at the same time: new ice-water interface is formed, product temperature is drastically reduced, water is progressively separated as ice crystals, and a cryo-concentrated amorphous phase is formed. The ensemble of these events can also impact the final product attributes such as the pore size of the lyophilised cake, the residual biological activity, and the residual moisture.

In this scenario, the cooling rate during freezing (Eckhardt et al., 1991) and the nucleation temperature (Fang et al., 2018) can be manipulated to control the average size of the ice crystals and, hence, the final pore size. A high cooling rate, resulting in pronounced undercooling, and a low nucleation temperature promote the formation of small ice crystals (Bald, 1986; Arsiccio et al., 2020). However, because of the intrinsic stochasticity of nucleation events, nucleation

temperature is widely distributed within a batch of vials (Capozzi and Pisano, 2018; Pisano et al., 2023).

In our recent work, the impact of different loading configurations of vials on the distribution of ice nucleation temperatures has been investigated (Pisano et al., 2023). The use of a rack system for housing vials simplifies the vials handling in sterile conditions, as vials can be used as received, i.e., in a sterile secondary packaging. However, the use of a rack system can affect the freeze-drying process, as vials are slightly raised above the shelf and are physically isolated by the rack system. The thermal evolution of the product differs from the configuration where vials are directly loaded on the shelf and needs to be investigated to identify optimal process conditions. A comparison between the freezing and drying behaviour of vials directly resting on the shelf and vials nested in a rack system is here reported.

2. Materials and Methods

2.1 Materials

All the freezing tests were performed on a 5 wt% sucrose (Merck, Milan, Italy, used without further purification) solution prepared with water for injection (Fresenius Kabi, Milan, Italy). 4 cc tubing vials (2R ISO, Stevanato Group, Piombino Dese, Italy) were filled with 2 mL of 0.22 µm filtered (syringe filters, PVDF, Merck, Milan, Italy) solution. All the operations were carried out inside a laminar hood to limit contamination.

The tests were conducted by loading the vials directly on the temperature-controlled shelf or into a rack system (SG EZ-fill® Nest, Stevanato Group, Piombino Dese, Italy). The rack system, also named *nest*, hosted 100 vials in hexagonal arrangement and raised approximately 1 mm above the shelf. A picture of the rack system used to house vials is shown in Fig.1.

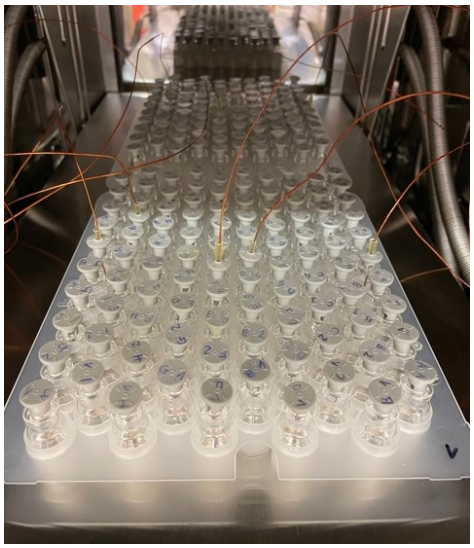

Fig. 1. Vials nested in the rack system.

For the residual biological activity tests, lactate dehydrogenase (LDH) from rabbit muscle (Merck, Milan, Italy) was used. LDH was dialysed against 10 mM citrate buffer pH 6.5 at 5 °C. The buffer was changed three times and, at the end of the dialysis, LDH concentration was measured with a spectrophotometer (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Milan, Italy). The absorbance was recorded at 280 nm, and an extinction coefficient of 1.44 mL/(mg cm) was used to calculate LDH concentration.

2.2 *Freezing tests*

The freezing tests were conducted in a lab-scale freeze-dryer (Revo, Millrock Technology, Kingston, New York, USA). Two loading configurations were considered: vials loaded directly on the shelf or nested in the rack system. For both configurations, the ice nucleation time (t_n) of each of the approximately 100 vials of the batch was evaluated with the help of two video cameras placed on the opposite sides of the shelf (frame acquisition rate = 5 fps). t_n was determined as the time instant in which the liquid became opaque. A reference time, t_0, was set as the time instant in which the temperature of a reference vial, monitored with a T-type thermocouple (Tersid, Milan, Italy), reached 0 °C. The reference vials and the edge vials were excluded from the analysis because of possible alteration of nucleation due to the presence of the thermocouple tips and edge effects, respectively.

Immediately after filling with 5 wt% sucrose solution, vials were loaded into the freezing chamber at room temperature. Samples were frozen at a cooling rate of 0.25 / 0.5 / 1 °C/min and held at −45 °C for 30 min. Then, they were thawed at a heating rate of 3 °C/min and held at +30 °C for 60 min to achieve complete melting. Tests were performed in triplicates.
The ice nucleation time resulted to be in the range of 0 to 125 min for all the freezing tests; the nucleation time frequency distribution $f_{n,i}$ was determined as:

$$f_{n,i}(t_{n,i}) = N_i\,(t_{n,i}) \tag{1}$$

where N_i is the number of vials of the i class, which nucleated between the time $t_{n,i}$ and $t_{n,i-1}$. The width of the distribution classes was set at 5 min.

Knowing the temperature profile during the freezing phase of a reference vial and assuming that all the vials follow the same evolution, each nucleation time $t_{n,i}$ can be transposed to a nucleation temperature $T_{n,i}$. The nucleation temperature frequency distribution results to be:

$$f_{n,i}(T_{n,i}) = N_i(T_{n,i}) \tag{2}$$

where N_i is the number of nucleated vials belonging to the i class, and $T_{n,i}$ is the nucleation temperature of the i vials. The width of the distribution classes was set at 5 min.

2.3 *Characterisation of the freeze-dried product*

Scanning electron microscopy (SEM, FEI type, Quanta Inspect 200, Eindhoven, the Netherlands) was used to investigate the cross-section of the lyophilised cake. Samples were frozen at a cooling rate of 0.5 °C/min and held at −45 °C for 2 h. Primary drying was performed at −25 °C and 10 Pa for 63 h, followed by secondary drying carried out at +20 °C (0.2 °C/min) and 10 Pa for 3 h. Samples were cut along the axial direction and metallized with a thin layer of platinum. SEM images were acquired considering the central area of the cake, and the average pore size was determined considering at least 100 pores.

2.4 *Determination of the overall heat transfer coefficient during drying*

The heat transfer coefficient K_v was determined by the gravimetric method (Pisano et al., 2011). Each vial was filled with 2 mL of deionized water and individually weighed before and after the test. Approximately 200 vials were monitored for each test. Vials were frozen at a cooling rate of 1 °C/min to −45 °C and then partial ice sublimation was performed at −10 °C and different chamber pressures (5, 10, 20, and 30 Pa). The ice temperature of central vials was monitored through 5 T-type thermocouples. Knowing the weight loss due to ice sublimation,

Δm, and the duration of the sublimation phase, Δt_{ice}, the heat transfer coefficient was calculated as:

$$K_v = \frac{\Delta m \Delta H_s}{S \int_0^{\Delta t_{ice}} (T_S - T_B) dt} \quad (3)$$

where ΔH_s is the ice sublimation enthalpy, S is the internal cross-sectional area of the vial, and T_S and T_B are the temperatures of the shelf and of the product at the vial bottom, respectively.

3. Results and discussion

3.1 Freezing

The frequency distribution of ice nucleation time for vials in contact with the shelf and nested in a rack system is reported in Fig. 2 considering three different cooling rates. For both configurations, the median nucleation time and the width of the distribution decreased as the cooling rate increased. For a given time, higher cooling rates lead to higher supercooling compared to lower cooling rates. This results in a higher probability of inducing nucleation. Consequently, most of nucleation events occurred in a shorter time as the cooling rate increased.

The onset of the time distribution was systematically smaller for vials nested in the rack system considering slow, i.e., 0.25 °C/min, and moderate, i.e., 0.5 °C/min, cooling rates. Conversely, the time distributions were comparable at high cooling rates, i.e., 1 °C/min.

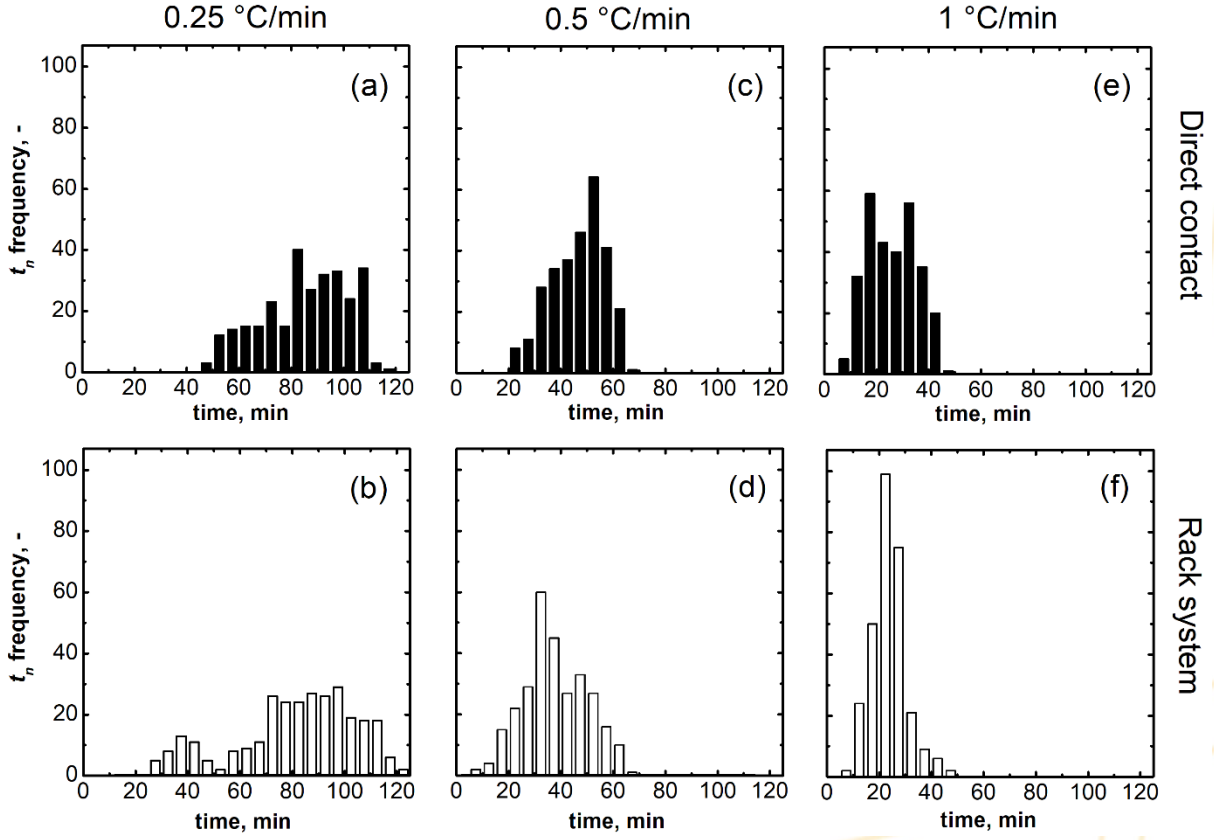

Fig. 2. Frequency distribution for the ice nucleation time of vials in direct contact with the shelf (black bars) and vials nested in a rack system (white bars). Data refer to various cooling rates: a), b) 0.25, c), d) 0.5, and e), f) 1 °C/min.

Knowing the temperature profile of the supercooled liquid, it is possible to obtain the nucleation temperature corresponding to any nucleation time and, finally, the frequency

distribution of nucleation temperatures. It has to be noted that the evolution of product temperature was monitored by means of thermocouples inserted in reference vials and is representative of the whole batch of vials as long as nucleation does not occur in the reference vials. When nucleation occurs, the temperature of the product suddenly increases following the release of heat due to the exothermicity of the nucleation event. It follows that, after nucleation, the thermocouples reading cannot be used to derive the temperatures of the vials that have not yet nucleated. To obtain a representative supercooling temperature profile at all the time instants of freezing, the thermal evolution of the product after nucleation was extrapolated by linear fitting of the signal before nucleation.

The frequency distribution of nucleation temperature for vials in contact with the shelf and nested in a rack system is reported in Fig. 3, considering the same three different cooling rates. In the range of 0.25 to 0.5 °C/min, the temperature distributions resulted to be slightly affected by the cooling rate, as most of nucleation events occurred within −10 °C and −25 °C. However, nucleation in vials nested in the rack system tended to occur at higher temperatures compared to vials in direct contact with the shelf.

At the highest cooling rate, the fraction of vials nucleated below −25 °C in direct contact with the shelf was significantly larger than in the rack system. For cooling rates higher than 0.5 °C/min, the narrower nucleation temperature distribution obtained for vials nested in the rack system highlighted a reduced vial-to-vial variability during freezing.

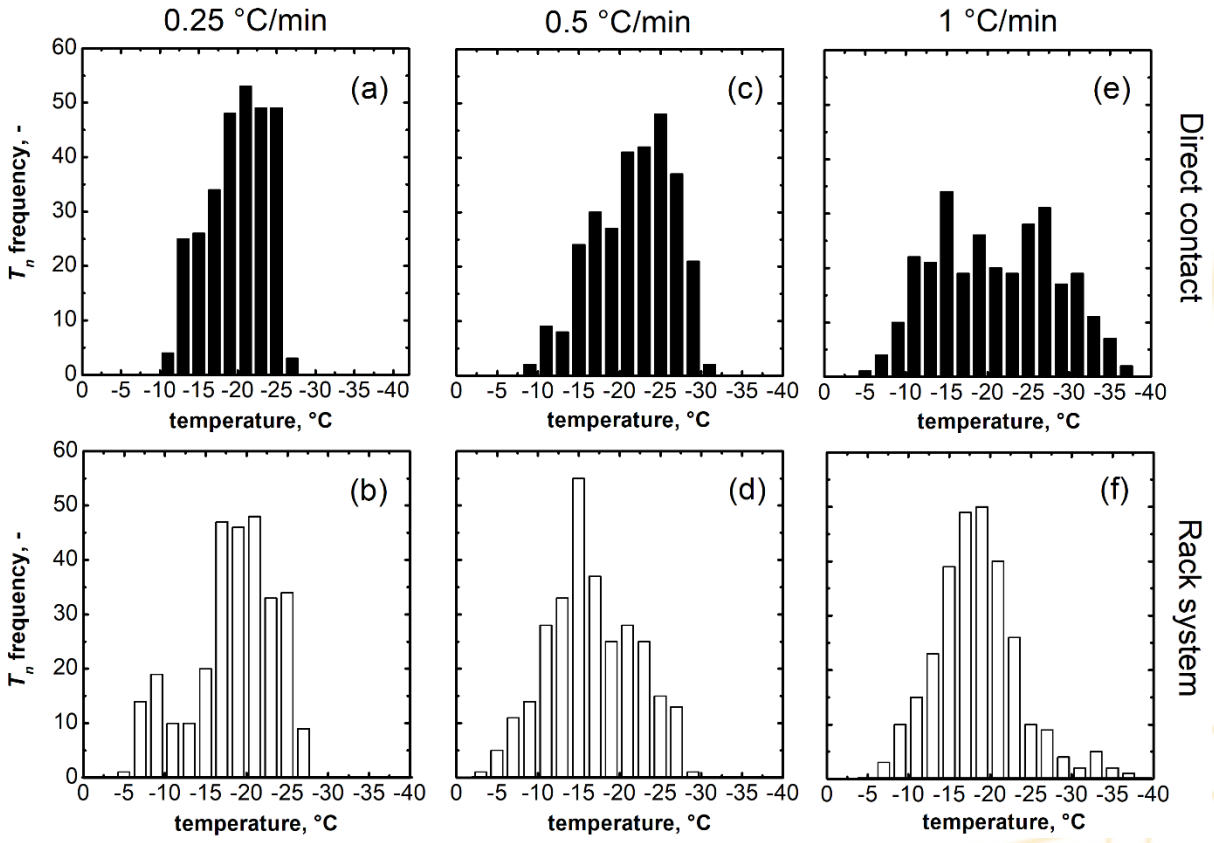

Fig. 3. Frequency distribution for the ice nucleation temperature of vials in direct contact with the shelf (black bars) and vials nested in a rack system (white bars). Data refer to various cooling rates: a), b) 0.25, c), d) 0.5, and e), f) 1 °C/min.

An explanation of the differences in the freezing behaviour of vials between the two loading configurations can be found in the thermal profile of the supercooled liquid. We have recently reported (Pisano et al., 2023) that the evolution of the temperature of the supercooled liquid

depended on the loading configuration. This difference was attributed to the different efficiency in heat transfer between the equipment and the vials. The heat transfer encounters an additional resistance in the case of vials loaded in the rack system since they are not in contact with the temperature-controlled shelf. It follows that a direct comparison between the nucleation time distributions of the two loading configurations cannot be rigorously conducted because of the variations in the reference time and the actual freezing rate. Conversely, this comparison is still valid for temperature distributions since they account for the actual thermal profile of the product. The differences in the distributions of ice nucleation temperatures obtained with the two loading configurations had an impact on the dimensional distributions of ice crystals and, hence, the pores of the lyophilised cake. Fig. 4a) and b) compare the microstructure of 5 wt% sucrose cakes obtained in vials in direct contact with the shelf and nested in the rack system, respectively. The average pore size was 75 µm for vials in direct contact with the shelf, whereas it increased to 170 µm when vials were nested in the rack system.

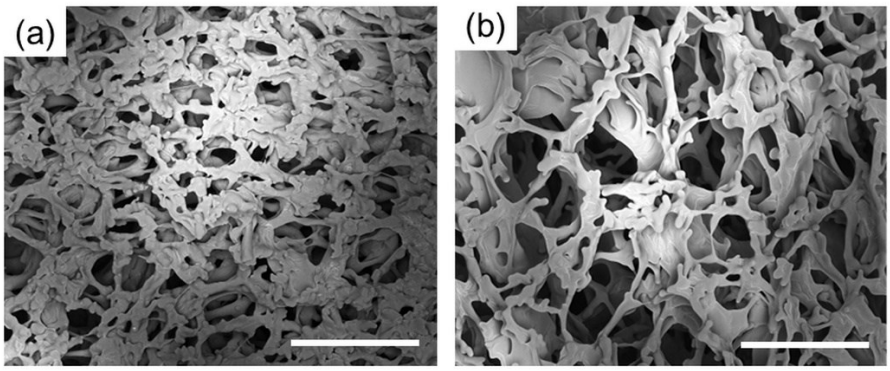

Fig. 4. SEM micrographs of the centre of the lyophilised cake in vials a) in direct contact with the shelf and b) nested in a rack system. Data refer to a cooling rate of 1 °C/min. The scale bar is 300 µm.

Based on the different behaviour of the vials during freezing, the residual activity of an enzyme prone to denaturation at the ice-water interface (Fang et al., 2018), i.e., lactate dehydrogenase (LDH), was tested after repeated freeze/thawing cycles. As reported in our previous work (Pisano et al., 2023), LDH residual activity was systematically higher for vials nested in the rack system than in direct contact with the shelf. These results agree with the smaller specific surface area displayed by vials nested in the rack system, thus allowing for reduced interface for LDH denaturation.

3.2 Drying

Considering the drying phase, the loading configuration also plays an important role as it can affect the heat transfer efficiency and, hence, the product temperature. It has been reported (Pisano et al., 2011) that vials resting on the shelf can be divided into three different groups, i.e., central, semi-border, and border vials, depending on their position within the batch. The temperature of the product will vary depending on the vials position as a consequence of the variable contribution of radiation from the chamber walls. In general, border vials receive additional radiative heat compared to the central ones. Fig. 5 reports the evolution of ice temperature in vials nested in the rack system during the ice sublimation phase. Central vials were approximately 2 °C colder than border vials, highlighting the heterogeneity in heat received by the vials depending on their position. Even if the vials nested in the rack system were partially shielded by the plastic secondary packaging, the effect of radiation was still not negligible and must be considered when designing cycle conditions.

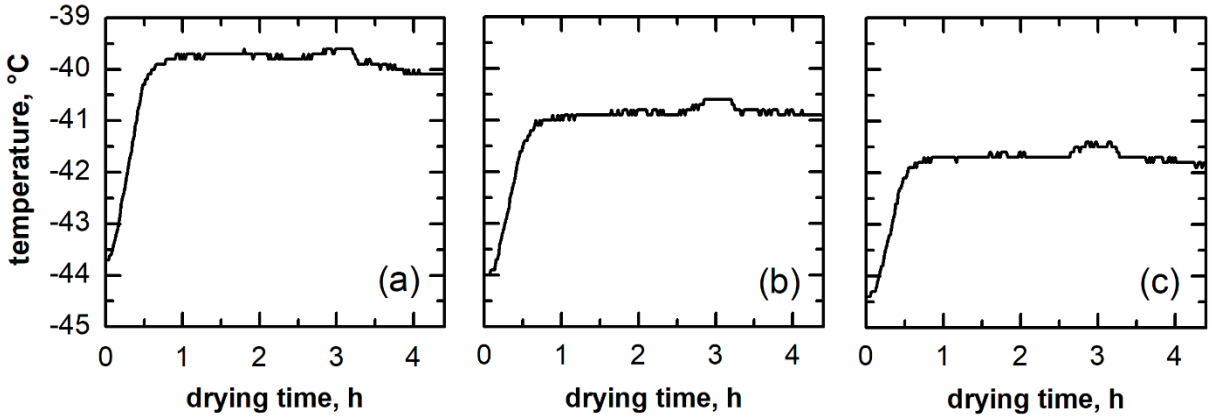

Fig. 5. The evolution of the ice temperature during the sublimation step for a) border, b) semi-border, and c) central vials nested in the rack system. Chamber pressure was 5 Pa.

The heat transfer from the equipment to the vials was also evaluated for the central and border vials considering the two loading configurations. The overall heat transfer coefficient, K_v, provides an indication of the heat transfer efficiency. As shown in Fig. 6, K_v values of vials directly resting on the shelf and vials nested in the rack system were comparable at lower pressures, i.e., 5 and 10 Pa. At higher pressures, i.e., 20 and 30 Pa, K_v was significantly higher for vials directly resting on the shelf. Such a behaviour can reasonably be explained considering the contribution of heat exchanged by gas conduction between the shelf and the vial bottom as a function of pressure. Vials directly resting on the shelf receive more heat by gas conduction at higher pressures because of the higher number of gas molecules trapped between the shelf and the vial bottom. Conversely, in the rack system, vials are slightly raised above the shelf (approximately 1 mm). The contribution of gas conduction becomes less important for this type of configuration, reducing the heat transfer efficiency at high pressures.

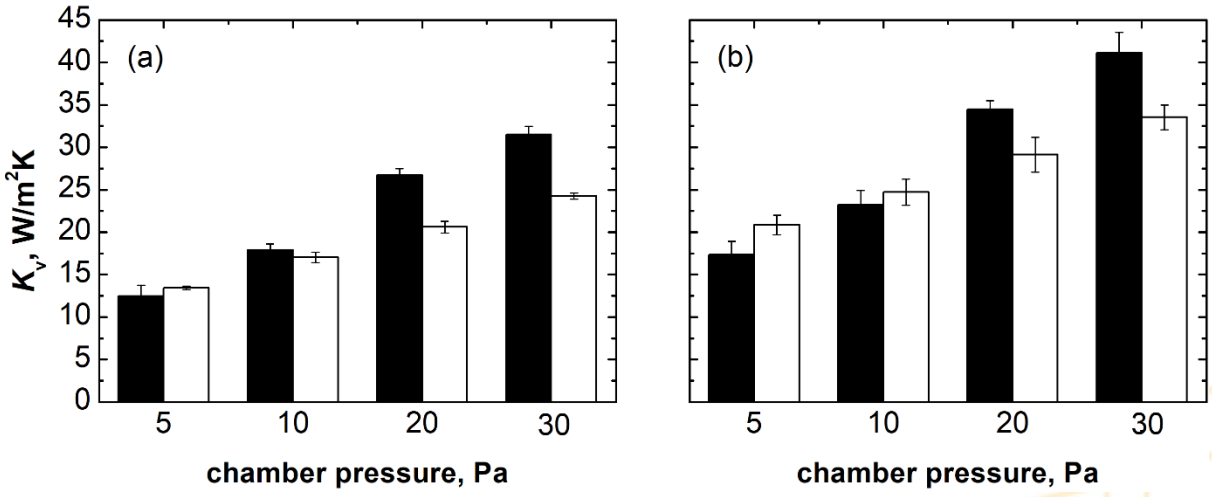

Fig. 6. K_v of a) central and b) border vials loaded in direct contact with the shelf (black bars) and nested in a rack system (white bars) at different chamber pressures.

At a given chamber pressure, K_v was always higher for border vials because of the higher heat flux received by these vials compared to the central ones.

4. Conclusions

The impact of two loading configurations, i.e., vials directly resting on the shelf or nested in a rack system, on the freezing and drying behaviour of vials has been studied. When a rack system was used, vials tended to nucleate at higher temperatures. Consequently, the formation of larger ice crystals was promoted, leading to larger pores in the lyophilised cake. This feature is desirable especially when dealing with active molecules prone to denaturation at the ice-water interface, since the reduced extent of the interface exerts a protective action. In addition, the loading configuration also impacts the product temperature and the heat transfer efficiency during drying. Vials nested in a rack system experience an additional resistance to heat transfer due to the lack of contact with the shelf. Therefore, the use of a rack system to load vials can impact the freeze-drying process in various manners, ranging from the final product morphology to the choice of optimal process conditions and the preservation of product activity. The data show a small effect also on the wideness of the nucleation temperature or time range, which can become larger or smaller depending on the cooling rate; the effect is anyway limited, but is currently under deeper investigation. The use of a rack system can also shorten the loading time of vials into the equipment and simplify the handling under sterile conditions.

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