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Research of inhomogeneous lyophilization and development of methods for the stabilization of samples

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Lyophilization is a multi-step process and is one of the most energy-disadvantageous and time-consuming methods. Nevertheless, various pharmaceutical companies use freeze-drying to extend the shelf life of products, such as injectables or vaccines. In addition, lyophilization is now becoming increasingly important due to the rapid development of biotechnology and the associated production of modern drugs such as therapeutic proteins or monoclonal antibodies. The freeze-drying cycle consists of freezing the substance and then removing the solvent (water) by sublimation, which passes directly from the solid to the gas phase. There are several possible reasons that affect the drying rate in the vials, such as the heat transfer through the walls of the lyophilizer, the different design of the heating shelves in the lyophilization equipment, and finally, the packing density of the vials.

The aim of the work was an experimental study of inhomogeneous lyophilization in lyophilizers of various sizes and the construction and development of methods of using lyophilization for the stabilization of samples from human biological fluids, as well as to compare the suitability of using different vials and Eppendorf tubes for drying and stabilizing samples. The experimental part was performed at the Institute of Chemistry of the Slovak Academy of Sciences (ChÚ SAV).

Lyophilizers used in experimental studies

Christ lyophilizer – The Christ Alpha 1-2 LDplus lyophilizer is a compact and high-performance model for efficient lyophilization. The weight of the lyophilizer is approx. 28 kg and consists of two unheated teflon-coated stainless steel shelves with a diameter of 200 mm. The capacity of the ice condenser is 2.5 kg, and the condenser temperature is approx. -55 °C, which allows drying of aqueous solutions. The diameter of the acrylic drying chamber is 240 mm with a weight of 3.6 kg. The pressure in the lyophilizer during drying is around 0.063 - 0.075 mBar.

Labconco lyophilizer – The Labconco lyophilizer is designed for drying smaller as well as larger amounts of aqueous solutions. This type of lyophilization equipment is very easy to operate. Compared to the Christ lyophilizer, it is much larger. It consists of a drying chamber, a vacuum pump and a cooling coil made of stainless steel. The LCD displays system operating parameters, setting parameters and alarm messages. The condenser temperature is approx. -48 °C, which allows the drying of aqueous solutions. The diameter of the drying chamber is approximately 270 mm, on which the vent valve is located. The typical pressure in the lyophilizer during drying is about 0.027 mBar.

Sublimation studies

We evaluated the experimental testing of the drying speed using gravimetric sublimation tests in different lyophilizers, by weighing the weight loss of the sublimed water in the individual vials. In sublimation tests, the drying rate in individual vials is determined experimentally at different locations in the lyophilizer. Subsequently, inhomogeneity was evaluated using the inhomogeneity ratio. The calculated inhomogeneity of the batches is defined as the ratio of the maximum and minimum amount of water sublimed from vials.

We performed several experiments with different types of vials, also comparing different protections (outflow barriers) such as parafilm with different numbers of holes, filter paper, and gauze.

Figure 8 Christ lyophilizer.

Figure 2 Labconco lyophilizer.

Evaluation of sublimation tests

Different types of vials or Eppendorf tubes and different uses of their protection were compared for example parafilm, filter paper, and gauze.

Before placing the vials/Eppendorf tubes in the lyophilization equipment, the vials were filled with deionized water, 3.5 % whole milk, or 3 % solution of salt and weighed without stopper and protection (m_1). Then, if necessary, they were covered with the used protection and placed in a freezer at -80 °C. After the sample solidified, the vials or Eppendorf tubes were then placed in a lyophilizer. At the end of the process, all vials were weighed again without stopper and protection (m_2). In the last step, the amount of sublimed water (Δm) was calculated as a difference of values m_1 a m_2.

Figure 3 shows colour drying rates in all comparative experiments. The colour representation is evaluated according to the colour scale, **Table 1**, where each colour represents the range of the amount of sublimed water - the highest amount of sublimed water is burgundy and, conversely, the lowest amount of sublimed water is dark green.

Table 3 Colour representation of drying rate for Figure 3.

	***Mass sublimed* [g]**
1	0.881 – 1.001
2	1.002 – 1.121
3	1.122 – 1.241
4	1.242 – 1.361
5	1.362 – 1.481
6	1.482 – 1.601
7	1.602 – 1.721
8	1.722 – 1.841
9	1.842 – 1.961
10	1.962 – 2.081

The resulting amounts of sublimed water, as well as the inhomogeneity ratio for all experiments, are summarized in **Table 2** and **Table 3**, where ↑ shows the placement of the rack with tubes on the top shelf and ↓ shows the placement of the rack with tubes on the bottom shelf.

Table 4 Comparison of maximal and minimal amount sublimed of water and inhomogeneity ratio for experiments A) - F).

Experiment	Δm_{max} [g]	Δm_{min} [g]	*Inhomogeneity ratio*
A) ↑	1.9822	1.9277	1.03
↓	1.9151	1.8022	1.06
B) ↑	1.9838	1.7908	1.11
↓	0.9906	0.8812	1.12
C) ↑	1.2960	1.1849	1.09
↓	1.2220	1.1282	1.08
D) ↑	1.2563	1.0087	1.25
↓	1.2183	0.9665	1.26
E) ↑	1.4967	1.2782	1.17
↓	1.3956	1.2346	1.13
F) ↑	1.3367	1.1326	1.18
↓	1.4705	1.2357	1.19

First, we performed several experiments comparing the drying rate in glass vials and plastic vials with different protection applications **Figure 3**. The highest amount of sublimed water was from glass vials and plastic vials without stopper and protection. In experiments, where we used a rubber band to fasten the vials so that all vials remained in their original arrangement during lyophilization, the results confirmed that the drying rate is slowest in the central vials, which are surrounded by another six vials. All results are shown in the **Table 2**.

Table 5 Comparison of maximal and minimal amount sublimed of water and inhomogeneity ratio for experiments G) - J).

Experiment	Δm_{max} [g]	Δm_{min} [g]	*Inhomogeneity ratio*
G) deionized water ↑	0.4359	0.2364	1.84
↓	0.3602	0.1970	1.83
H) deionized water ↑	0.7190	0.3389	2.12
↓	0.6528	0.3878	1.68
I) 3.5 % whole milk ↑	0.9067	0.8961	1.01
↓	0.9152	0.9011	1.02
J) 3 % solution of salt ↑	1.0017	0.9662	1.04
↓	0.9912	0.9558	1.04

In other sublimation tests, unlike glass and plastic vials, we used polypropylene Eppendorf tubes. In these experiments, the drying rate in the used Eppendorf tubes with a parafilm with different numbers of holes or without a parafilm was also compared. In the first two experiments, we worked with deionized water, where the results of experiments showed high inhomogeneity even when using Eppendorf tubes, with factors from 1.68 - 2.12. In the last two experiments, our goal was to monitor whether solid-phase escape

occurred during the lyophilization process. The results showed that with a 3 % solution of salt it is necessary to use protection (most often parafilm is used) against solid-phase escape. This is due to the fact that the 3% solution of salt formed much finer and lighter particles that could not be retained at the bottom of the Eppendorf tubes. All results are summarized in the **Table 3**.

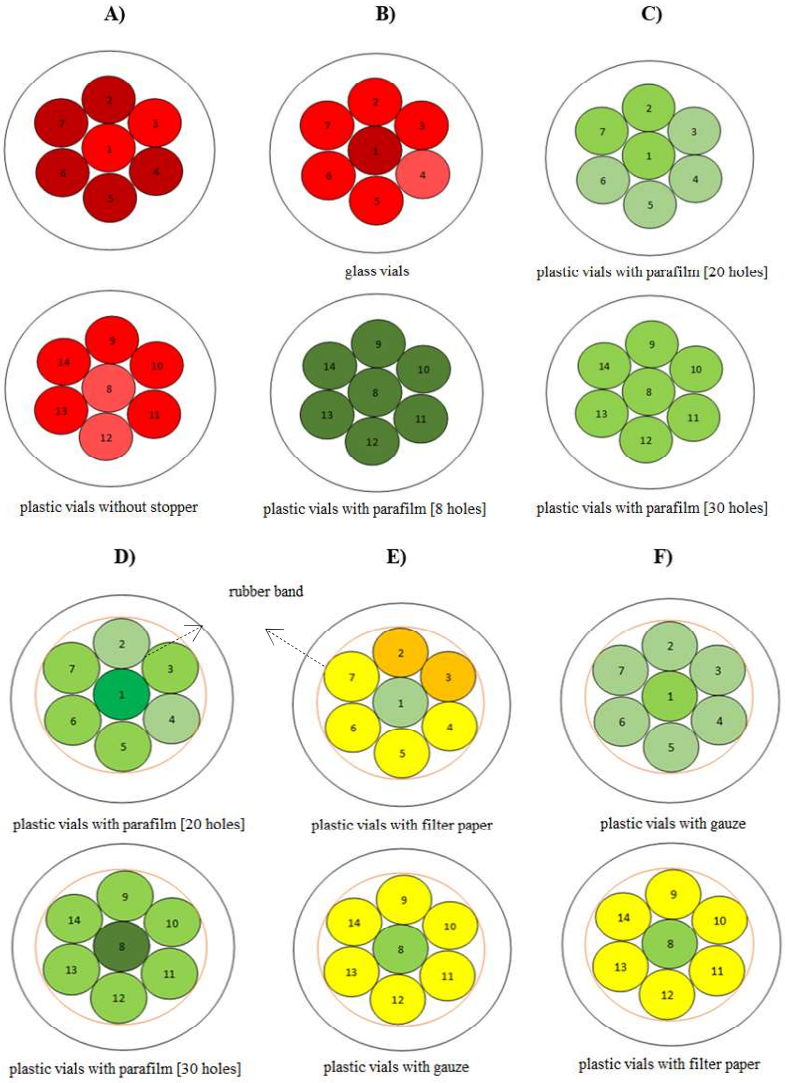

Figure 3 Colour representation of drying rate in vials with different types of their protection.

Figure 4 Final products from the experiment with 3.5 % whole milk.

Figure 5 Final products from the experiment with 3 % solution of salt.

To eliminate the problem (solid-phase escape), we found and purchased commercially available vials with sterile-venting membrane screw caps. These vials are special in that they contain permeable membranes, thus being able to prevent contamination and solid-phase escape while allowing the product to dry. The aim was to compare the drying rate in standard glass and plastic vials, **Figure 6** and glass vials with/without membrane screw caps, **Figure 7**.

Figure 6 Standard plastic and glass vials.

Figure 7 Vials with sterile-venting membrane screw caps.

Table 6 Comparison of the drying rate in three different types of vials.

Type of vials	Δm [g]
	2.4393
Glass vials with rubber stoppers	2.2108
	1.7496
Glass vials with membrane screw caps	1.8776
	3.2511
Glass vials without membrane screw caps	3.2053
	2.0914
Plastic vials	2.1117

The results, **Table 4** showed that the drying rate is 1.28-fold slower in glass vials with membrane screw caps compared to glass vials with a rubber stopper and 1.16-fold slower than in plastic vials. It follows that if we did not want to risk solid-phase escape during freeze-drying and we would like to use this type of vials, the drying time would have to be extended, which is associated with higher investment and operating costs for the lyophilization process.

Lyophilization of biological material

Finally, we proposed optimal conditions for the drying and stabilization of human serum for analysis of N-glycoprofile. Reference serum samples (obtained from Sigma-Aldrich, MA, USA) were stored for the period of 10 days at room temperature with and without lyophilization and then the N-glycoprofile of serum proteins was analyzed as described before (Ziburová et al., 2021). Samples of lyophilized human serum are shown in **Figure 8**.

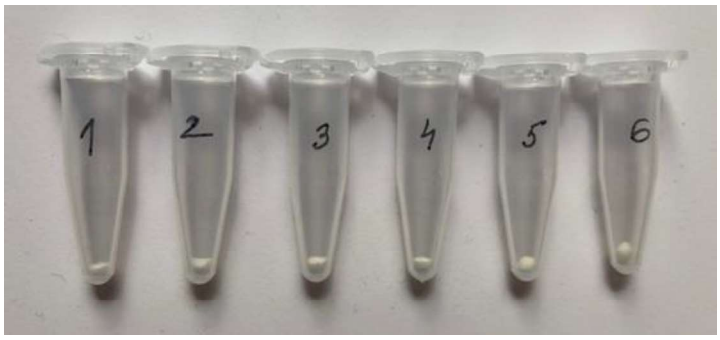

Figure 8 Human serum after the lyophilization.

Analysis of serum N-glycoprofile by mass spectrometry revealed significant effect of lyophilization on obtained relative intensities of single glycan structures. Relative intensity of the most abundant disialo diantennary N-glycan in human (m/z 2792) was maintained in lyophilized sample, even when stored at room temperature, and its level was similar to the one in sample stored at -80 °C (data not shown). These results suggest lyophilization as a suitable option for storage of biological samples, when deep freezing equipment is not available.

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