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INSIGHTS ON THE TIGHTNESS AND LONG-TERM STORAGE SUITABILITY OF CRYOVIALS AND STRAWS IN BIOBANKING

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ABSTRACT

The long-time storage of biological specimen is very important for biomedical research, e.g. for the investigation of common diseases. Since many research activities are based on such samples, their integrity is a critical task. Under common storage conditions biodegradation processes are slowed, but low temperatures also pose risks to the sample by material fatigue of the storage container with increasing storage time. This can result in leaks, which can lead to contamination or to chemical reactions (e.g. oxidation).

The CO_2 leak test enables a statement on the risk of contamination during transportation on dry ice. The LN_2 leak test differentiates between temporary and permanent leakage after LN_2 contact. Furthermore the tightness under both conditions, at negative pressure and at temperatures from −40 °C to +55 °C, is evaluated. In order to characterize the storage stability, tensile tests and DSC measurements of fresh, real-time stored and cyclically thermal stressed packaging materials are compared. The objective is a simulation of long-term storage by cyclical alternating loads and to evaluate the impact on the integrity of the vials.

Keywords: Cryotubes, Cryovials, Transport safety, Biobanking, Storage, Tightness, Leak test

1. INTRODUCTION

Storage and transport of biological materials (e.g. blood or urine) play a main role in the context of many clinical and diagnostic studies, for example to investigate the causes and mechanisms of cancer, diabetes and cardiovascular diseases. Samples for comparative long-term studies usually have to be stored for long periods (storage periods of more than 30 years are possible, for example in the NAKO health study). During this process, the samples must remain free of contamination and must not change in their biological and physical properties. Storage above liquid nitrogen at temperatures below −180 °C has proven effective in reducing biodegradation processes to a minimum. Cryovials or so-called straws made of polypropylene are frequently used as primary packaging materials.

In principle, cryogenic storage and transport of biological samples involve various risks and place high demands on the primary packaging materials used. With regard to material selection and design, basic aspects of sample safety (protection against chemical and biological contamination, protection against sample loss due to leakage problems) and occupational safety (protection against leakage of biological material as well as bursting safety) must be taken into account under the planned working and storage conditions.

To replicate some of the possible exposure scenarios and evaluate leak tightness under various conditions, three methods of leak-tightness evaluation are discussed below.

Testing for tightness against liquid nitrogen is used to evaluate the tightness of cryovials for the scenario of storage in cryobanks. There are various LN_2 related risks associated with leakage of primary packaging materials. The sample can be contaminated by penetrating nitrogen or the leaking sample contaminates the entire storage container. From an occupational health and safety perspective, LN_2 ingress is also hazardous. Due to the approximately 700-fold volume expansion during the phase transition from liquid to gaseous, a large pressure can build up in the vial, which can lead to spontaneous bursting of the vial when it is removed from the storage tank.

The test for tightness against CO_2 is used to evaluate the cryovials in a transport scenario on dry ice. The main problem with CO_2 penetration is the reduction in pH value. This is particularly important for sensitive and low buffered biological samples, whose properties in some cases have a large dependence on pH or the samples where pH is an important property.

The gravimetric leak test is based on the specifications of the International Air Transportation Association (IATA) and tests the leak tightness in the temperature range from −40 °C to +55 °C at a pressure difference of at least 95 kPa between the test specimen and the environment. This test is used to simulate a pressure drop during transportation.

A further important aspect is the tightness during long-term storage. The low temperatures during storage can cause changes in the material, which may also have an effect on the integrity of the specimens. For example, if the stability of the material decreases during storage, the possibility of leakage increases and, in the worst case, can lead to changes in the sample's properties or even to the destruction of the sample. Since real-time storage over several years is difficult to test, an attempt to induce accelerated aging by multiple temperature changes has been made. The material properties of glass transition temperature and melting or solidification temperature of the plastic, as well as the tensile strength of the specimens for fresh and thermally aged specimens, determined by DSC, are compared here. Real-time stored packaging materials kindly provided by various research institutions or biobanks serve as reference material here.

2. MAIN SECTION

2.1. Materials and Methods

2.1.1. CO_2-Leak test

For the transport tightness test, the vials were filled up to the specified working volume with 1 mM TRIS-HCl buffer solution (Merck, pH 8.2 at 25 °C) and sealed with torques according to the manufacturer's specifications. The lidded racks were cooled at a rate of 5 $Kmin^{-1}$ to −50 °C in a controlled-rate freezer and then stored at a temperature of −20 °C until the start of the experiment.

To simulate transport on dry ice, the frozen samples were overlaid with dry ice pellets (3 mm, IceProducts, Germany) and incubated for 24 h in a suitable sealed transport box. Following incubation, the racks of 96 vials each were stored for 18 h at 4 °C. Before determining the pH value, the samples were tempered at room temperature for 30 min.

2.1.2. LN_2-Leak test

For the proof of tightness against LN_2, the test specimens were filled under standard conditions with approx. 200 µl of an insoluble, white, microgranular solid. This was followed by overlaying with a red azo dye, readily soluble in non-polar solvents, which was bound to an insoluble microgranular carrier substance at a mass fraction of 6 %. After sealing the vials with torque specified by the manufacturer, the racks were closed with lids and locked in a burst-proof cage. Precooled to −20 °C by defined temperature control in a controlled rate freezer (SyLab IceCube M14). The cage was then placed in a thermally insulated test container containing approx. 3.5 l LN_2. After a 24 hour incubation period, the samples were removed from the LN_2 and tempered at 25 °C. This was followed by the evaluation of the test specimens. In the first step, clean layer separation between white solid and red azo dye was evaluated. For each mixing (positive evaluation), the vial was evaluated as leaky. In the second step, the test specimen was opened and the residual pressure was evaluated. A visible layer lifting of the solid (positive evaluation) leads to an evaluation of the vial as leaky.

2.1.3. Thermal Aging

Thermal aging was performed manually. For this purpose, an appropriate quantity of test specimens was placed in the rack or in a suitable frame and secured by a metal mesh in order to stop parts of the test specimens escaping in the event of a burst. The specimens were immersed in liquid nitrogen until no further gas formation could be detected visually and the temperature reached below −180 °C. The specimens were then placed in a temperature-controlled drying oven at 60 °C until the test specimens had reached room temperature again. This process was repeated in total 50 times.

2.1.4. Gravimetric Tightness test

The cryotubes and the suitable screwcaps are first pre-dried at 20 °C in a vacuum chamber and equilibrated under laboratory atmosphere to achieve a constant sample moisture. After determining the empty mass of tubes with screwcaps, the tubes are filled with antifreeze to 100 % of the working volume specified by the manufacturer. The cryotubes are sealed with the appropriate screw caps using a factory-calibrated torque tool (Bestool canon Japan, model CN30LTDK-H). After programming the test regime in the vacuum chamber, the cryotubes are pre-cooled in the laboratory freezer to a sample temperature of −40 °C ± 1 °C and transferred to the vacuum chamber. After evacuating the sample chamber to a test pressure of 99 ± 1 kPa, the samples are tempered for 0.5 h at −40 °C ± 1 °C. The samples are then heated to the final sample temperature of +55 ± 1 °C at a heating rate of 1 K min^{-1} by direct thermal contact. As soon as the cryotubes have reached the final temperature, the temperature is maintained for 2 h. At the end of the test, the cryotubes are cooled to room temperature in the laboratory freezer.

The test is then evaluated by a visual leak check (level check, test for leaked test liquid) and the backweighing of the cryotubes with an accuracy of ±0.1 mg on a calibrated balance. The cryotubes are considered to be leak-tight if the determined net mass loss is less than 0.1 % of the test liquid weight. The leak test is passed if all tested cryotubes meet this criterion.

2.1.5. DSC Measurement

All DSC measurements were performed at a DSC Q100 (Fa. TA Instruments, New Castle (DE), USA) which was provided by the TA Instruments LNCS cooler with liquid nitrogen, and helium was used as purging gas. Due to the large measurement range and small effect values, baseline optimization was performed using Tzero calibration. The measurement parameters are summarized in Table 1.

Table 1: Measurement parameter for DSC-measurements

Pan	20 µl Al Standard Pan, non-hermetic
Sample mass	8 … 10 mg (±5 %)
Temperature program	First heating run: Heating from -180 °C to 220 °C at 10 K/min. Cooling run: Cooling from 220 °C to -180 °C with 10 K/min Second heating run: Heating from -180 °C to 220 °C at 10 K/min
Atmosphere	Helium 5.0

The obtained and dried sample material is compacted into discs with a diameter of 4.5 mm and a height of approx. 1 mm using a manual tablet press (ILK in-house production). The obtained pellets with a sample weight of approx. 8 to 10 mg are transferred into weight-selected aluminum standard crucibles (TA Instruments Standard Pan/ Standard Lid, non-hermetic) and sealed with a suitable crucible press. For each measurement, a sample and reference crucible pair is prepared with a net mass difference of < 0.05 mg.

For melting and crystallization effects, melting or crystallization temperature T_m or T_c (respectively as effect maximum) and for glass transitions, the glass transition temperature T_g (inflection point method) and the step height Δc_p (change in heat capacity) are determined.

2.1.6. Tensile Tests

Tensile tests were performed with a tensile testing machine (Fa. Zwick/Roell, Ulm) and a 2.5 kN tongs specimen holder. The tube samples were prepared according to its shape. Straws were cutted on each side to a total length of 100 mm. For tubes the area of the thread and the foot were removed. For both types of samples the clamping length between the tongs specimen holders was 1 mm. Tests were performed at a feed speed of 10 mm×min^{-1} at room temperature. The maximum tensile force was measured and the tensile strength was calculated from known geometry.

2.2. Results and Discussion

2.2.1. Experimental results of the CO_2-tightness test for tubes of two different manufacturers

For a 1 mM absorbance buffer, the pH values set after incubation were measured. Based on the calibration, shown in Figure 2, the CO_2 content could be calculated according to Equation 1.

By fitting the measured data shown in Figure 1, a logarithmic relationship was found between measured pH of the sample and corresponding CO_2 content of the sample.

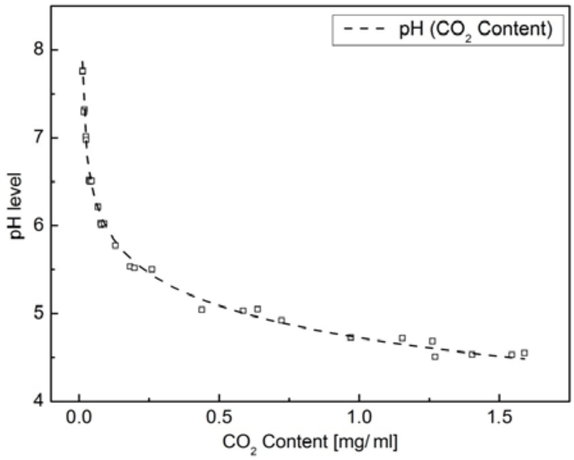

Figure 1: Fitted correlation between measured pH value of the sample and CO_2 content of the absorption buffer

Especially in the range of small contents of free carbonic acid, a relatively large change of the pH value is measurable. With increasing CO_2 content, the pH value decreases only slowly. However, due to the increase in concentration of the absorption buffer, a shift of the sensitivity into the range of large CO_2 contents is possible. Below a pH value of 4.3 the solubility limit for CO_2 in the absorbance buffer is reached. The maximum CO_2 capacity thus correlates with the molarity of the absorbance buffer used, so that even large amounts of CO_2 could be detected.

$$\beta_{CO2}[\tfrac{mg}{ml}] = exp\left(\frac{4.72 - pH}{0.52}\right) - 0{,}01 \qquad \textit{Equation (1)}$$

The results are presented in a scale with variable class width in figure 2.

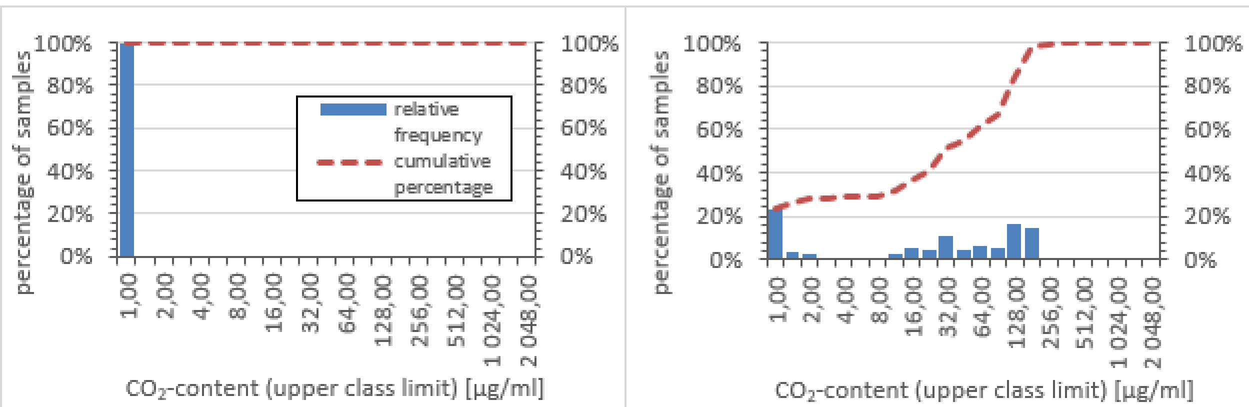

Figure 2: Comparison of the CO_2 content for 96 vials each from two different manufacturers.

Figure 2 clearly shows that there can be significant differences in the sealing behaviour of different manufacturers. While the tubes in the left part of the figure fully meet the leak-tightness criteria, the tubes in the right part of the figure are leaking. In some cases, a high amount CO_2 penetrates into the sample so that sensitive samples can be destroyed. Furthermore, the leaky tubes are not evenly distributed, which suggests in this example that the sealing effect is not equally good for every tube. Possible reasons could be too low sealing torque or too large tolerances in the production process.

2.2.2. Results of LN_2-Tightness-Test

The assessment of a vial as leaking is made when a mixing of the colored layers and / or a layer lifting is visible. Figure 3 shows examples of the different scenarios and the initial state of the samples.

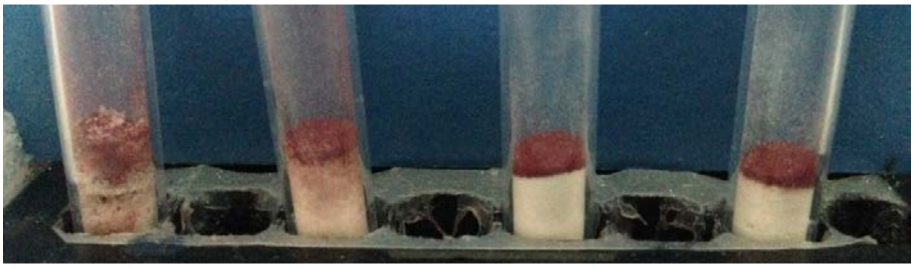

Figure 3: Result of the LN_2 leak test. From left to right: residual pressure and mixing effect positive, mixing effect positive and residual pressure negative, mixing effect and residual pressure negative (tight vial), mixing effect negative and residual pressure positive.

A characterization of the sealing problem can be estimated based on the following criteria:

1) Residual pressure positive, mixing effect negative.

A small temporary leakage in the low-temperature range leads to the penetration of small amounts of LN_2; on heating, the vial seals again and a high pressure develops, which can even lead to spontaneous bursting of the vial.

2) Residual pressure and mixing effect positive

A pronounced temporary leak leads to the ingress of relatively large quantities of LN2, which evaporate abruptly on heating and mix the layers. The leakage seals again at low residual pressures, only.

3) Residual pressure negative, mixing effect positive

A permanent leak leads to evaporation of the LN2 when the vial is heated, a mixing effect occurs. Due to the lack of resealing, no pressure can build up in the vial.

With the method used, a comprehensive sample and work safety evaluation of cryovials is possible. Due to the two-stage evaluation of the results, conclusions can be drawn about the leak-tightness behaviour and allow the design of the vials to be adapted by the manufacturers. Complete leak tightness against LN_2 could not be determined for any of the tested tubes.

2.2.3. Experimental results of gravimetric leak test for fresh and thermally aged samples with external thread

The gravimetric leak test is performed for fresh and thermally aged tubes of the same manufacturer. The tubes are tested twice and the results are shown in figure 4.

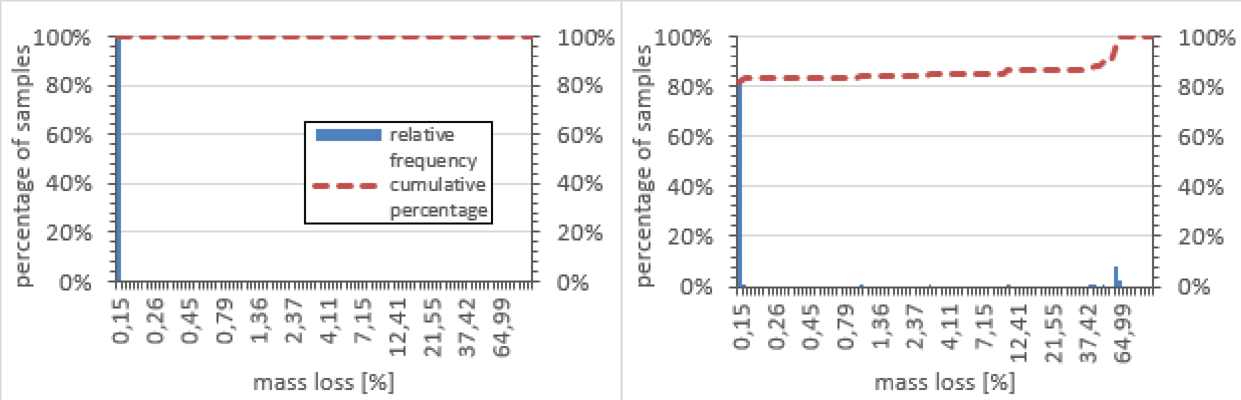

Figure 4: Comparison of the mass loss after gravimetric leak test for 96 vials each from fresh and thermally stressed tubes

The left part of figure 4 shows the test results for fresh tubes, in the right part the results for thermally stressed tubes are shown. Some of the previously tight tubes now show leakages, which leads to a loss of mass. However, it cannot be excluded that this effect could also be related to the multiple opening and closing of the lid. Within the tested tubes, there were tubes that remained sealed and tubes that became leaky due to the thermal load.

2.2.4. Experimental results of DSC-measurements for fresh and thermally aged samples

The results of DSC-measurements to compare fresh und thermally aged samples are shown in table 2 whereas only the glass transition temperature and the corresponding step in heat capacity is shown. There were no significant differences in melting or crystallization temperatures detectable.

Table 2: Glass transition temperature and change in heat capacity at glass transition temperature for fresh and thermally stressed samples

Sample	Thermally aged	Glass transition temperature T_g [°C]	Change in heat capacity Δc_p [J×g^{-1}×°C^{-1}]
A	yes	−17.4	0.124
A	no	−14.7	0.192
B	yes	−15.6	0.215
B	no	−12.5	0.151
C	yes	−13.2	0.241
C	no	−11.4	0.280
D	yes	−16.8	0.128
D	no	−12.9	0.141
E	yes	−18.1	0.302
E	no	−18.9	0.253

Although there are small differences between the fresh and the aged samples, there is no significant trend in the change of the heat capacity. But the results show a slightly decreasing glass-transition temperature for thermally aged samples.

2.2.5. Experimental results of tensile tests for fresh and aged samples

As an example, Table 3 gives an overview of studies on tensile strength. There is a systematic reduction of the strength properties. The value for 25 years of storage is almost 15 % decreased compared to fresh samples. These test results were obtained for straws.

Table 3: Dependence of tensile strength of unused straws stored in liquid nitrogen for different lengths of time.

Storage since	Cross section area	Tensile strength
1995	2.39 mm²	14.5 ± 0.3 N
2006	2.39 mm²	14.3 ± 0.3 N
2012	2.39 mm²	15.1 ± 0.7 N
2017	2.39 mm²	16.2 ± 0.8 N
fresh	2.39 mm²	16.4 ± 0.7 N

The investigation of cryotubes showed large differences between fresh and thermally aged tubes, depending on the product and the manufacturer. While no significant changes in tensile strength were observed for tubes from some manufacturers after 50-fold cycling, a reduction in maximum tensile strength from (410.3 ± 3.3) N to (392.3 ± 5.4) N for thermally stressed tubes was determined for other products.

3. CONCLUSIONS

The developed methods for the tightness evaluation of cryovials allow a differentiated and objective evaluation of the tightness according to defined test conditions. In this context, the LN_2 leak test enables an evaluation of the sample and work safety of the primary packaging materials during storage above or in LN_2 and the removal of the samples. The verified CO_2 tightness test documents the tightness behaviour during transport of frozen samples with dry ice.

With the developed method, the amount of CO_2 in an absorption buffer can be quantitatively determined. Furthermore, by determining the amount of CO_2 absorbed, a conclusion can be drawn about the leak tightness behaviour of cryovials - this allows identification of the weak point of the seal and thus opens up the possibility of minimizing or eliminating this through suitable quality assessment or an improvement in the design.

Thermal aging of the packing material sometimes leads to measurable changes in material structure and strength. These material changes can be shown with the predefined leakage tests and by a decreasing leakage behaviour. This compromises sample reliability and should be considered in the future selection of the packing material. However, there also appears to be some tolerance, depending on the manufacturer, for leak tightness with degrading material properties. While tubes from some manufacturers do not show a decrease in tightness, such is detectable for others.

ACKNOWLEDGEMENTS

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NOMENCLATURE

β_{CO2}	CO_2 content ($mg \times ml^{-1}$)	T_g	Glass transition temperature (°C)
Δc_p	Change in heat capacity at glass transition temperature ($J \times g^{-1} \times °C^{-1}$)		