Cryogenic preparation of sample materials

Within the context of sample preparation, size reduction plays an important role as it has a substantial influence on the results of the subsequent analysis. If the particles are too coarse or inhomogeneous the results of the analysis may turn out to be incorrect, especially if there is only a very small amount of sample material which represents the total amount.

Brittle materials like minerals, salt or slag can be easily crushed by applying high mechanical stress through impact, pressure or friction from outside.
However, what can be done when the mechanical forces alone are not able to reduce the sample material to particles that are as small as possible?

One solution to this problem is provided by the use of grinding aids such as **liquid nitrogen** (LN2 ;T =-196 °C) or **dry ice** (CO2 ;T =-78 °C) which promote the breaking behavior of such materials.

For which materials is cold grinding advisable?

- **Samples with elastic behavior**
  With many polymers (plastics such as PP, PET, PA, etc.), as well as other materials, their viscoelastic behavior during grinding only results in a plastic deformation, i.e. crack initiation and therefore break-up does not occur.

If objects such as **elastic plastics** are immersed in liquid nitrogen then their temperature falls below the so-called glass-transition temperature; this reduces the ability of the material to resist a high mechanical stress by elastic-plastic behavior or viscous flow. If this precooled material is now placed in a suitable mill there is a build-up of stress peaks in the material matrix which results in **brittle breaking behavior of the sample,** i.e. the sample breaks like glass.

- **Samples with highly volatile constituents**
  Because of their thermal sensitivity materials that contain highly volatile constituents such as solvents (benzene, toluene, PCB, PCP, etc.) are difficult to prepare properly and reliably for analysis.

These samples can be prepared in a similar way as elastic material. The influence of the low temperature considerably reduces the generally high vapor pressure of the constituents at room temperature and **the sample matrix becomes embrittled.** The increase in temperature that occurs during the size reduction process has no lasting influence on the analytical results through evaporation and loss of concentration. Cooling also counteracts the increased emission of the highly volatile constituents which would otherwise be favored by the larger surface area of the sample resulting from the size reduction process.

- **Cell extraction**
  In the medical or biotechnical sectors for the extraction of DNA sequences from single cells or groups of cells (plant, human and animal tissues) the problem must be faced that their fragments react with extreme sensitivity to heat during and, in particular, after preparation and could be destroyed.

These applications require low-temperature grinding both in order **to embrittle groups of cells and cell walls** for easier disruption as well as to greatly slow down the rapid decomposition of the cell fragments. The higher the ambient temperature, the quicker the reaction kinetics of the oxidation and decomposition processes; this renders the samples unusable.

In all the methods mentioned above we are talking about "cold grinding" or "cryogenic grinding".

Suitable laboratory mills for cryogenic grinding

Mixer Mill MM 400

With the Mixer Mill MM 400 2 to 20 samples can be prepared within one single operation cycle. Pulverization is effected through the impact of the grinding ball on the sample material, homogenization is achieved through the horizontal oscillations of the jars in combination with the movement of the balls inside the jars. The oscillation frequency can be set continuously between 3 and 30 Hz.

Fig. 1: Mixer Mill MM 400 with grinding jars of steel

Fig.2: Kryo-Kit with containers, tongs and goggles

The Mixer Mill MM 400 is outstandingly suitable for cryogenic applications. RETSCH offers a "Kryo-Kit" as an accessory which consists of two differently-sized insulated containers, two pairs of crucible tongs and protective goggles.
For grinding with the aid of liquid nitrogen, grinding jars made from stainless steel and Teflon are suitable.

- **Using stainless steel grinding jars**

If steel grinding jars are used they are first filled with the ball charge and sample material, the cover is screwed on and the grinding jar is then immersed in the liquid nitrogen contained in one of the insulated containers for approx. 2 – 3 minutes using the crucible tongs.
When nitrogen boiling has noticeably diminished (temperature equalization), the grinding jar is removed with the help of the tongs and safely clamped in the self-locking device (Fig.4).
As the preparation time only takes approx. 1 – 3 minutes, the temperature in the jar hardly increases at all as a result of frictional heat, which means that the sample cannot be damaged by “thawing”. Additional intermediate cooling or post-cooling, such as is necessary with other mills, can normally be dispensed with. **The short length of time required and the easy handling represent considerable advantages, which allow an extremely cost-saving use of the expensive liquid nitrogen.**

Fig. 3: Cooling the jars in liquid nitrogen

Fig. 4: Clamping the cooled jars into the MM 400

Fig. 5 shows granulated rubber before it is ground in the MM 400. It was pulverized to a final fineness of 0,1 – 0,5 mm (Fig. 6) within a few minutes for the subsequent analysis of harmful substances. This would not have been possible without the use of cryogenic grinding aids.

Fig. 5: Granulated rubber before grinding

Fig. 6:...and after grinding with LN_2

If direct contact with the cooling medium is not harmful to the sample, grinding jar and sample material can be immersed into liquid nitrogen for a few minutes. After that, the material together with the balls is cooled down to the final temperature inside the jar by immersing it into liquid nitrogen and is then ground to the required fineness. Plant materials such as leaves (Fig. 7) can be prepared like this for a subsequent cell extraction within the MM 400 (Fig. 8).

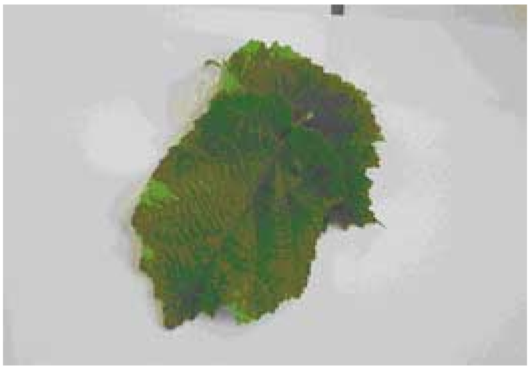

Fig. 7: Plant leaves before grinding

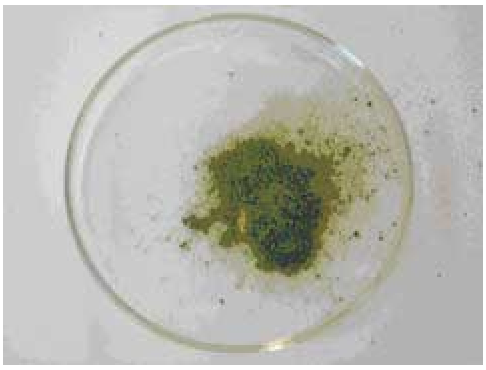

Fig. 8: ...and after grinding with LN_2

- **Using Teflon grinding jars**

If for contamination reasons it is not possible to use steel grinding jars, then grinding jars and balls made from Teflon (PTFE) can be used. However, because of their poorer thermal conductivity when compared with steel, it is advisable to carry out a separate pre-cooling process. If the sample is allowed to come into contact with the liquid nitrogen, then the grinding jar and sample material can also be directly cooled before the grinding process. After a few moments the sample and the grinding balls can be placed in the grinding jar which is then tightly sealed and cooled down to the final temperature before starting the grinding process.

- **Using disposable reaction vials**

Teflon (PTFE) adapters (Fig. 9) are available for preparing samples (e.g. tissue samples) in disposable reaction vials; these are also resistant to the low temperature of liquid nitrogen. The reaction vials containing the sample (and frequently also a digestion buffer solution) can be briefly precooled together with the adapters in the insulated container and then clamped in the MM 400.

Fig. 9: Adapters for disposable reaction vials

The advantage of using disposable reaction vials is that no cross-contamination between the individual samples can occur. The heavier cell fragments are concentrated on the base of the reaction vial by centrifugation and in this way are separated from the lighter fragments in the lysate so that they can be easily extracted.

Ultra-Centrifugal Mill ZM 200

Another mill that is ideally suited for the cryogenic pulverization even of larger sample amounts is the Ultra-Centrifugal Mill ZM 200. Although with this high-speed rotor mill it is possible to pulverize some elastomers without cooling (e.g. polystyrene), in most cases pre-embrittlement with liquid nitrogen is necessary. This is done by immersing the sample in the form of granules or pre-crushed molded parts directly in the box containing LN2 und then grinding it. As a result of the temperature falling below the glass-transition temperature, the sample then “explodes” into particle sizes of 100-200 µm or less. For samples with a high fat content or thermally sensitive samples, embrittlement often results in improved grinding behavior as the frictional heat produced is considerably reduced. **For powdery samples dry ice is often a more suitable grinding aid than liquid nitrogen.** The dry ice is mixed with the sample at a ratio of 2:1 by volume and the whole mixture is ground in the ZM 200. As pure CO2 is involved the dry ice gradually evaporates from the sample without leaving any residue.