Umwelt Proben Bank
Des Bundes

Guidelines for Sampling and Sample Processing

Pulverisation and Homogenisation of Environmental Samples by Cryomilling

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Umwelt Bundes Amt
Für Mensch und Umwelt

Contents

Guidelines for sampling, transport, storage and chemical characterisation of environmental and human samples

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1 German Environmental Specimen Bank

The German Environmental Specimen Bank (ESB) is an instrument for the monitoring of the environment. It is in the responsibility of the Federal Ministry for the Environment, Nature Protection and Reactor Safety (BMU) and technically and administratively coordinated by the Federal Environment Agency (Umweltbundesamt). The ESB collects ecologically representative environmental specimens as well as human samples, stores them and examines the archived material for environmental relevant substances.

The long-term storage is performed under conditions that exclude a change of state or a loss of chemical characteristics as far as possible during a period of several decades. By this means the archive provides specimens for a retrospective monitoring of such substances, whose hazard potential for the environment or human health are not yet known.

Comprehensive information on the German ESB is available at www.umweltprobenbank.de (English language pages available).

2 General information

This protocol applies to biological environmental samples like bladder wrack, bream muscle, earthworm, spruce shoots as well as abiotic samples such as soil and suspended particulate matter.

For the storage in the ESB archive all samples are frozen immediately at the time of the sampling and the cooling chain is not interrupted at any time afterwards. Thus transport, storage as well as grinding and homogenization of the sample material takes place under cryogenic conditions (temperature below approx. -130°C) considering special safety regulations.

The particularly high quality assurance requirements result from the extraordinary importance of the samples as archive material. Representativeness and reproducibility of the specimens are prerequisites for the comparability of the analytical data in time and space.

The processes defined are the pulverisation and homogenisation of deep-frozen material by milling under cooling with liquid nitrogen. The low temperatures render the water containing biological material brittle, thereby allowing it to be reduced to particles > 200 µm without damage to its contents.

3 General notes

For samples weighing more than 1 kg, the material is pulverised and homogenised by means of milling in an oscillating mill. For homogenisation of smaller sample quantities (max. 1 kg) a planetary ball mill is used.

To prevent contamination of the sample material by dust particles and gaseous substances, the material should be prepared only in clean rooms which are ventilated with purified air (e.g. operation of the cryomill in a laminar-flow field with filtered air; bottling of samples in a laminar flow clean bench).

During the processing stages, it must be ensured that the sample material does not become warm. Before use, all apparatus and vessels must be cooled to a temperature of at least -150°C with liquid nitrogen. If necessary, liquid-nitrogen cooling should also continue during processing. If this is the case, it should be ensured that as little liquid nitrogen as possible comes into direct contact with the sample.

4 Apparatus and materials

- Cryo-oscillating mill KHD Humboldt Wedag Palla VM-KT with titanium milling cylinder and titanium milling rods (manufactured by KHD, Cologne).
- Planetary ball mill (e.g. Pulverisette 5, manufactured by Fritsch, Idar-Oberstein, with 500 mL milling bowls and 3 cm milling balls of zirconium oxide).
- Mortar and pestle of teflon, titanium or ceramic material.
- Particle-size analyser (e.g. Mastersizer 2000, manufactured by Malvern, Herrenberg).

- Laminar flow clean bench (standard type).

- Instrument for monitoring the oxygen content in the room air (e.g. gas-alarm unit type Micro III, GfG, Dortmund).

- Glass vials (e.g. 20 mL scintillation vials purchased from PerkinElmer, Rodgau-Jügesheim) of high-quality glass with following standard dimensions: height approx. 60 mm, diameter approx. 25 mm. The screw caps are of plastic with metallic foil on the inside.

- Cold-resistant plastic adhesive labels (e.g. glossy white polyester labels, size approx. 2 x 4 cm, ESTO GmbH) for printing with a thermoprinter (e.g. Eltron TLP 3642, with resin based thermal-transfer foil; ESTO GmbH, Potsdam).

- Liquid nitrogen (99.999 %).

5 Preparation of material

5.1 Storage

Immediately after sampling, the material is stored at temperatures below -150°C until processing takes place. Stainless-steel containers are used for storing material (e.g. the type of containers used in gastronomy, with volumes of 1.5 litres, 3.5 litres or 5.5 litres and lids fastened by a clasp). Each container must be clearly identified (e.g. by a punched or engraved serial number). The type of sample must also be stated on the metal container.

5.2 Manual size reduction

The cryomilling process described here can be used for biological material with a grain size of < 1 cm edge length. If necessary, prior reduction should be carried out as a preparatory step.

In most cases, the material can be manually reduced to smaller pieces with a pre-cooled pestle of ceramics, Teflon or titanium. It can be carried out in a pre-cooled ceramics mortar of suitable size. It may be necessary to prepare the material in a larger stainless-steel tray (e.g. if a larger quantity of sample material is being used at the same time), or in a metallic container in which the material is stored.

6 The cryomilling process

6.1 Cryomill KHD Palla VM-KT

When working with the cryomill KHD Palla VM-KT always comply with the operating instructions.

The cryomill KHD Palla VM-KT consists of the actual mill itself, a milling cylinder and milling rods. All the parts of the mill coming into contact with the sample and which are subject to mechanical strain are made of titanium (with Teflon for the bellows which connect the vibrating milling cylinder with the sample-infeed device and the collecting container). The cryomill also has a dosage groove for ensuring that identical sample quantities enter the mill.

SAFETY NOTE: Large quantities of liquid nitrogen are required to cool the cryomill KHD Palla VM-KT. This may cause a displacement of oxygen in the room atmosphere. Adequate ventilation is therefore essential. The oxygen content of ambient air should be monitored by a sensor which gives an audible alarm signal when the content drops below 19%. Contact with liquid nitrogen may cause serious injury. Always wear protective clothing (insulated safety gloves, goggles, lab coat, strong shoes). Instructions for working with liquid nitrogen are given in appendix C.

Fill the milling cylinder of the cryomill KHD Palla VM-KT up to approximately two thirds with the milling rods. Then assemble the unit and tighten all screws firmly. Before starting the milling operation, cool the mill and the dosage groove for at least two hours with liquid nitrogen. The temperature at the start of the milling operation must be less than -170°C when measured at the dosage groove and the milling cylinder.

After cooling of the cryomill, the sample material itself is milled. The grinded material is collected in a stainless-steel container which is cooled with liquid nitrogen. In particular, when the mill is idling titanium particles may be dislodged by the mechanical stress. In order to minimise contamination of the sample material, the dosage groove is switched on first and then (after about one minute) the mill. When sufficient sample material is available, the first part of the milled material can also be discarded to avoid unnecessary contamination.

The cryomilling process should be continued until the sample material has reached the desired grain size. The aim is to achieve a homogeneous substance with a grain size of < 200 µm (for > 90% of the particles). For most of the particles, this figure is achieved after approx. 5 milling operations.

Between the individual milling operations the sample material should be cooled (i.e. collection in a stainless-steel container, intermediate storage in a cryogenic-storage container cooled by liquid nitrogen).

Should the temperature at the end of a milling operation exceed -130°C, the mill and the dosage groove have to be re-cooled to temperatures below -170°C with liquid nitrogen.

6.2 Planetary mill

For the homogenisation of smaller sample quantities (less than approx. 1 kg), a planetary mill should be used.

When operating the planetary mill, always comply with the operating instructions.

SAFETY NOTE: Due to the extremely low temperature of the milling bowls and their high weight, there is a considerable risk of frostbite to the hands. Always wear safety clothing during cryomilling operations (insulated safety gloves, goggles, lab coat).

The milling bowls and balls are cooled overnight in a cryostorage container in the gas phase above liquid nitrogen. Each of the bowls is then filled with several milling balls and the sample material is then added to the balls. The bowl is then closed and clamped into the mill. The sample is then milled for approx. 1-2 min. Following intermediate cooling of the milling bowls for at least 2 hours in the gas phase above liquid nitrogen, the milling operation can be repeated 2 to 4 times before the desired particle size distribution is achieved.

Currently, a planetary ball mill of type Pulverisette 5, manufactured by Fritsch, Idar-Oberstein or an equivalent system is used. Milling bowls and balls of zirconium oxide are used (500 mL milling bowls and 3 cm milling balls). The milling bowls are normally filled with 10 balls and 250 g of sample material. The milling operation takes place for 1-2 minutes at 400 rpm. If more or less sample material is used, the number of milling balls is reduced or increased as necessary. The quality of the milling operation is checked for every sample.

6.3 Particle-size analysis

After every milling operation, the quality of the results must be verified. This is done by analysing particle dimensions, e.g. by laser-diffraction or light-diffusion processes. All pulverised and homogenised samples are examined using a particle-size analyser (e.g. of type Mastersizer 2000 with a Hydro MU sample-dispersion unit; manufactured by Malvern, Herrenberg). The particle-size analysis of a typical ESB beech-leaves homogenate is shown in appendix B.

The cold material is examined directly after milling (e.g. after the fourth milling operation). Analysis is carried out by using water as the dispersion medium (for non-plant samples following addition of approx. 30% 2-propanol). If the desired particle size is not yet achieved, further milling operations are carried out.

The results are compared with those achieved with similar samples analysed under the same conditions in previous years.

7 Cleaning of apparatus

Since the homogenised samples are examined for traces of organic substances and trace elements, it is essential to avoid any contamination of the samples. All the apparatus and receptacles used must be cleaned before use with high-purity solvents (e.g. ethanol) and/or high-purity water. Before cleaning, the apparatus should be allowed to warm up to room temperature after use. Removable parts of the cryomill can be washed in a machine (temperature approx. 90°C).

In cases where a number of similar materials containing similar substances are being processed (e.g. individual samples of one type of material from one area), it may be sufficient just to clean the cryomill KHD Palla VM-KT mechanically

or to allow it to idle before adding the next sample.

8 Documentation

All the data pertaining to the pulverised material must be documented (form shown in appendix A). The sample is identified by means of the ESB code stating type of sample, and year and location of sampling and complies with the specifications of the ESB code system. For each sample processed, the weight must be recorded with a precision of 0.1 g before and after processing.

The completed forms are filed and retained for at least five years.

9 References

BMU (Bundesministerium für Umwelt, Naturschutz und Reaktorsicherheit, Hrsg.) (2008): German Environmental Specimen Bank – Concept (Status: October 2008); www.umweltprobenbank.de

Umweltbundesamt (1996): Umweltprobenbank des Bundes – Verfahrensrichtlinien. Herausgeber: Umweltbundesamt, Berlin. Erich Schmidt Verlag, Berlin.

Appendix A: Milling Documentation Form

Environmental Specimen Bank **Form Cryomilling**

Sample identification

ESB code of sample: ☐☐☐☐/☐/☐☐☐☐/☐☐☐☐☐/☐

Comments:..... .....

Identification of stainless-steel vessel from which the sample was taken:

1	2	3	4
5	6	7	8

Total weight before cryomilling: ☐☐☐☐ g

Cryomilling

Operator: _____ **Date:_____**

Milling conditions KHD Palla VM-KT:

Temperature of cryomill	before milling	after milling
Dosage groove device	**°C**	**°C**
Milling cylinder	**°C**	**°C**
Sample material	**°C**	**°C**

Setting of dosage groove	**mm**
Forward feed of dosage groove	**scale units**

Milling conditions for planetary mill Pulverisette 5:

No. of balls: _____ No. of bowls: _____

Duration of milling operation: _____ sec.

No. of milling operations:

(tick after each milling cycle): 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐ 8☐

Intermediate cooling of cryomill: **yes** ☐ / **no** ☐

Total duration of milling: _____ **min.**

Total sample weight after cryomilling: ☐☐☐☐ g

ID no. of stainless-steel vessels in which material is temporarily stored:

1	2	3	4
5	6	7	8

Temporal storage until next processing phase: in cryo-storage container ☐☐☐

Determination of particle size distribution after milling

Operator: _____ Date:_____

Percentage smaller than 200 µm: ☐☐ %

Appendix B: Particle size distribution of a beech-leaves homogenate

Mastersizer 2000 — **Fraunhofer IME / Umweltprobenbank**

P2 B 318 K-UBA-011/7-30

Result Analysis Report

Measured: Montag, 30. Oktober 2006 09:18:19

Sample Name: Buchenblätter Hochharz 2006 **SOP Name:**

Measured by: Unbekannt

Sample Source & type: **Result Source:** Messung 30.10.06

Sample bulk lot ref:

Particle Name: Default **Accessory Name:** Hydro 2000MU (A) **Obscuration:** 20,11 %

Particle RI: 1,520 **Absorption:** 0,1 **Analysis model:** Universal

Dispersant Name: Water **Size range:** 0,020 to 2000,0... um **Weighted Residual:** 0,695 %

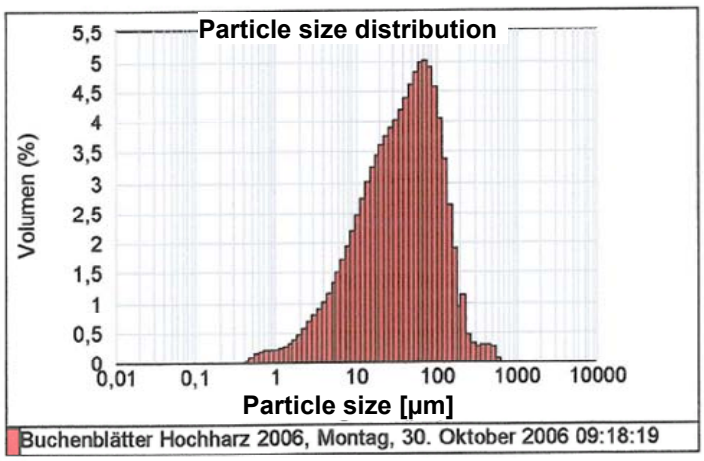
Dispersant RI: 1,330 **Result Emulation:** Aus

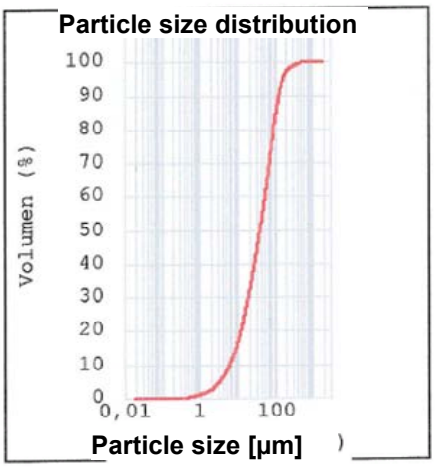
Concentration: 0,0495 %Vol **Vol. Weighted Mean D[4,3]:** 58,655 um **Specific Surface Area:** 0,451 m²/g

Span : 3,169 **Uniformity:** 1,08 **Surface Weighted Mean D[3,2]:** 13,294 um

Result units: Volumen

D(0,10): 6,27 µm D(0,25): 15,43 µm D(0,50): 38,89 µm D(0,75): 80,40 µm D(0,95): 168,18 µm





size fraction	% passed	size fraction	% passed	size fraction	% passed	size fraction	% passed	size fraction	% passed	size fraction	% passed
0,010	0,00	0,105	0,00	1,096	1,05	11,482	18,84	120,226	88,09	1258,925	100,00
0,011	0,00	0,120	0,00	1,259	1,28	13,183	21,57	138,038	91,47	1445,440	100,00
0,013	0,00	0,138	0,00	1,445	1,54	15,136	24,57	158,489	94,10	1659,587	100,00
0,015	0,00	0,158	0,00	1,660	1,84	17,378	27,80	181,970	96,00	1905,461	100,00
0,017	0,00	0,182	0,00	1,905	2,22	19,953	31,25	200,000	96,91	2187,762	100,00
0,020	0,00	0,209	0,00	2,188	2,69	22,909	34,86	239,883	98,02	2511,886	100,00
0,023	0,00	0,240	0,00	2,512	3,25	26,303	38,62	275,423	98,48	2884,032	100,00
0,026	0,00	0,275	0,00	2,884	3,92	30,200	42,51	316,228	98,79	3311,311	100,00
0,030	0,00	0,316	0,00	3,311	4,70	34,674	46,54	363,078	99,06	3801,894	100,00
0,035	0,00	0,363	0,00	3,802	5,60	39,811	50,72	416,869	99,35	4365,158	100,00
0,040	0,00	0,417	0,00	4,365	6,62	45,709	55,11	478,630	99,65	5011,872	100,00
0,046	0,00	0,479	0,00	5,012	7,78	52,481	59,72	549,541	99,92	5754,399	100,00
0,052	0,00	0,550	0,10	5,754	9,09	60,256	64,55	630,957	100,00	6606,934	100,00
0,060	0,00	0,631	0,26	6,607	10,58	69,183	69,53	724,436	100,00	7585,776	100,00
0,069	0,00	0,724	0,44	7,586	12,28	79,433	74,56	831,764	100,00	8709,636	100,00
0,079	0,00	0,832	0,64	8,710	14,20	91,201	79,46	954,993	100,00	10000,000	100,00
0,091	0,00	0,955	0,85	10,000	16,38	104,713	84,04	1096,478	100,00		

Operator notes: *dest. Wasser*

Appendix C: Instructions for working with liquid nitrogen

DANGER FOR PERSONS AND ENVIRONMENT

On contact with eyes or skin, cryogenic, liquid nitrogen causes serious frostbite with injuries similar to burns (i.e. inflammation, swelling, blistering) as well as severe damage to tissue. The cold gas is heavier than air, accumulates at floor level and may displace the oxygen in low-lying rooms. One litre of liquid nitrogen produces approximately 650 litres of gas! At concentrations of more than 85% in the air, severe oxygen deficiency occurs causing symptoms such as drowsiness, nausea, increase in blood pressure and difficulty in breathing. Concentrations of 88% and more, lead to immediate loss of consciousness and risk of asphyxiation.

SAFETY MEASURES AND PROCEDURES

Liquid nitrogen should be transported and handled only in suitable cryogenic vessels or apparatus which is resistant to cold. Never handle larger quantities in small poorly ventilated rooms. Always avoid contact between the cryogenic liquid or gas and the skin and eyes. Always wear protective clothing, impermeable shoes, leather safety gloves and goggles. If release of large quantities of gas is anticipated or unavoidable, also use insulating equipment. When working in confined or inadequately ventilated rooms, a second person is required for supervision outside the danger zone who can raise the alarm if necessary.

EMERGENCY PROCEDURE

Fire: Nitrogen is not inflammable. Take measures appropriate to the surroundings. If a fire cannot be extinguished immediately, leave the area immediately. Never attempt to extinguish flames with liquid nitrogen. On release of larger quantities of nitrogen, warn all other persons, leave the danger zone and re-enter it only with insulating equipment. If possible, repair the leakage. Do not re-enter the danger zone without isolating equipment and before it has been thoroughly ventilated. If necessary, measure the concentration of nitrogen in the air.

FIRST AID

Aspiration: Take affected person out of doors immediately. If necessary assist respiration with mask and bag to provide ventilation. Seek medical assistance! Eye contact: do not move or rub parts of the body which are frozen rigid. Thaw carefully with (cold) water. Remove clothing and cover the body loosely with sterile bandaging material. Seek medical assistance!

DISPOSAL

Allow to evaporate slowly in the open air. Loosely cover the opening of the vessel to avoid condensation and concentration of atmospheric oxygen in the remaining liquid.