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SAMPLE PRETREATMENT PROTOCOL

Muffle furnace dry ashing of animal and plant samples

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1. Purpose

This protocol describes the removal of organic matter from samples of animal and plant origin by dry ashing. **15 g** of sample are weighed, dried at **70 °C for 24 h**, ashed in a muffle furnace at **450 °C ± 25 °C** until **white ash** is obtained (≈ 48 h), and the ash is dissolved in **1.5% HNO_3** to a final volume of **25 mL**.

- **Scope:** the procedure is common to all food matrices, both of animal origin (fish, invertebrates, meat, ready meals) and of plant origin (legumes, cereals, medicinal plants, supplements).
- **Analytes:** Al, B, Ba, Cd, Co, Cr, Cu, Fe, Li, Mn, Mo, Ni, Pb, Sr, V, Zn and Na, K, Ca, Mg.
- **Limitations:** the method is not suitable for Hg, As, Se or other elements volatile at 450 °C, for which microwave acid digestion is used.
- **Selection criterion:** recommended when ample sample is available and trace-level elements are determined in matrices with high organic content. The large sample mass compensates for low concentrations and, since only minimal amounts of reagents are used, blanks are very low.

2. Principle

At 450 °C and in the presence of air, organic matter is oxidised to CO_2 and H_2O, and the metals remain in the ash as oxides, carbonates and salts, which are subsequently dissolved in dilute nitric acid. Gradual heating prevents flaming combustion and spattering, while not exceeding ≈ 450 °C limits volatilisation losses and the formation of poorly soluble refractory oxides.

Aspect	Muffle furnace ashing (this protocol)	Microwave digestion (complementary protocol)
System	Open, dry (450 °C, air)	Closed, wet (HNO_3 + H_2O_2, 180 °C)
Sample mass	15 g	1 g
Duration	24 h drying + ≈ 48 h ashing	≈ 1 h 30 min
Reagents	Dilute HNO_3 (concentrated HNO_3 only for very fatty samples)	4 mL 65% HNO_3 + 2 mL 30% H_2O_2
Final volume	25 mL	10 mL
Hg, As, Se	Lost	Retained
Capacity	Dozens of dishes per batch	12 vessels per batch

3. Equipment, materials and reagents

Equipment	Function / requirement
Muffle furnace	Programmable ramp and hold; 450 °C ± 25 °C
Drying oven	70 °C, 24 h
Hot plate	100 °C, for HNO_3 pretreatment and re-ashing

Equipment	Function / requirement
Porcelain dishes or crucibles	With lid or labelled on the base
Analytical balance	Resolution 0.1 mg

Materials

- 25 mL volumetric flasks (class A) and polypropylene bottles with caps.
- Funnels and ashless filter paper, or 0.45 µm syringe filters.
- Long crucible tongs, heat-resistant gloves and desiccator.

Reagents

- 65% nitric acid, trace-analysis grade.
- **1.5% HNO_3:** prepared by diluting 15 mL of 65% HNO_3 to 1 L with ultrapure water (1.5% v/v). Weekly preparation is recommended.
- Ultrapure water (18.2 MΩ·cm).

4. Crucible cleaning and contamination control

Porcelain crucibles are reused, so their cleaning determines the blank value. The procedure is as follows:

1. Ash residues are removed with water and a soft brush, without metal scourers.
2. They are immersed in a 10% HNO_3 bath for at least 24 h.
3. They are rinsed three times with ultrapure water and dried in an oven.
4. Before first use, or after each ashing, the crucibles must undergo a cleaning treatment at 800 °C for 8 hours (the ramp used to reach this temperature should last at least 8 h so as not to subject the material to unnecessary stress).
5. They are stored in a desiccator and handled only with tongs.

5. Sample preparation

The procedure starts from the sample already homogenised in accordance with the sampling and sample preparation manual. Regardless of their nature, all samples are treated in the same way: **15 g** of fresh sample are weighed into a previously tared and labelled porcelain dish, in triplicate. Results are expressed on a **fresh-weight basis** (mg/kg f.w.), the usual basis for intake estimation.

Fresh weight and moisture. If the mass of the empty dish, of the dish with fresh sample and after drying is recorded, the moisture percentage is obtained and the result can also be expressed on a dry-weight basis.

6. Procedure

The complete cycle takes approximately 72 h: 24 h of drying and ≈ 48 h in the muffle furnace. The control point is the colour of the ash: only a white ash indicates the absence of residual carbon that could retain metals or interfere with subsequent analysis.

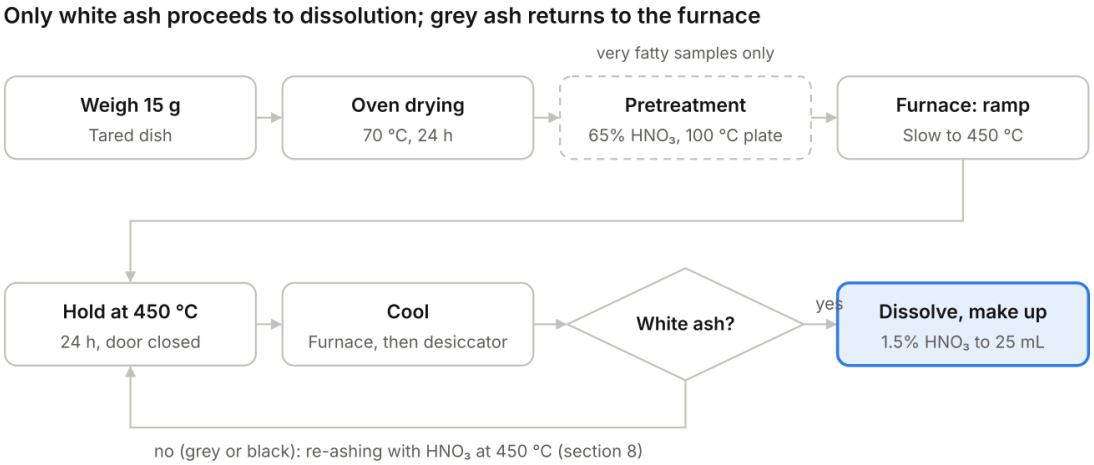

Figure 1. Ashing sequence and ash acceptance criterion.

A. Drying

1. The empty dish is weighed (m_0), 15 g of sample is added and it is weighed again (m_1). Each sample is analysed in triplicate.
2. The dishes are dried in an oven at **70 °C** for **24 h**.
3. They are allowed to cool in a desiccator and weighed (m_2) to determine moisture.

B. Nitric acid pretreatment (very fatty samples only)

4. This step is only considered for samples with a very high fat content that present difficulties during ashing (flaming combustion, spattering or fused, black ash). In that case, 2–5 mL of 65% HNO_3 is added to the dry sample until it is covered and heated on a hot plate at **100 °C** until the acid has completely evaporated. All other samples proceed directly to ashing.

C. Ashing

5. The dishes are placed in the cold muffle furnace with the door closed.
6. The temperature is raised gradually to **450 °C**, with a ramp of ≈ 50 °C/h or spreading the rise over ≈ 24 h, so that the sample chars without flaming.
7. The temperature is held at **450 °C for 24 h** (or until white ash is obtained), giving ≈ 48 h in the muffle furnace.
8. The furnace is switched off and allowed to cool inside to < 100 °C; the dishes are transferred to the desiccator with tongs.

D. Ash check

9. If the ash is **white or very light grey**, proceed to dissolution. If it is **dark grey or shows black specks**, it is re-ashed according to section 8.

E. Dissolution and make-up to volume

10. A few millilitres of **1.5% HNO_3** are added to the dish and the ash is dissolved by gently scraping with a plastic rod.
11. The solution is filtered into a **25 mL** volumetric flask; the dish and filter are rinsed with 1.5% HNO_3 and the flask is made up to the mark.
12. The solution is transferred to a **polypropylene** bottle and kept at room temperature until analysis.

7. Thermal programme

The thermal programme is the same for all samples: a slow ramp to **450 °C** and a hold of **24 h**, with a total time of **≈ 48 h** in the muffle furnace.

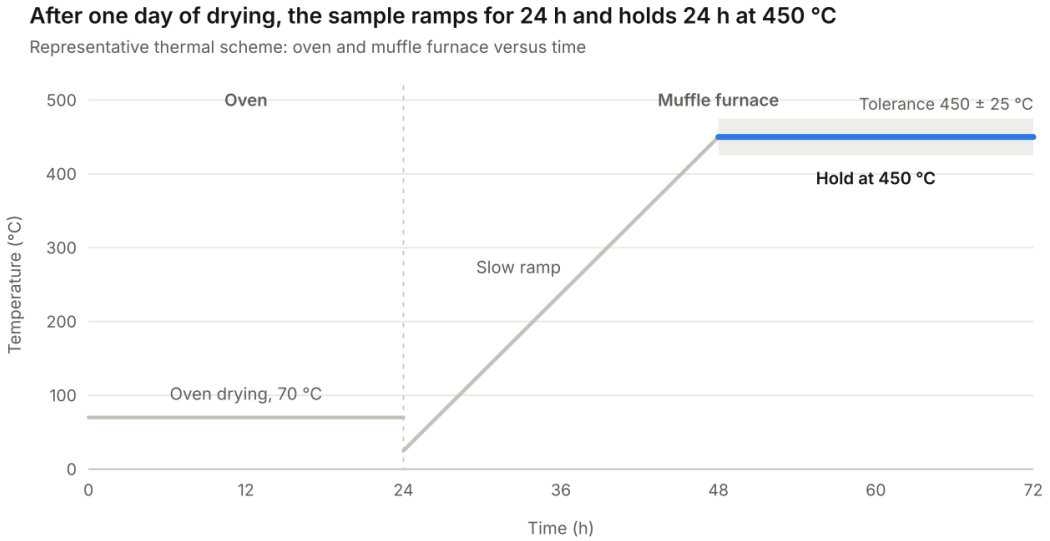

Figure 2. Thermal scheme (ramp drawn as linear; initial furnace temperature of 25 °C assumed).

Stage	Conditions
Drying (oven)	70 °C, 24 h
Ramp (muffle furnace)	≈ 50 °C/h to 450 °C (≈ 24 h)
Hold	450 °C ± 25 °C, 24 h
Cooling	Free, inside the furnace, to < 100 °C

If a sample smokes or ignites during the ramp, an intermediate hold of 2–4 h at 200–250 °C can be added to complete charring before heating continues. 500 °C must not be exceeded, since above this temperature Cd and Pb losses increase and Cr, Fe and Ni oxides that are difficult to redissolve are formed.

8. Re-ashing procedure

Re-ashing is applied when, after the 24 h hold at 450 °C, the ash is not white. The temperature **is not increased**: oxidation of residual carbon is promoted with a few drops of HNO_3 and the sample is returned to 450 °C for a few hours.

When it is required

- Dark grey or black ash, or ash with black specks or cores.
- White surface crust which, when broken with a rod, reveals black material underneath.
- Fused or vitrified ash with trapped carbon.

It is more frequent in samples with high fat or sugar content.

Procedure

1. **Cooling.** The dishes are cooled in a desiccator to room temperature. Acid must not be added to hot ash, as it spatters.
2. **Wetting and break-up.** In a fume hood, the ash is wetted with 0.5–1 mL of ultrapure water and lumps and crust are broken up with a plastic rod, without removing material from the dish. The rod is rinsed into the dish with a few drops of water.
3. **Acid addition. 1–2 mL of 65% HNO_3** is added dropwise until the whole ash is soaked.
4. **Evaporation.** The dishes are heated on a hot plate at **≈ 100 °C** to complete dryness, avoiding vigorous boiling. No liquid must remain, as unevaporated acid corrodes the furnace.
5. **Return to the furnace.** The dishes are placed in the furnace when cold or below 200 °C and the temperature is raised to **450 °C** at 50–100 °C/h.
6. **Hold.** The temperature is held at **450 °C for 4 to 12 h** (usually overnight).
7. **Cooling and inspection.** After cooling inside the furnace, the dishes are transferred to the desiccator and the colour of the ash is examined.
8. **Repetition.** If the ash remains grey, steps 1–7 are repeated. If after **three cycles** white ash is not obtained, the analysis is repeated applying the HNO_3 pretreatment (section 6.B) or microwave digestion is used instead.

Precautions

- 450 °C must not be exceeded to speed up the process, as Cd and Pb losses increase and poorly soluble oxides are formed.
- All replicates of the same sample are re-ashed following the same scheme, even if only one shows grey ash.
- The number of re-ashing cycles required is recorded on the sheet for each dish.
- A white ash with an occasional isolated grey speck is considered acceptable if it dissolves completely in 1.5% HNO_3.

9. Safety

- The furnace is loaded and unloaded with long tongs, heat-resistant gloves and goggles; the door must not be opened above ≈ 200 °C.
- The first phase of ashing gives off smoke, so the furnace must have extraction or be placed under a fume hood.
- Dishes containing unevaporated acid must not be placed in the furnace: nitric fumes corrode the chamber and heating elements.
- 65% HNO_3 is corrosive and oxidising; it is handled in a fume hood with nitrile gloves.
- Dishes are cooled in the desiccator on a heat-resistant surface.

10. References

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