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SAMPLE PRETREATMENT PROTOCOL

Microwave-assisted acid digestion of animal and plant samples

Área de Toxicología de la ULL

(Grupo Interuniversitario de Toxicología Ambiental y Seguridad de los Alimentos y Medicamentos)

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MAC

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1. Purpose

This protocol describes the mineralisation of samples of animal and plant origin by acid digestion in closed PTFE-TFM vessels, using **4 mL of 65% HNO_3 and 2 mL of 30% H_2O_2**, in a **microwave digester** with a stepwise programme up to **180 °C**. The digest is made up to **10 mL**.

- **Scope:** the procedure is common to all matrices, both of animal origin (muscle, viscera, molluscs, crustaceans, biological fluids) and of plant origin (algae, cereals, legumes, medicinal plants, supplements).
- **Analytes:** toxic metals (Cd, Pb, Hg, Al, Ni, Cr…), trace elements (Cu, Zn, Fe, Mn, Co, Mo, V, B, Ba, Li, Sr) and macroelements (Na, K, Ca, Mg).
- **Choice versus muffle furnace:** the method is preferable when determining **Hg** or other volatile elements, when the amount of sample is small (1 g) or when speed is required (≈ 1.5 h per batch of 12 vessels).

2. Principle

Microwave energy directly heats the acid mixture inside a closed vessel. The pressure generated raises the boiling point of HNO_3 and allows organic matter to be oxidised at 180 °C without volatilisation losses. HNO_3 breaks the C–H bonds, while H_2O_2 supplies additional oxygen, regenerates nitric acid and reduces residual carbon:

$$(CH_2)_n + 2n\ HNO_3 \rightarrow n\ CO_2\uparrow + 2n\ NO\uparrow + 2n\ H_2O$$

$$2\ NO + 3\ H_2O_2 \rightarrow 2\ HNO_3 + 2\ H_2O$$

Aspect	Microwave digestion (this protocol)	Muffle furnace ashing (complementary protocol)
System	Closed, wet (HNO_3 + H_2O_2)	Open, dry (450 °C)
Sample mass	1 g	15 g
Duration	≈ 1 h 30 min	≈ 72 h (drying + ashing)
Final volume	10 mL	25 mL
Hg and volatiles	Retained	Lost
Contamination risk	Low (closed vessel)	Higher (open crucible, prolonged exposure)

3. Equipment, materials and reagents

Equipment

Equipment	Function / requirement
Closed-vessel microwave digester	Temperature control of each vessel; automatic overpressure release (≈ 20 bar); up to 12 positions
Digestion vessels	50 mL PTFE-TFM, three-piece closure; one vessel per replicate
Analytical balance	Resolution 0.1 mg
Ultrapure water system	18.2 MΩ·cm

Materials

- 10 mL volumetric flasks (class A) and polypropylene bottles with airtight caps.
- Automatic pipettes (1–5 mL) with metal-free tips; plastic or titanium spatulas.

Reagents (trace-analysis grade)

- 65% nitric acid.
- 30% hydrogen peroxide.
- Ultrapure water (18.2 MΩ·cm).

4. Cleaning of materials and contamination control

PTFE vessels are washed with laboratory detergent and rinsed with ultrapure water before each use. For the determination of trace levels (Cd, Pb, Hg), cleaning is reinforced as follows:

1. The vessel is emptied and rinsed with tap water and then with ultrapure water.
2. It is washed with phosphate-free laboratory detergent and rinsed three times with ultrapure water.
3. A cleaning cycle is run in the digester itself with 5–6 mL of 65% HNO_3 (short programme at 150–180 °C) or, alternatively, a 10% HNO_3 bath for 24 h.
4. It is rinsed with ultrapure water, left to dry upside down in a clean area and stored closed.
5. Volumetric glassware and polypropylene bottles are kept for 24 h in a 10% HNO_3 bath and rinsed with ultrapure water.

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: **1 g of sample** is weighed directly into the digestion vessel, in triplicate (1 mL in the case of fluids). This amount allows complete oxidation without overpressure.

High-fat samples (oily fish, egg, cured meats): 1 g is also weighed, but the open pre-digestion is extended to 1 h, since fat generates high pressure and the relief valve could act before 180 °C is reached.

6. Digestion procedure

Each sample is digested in triplicate.

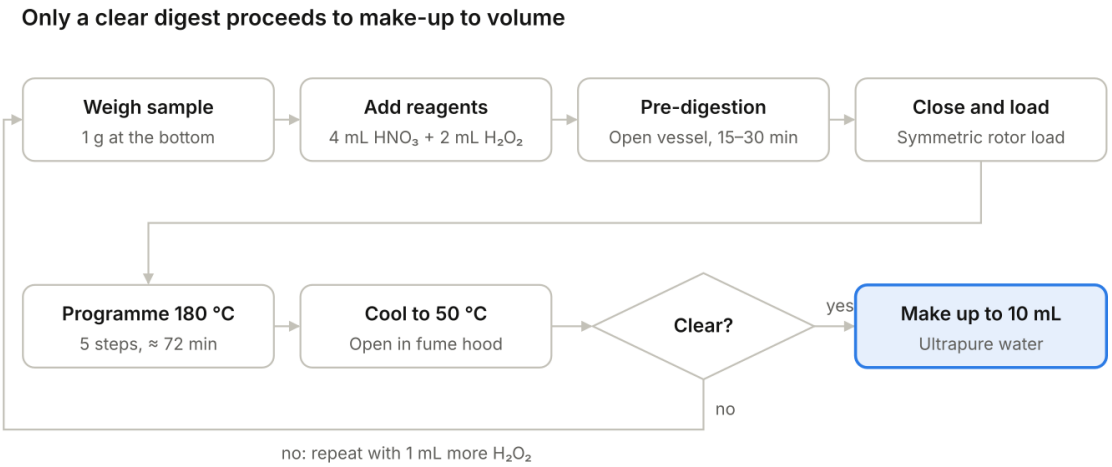

Figure 1. Digestion sequence and digest acceptance criterion.

1. **Start-up and checks.** The digester is switched on and it is checked that the IR sensor is clean and that vessels and lids show no cracks or deformation.
2. **Weighing.** The dry vessel is tared and the sample is weighed directly onto the bottom, recording the mass to 0.1 mg. Particles must not remain on the walls or the thread.
3. **Nitric acid. 4 mL of 65% HNO_3** are added, washing down any material adhering to the walls.
4. **Hydrogen peroxide. 2 mL of 30% H_2O_2** are added slowly, dropwise at first, since the reaction with organic matter is exothermic and produces foam.
5. **Open pre-digestion.** The vessel is left open for 15–30 min in a fume hood until foaming and brown fumes stop. For fatty or sugar-rich samples, this time is extended to 1 h.
6. **Closing.** The three-piece lid is assembled up to the closing point, without overtightening.
7. **Rotor loading.** The vessels are placed symmetrically in the rotor.
8. **Programme.** The programme described in section 7 is run and the door is not opened until the temperature has fallen to 50 °C.
9. **Opening.** In a fume hood, the lid is loosened gradually to release the nitrous gases.
10. **Inspection.** The digest must be liquid, clear (colourless or pale yellow) and free of dark particles and floating fat. Otherwise, the digestion is repeated with an additional 1 mL of H_2O_2. A fine white residue usually corresponds to silica, which does not retain the metals of interest and is removed by filtration (section 8).

7. Temperature programme

The programme is the same for all samples: it rises in five steps to 180 °C and holds this temperature for 10 min, with 72 min of heating and ≈ 1 h 26 min in total including cooling to 50 °C. The holds at 50, 60, 70 and 90 °C allow organic matter to react progressively and prevent pressure peaks.

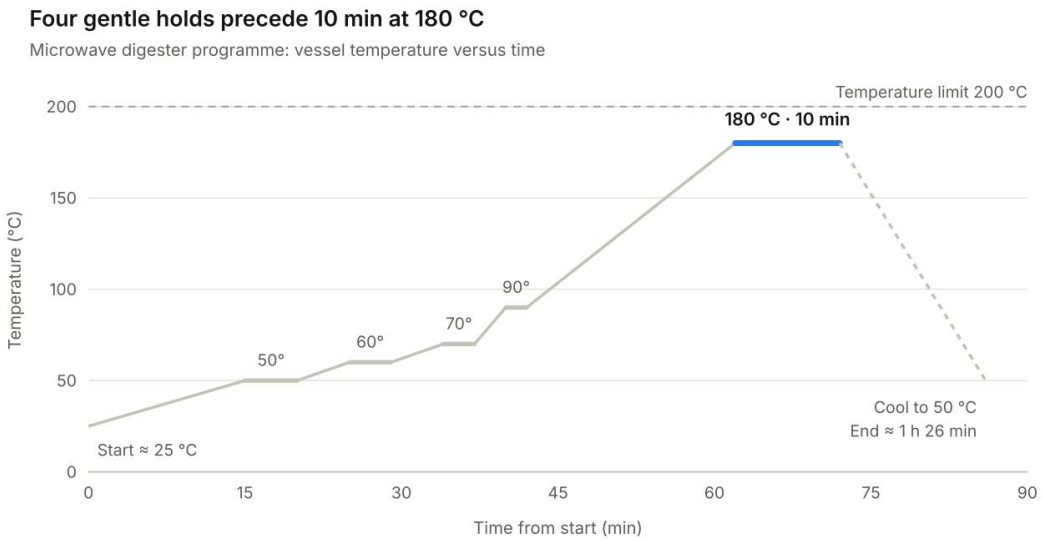

Figure 2. Digestion programme (starting temperature of 25 °C assumed).

Parameters entered in the instrument:

Step	Ramp (min)	Temperature (°C)	Hold (min)
1	15:00	50	5:00
2	5:00	60	4:00
3	5:00	70	3:00
4	3:00	90	2:00
5	20:00	180	10:00

Limit temperature: 200 °C. **Cooling temperature:** 50 °C.

Power: it need not be set; the digester regulates it automatically to follow the temperature ramp.

8. Post-digestion: make-up to volume and storage

The digest is made up to **10 mL with ultrapure water in a volumetric flask** immediately after digestion and stored in an airtight bottle until analysis.

1. The digest is transferred to the 10 mL flask using a small funnel.
2. The vessel and lid are rinsed 2–3 times with small volumes of ultrapure water (≈ 1 mL) and the rinses are added to the flask without exceeding the mark.
3. It is made up to 10 mL, stoppered and homogenised by inverting the flask ten times.
4. If insoluble residue (silica) remains, the solution is filtered through ashless paper or a 0.45 µm PTFE/PES syringe filter.
5. The solution is transferred to a properly labelled polypropylene bottle with an airtight cap.

Final acidity. Diluting 4 mL of 65% HNO_3 to 10 mL yields a solution containing ≈ 40% v/v concentrated HNO_3 (somewhat less after the reaction). This should be stated on the sample submission form, as it may be necessary to dilute the digest or prepare the standards in the same acid matrix.

9. Safety

- Work is carried out in a fume hood wearing a lab coat, goggles or face shield and nitrile gloves. 65% HNO_3 is corrosive and oxidising; 30% H_2O_2 is oxidising and causes skin burns.
- Peroxide is always added after the acid and slowly; no vessel is closed while it is still foaming.
- Organic solvents, alcohols or samples containing explosives or perchlorate are not digested in these vessels.
- Vessels are opened only below 50 °C and pointing towards the back of the fume hood, as toxic nitrous fumes (NO_2) are released.
- Before each batch, vessel lids and bodies are inspected and any with cracks, deep scratches or deformation are withdrawn.
- Acid waste is collected in the laboratory inorganic acid waste container.

10. References

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[2] Gómez-Rodríguez J, Rodríguez-Díaz R, Blanes-Zamora R, Paz-Montelongo S, et al. Influence of Follicular Fluid Metals on Female Sexual Hormones. *J Clin Med Sci* 2025; 9: 304.

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