European Mountain lake Ecosystems: Regionalisation, diaGnostics & socio-economic Evaluation

EMERGE

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PROTOCOLS FOR THE ANALYSIS OF ORGANIC MICROPOLLUTANTS IN FISH

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Protocol for organic micropollutants in fish

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1. ANALYSIS OF ORGANIC COMPOUNDS IN FISH TISSUES (MUSCLE, LIVER AND BILE) AND GUT CONTAIN

1.1 Compounds to be determined.

* Hexachlorobenzene
* Hexachlorocyclohexanes (namely a and g isomers)
* DDTs (namely, pp'-DDE and pp'-DDT)
* Polychlorobiphenyls (congeners Nos. 28+31, 52, 101+84, 118+149, 153, 138+163+160 and 180)
* Polycyclic aromatic hydrocarbons (PAH; parent compounds)
* Hydroxilated polycyclic aromatic hydrocarbons

1.2 Other measurements.

- Lipid and water content in muscle

1.3 Sample amount.

10 g. of muscle. 1 g or more of liver. The complete gall bladder

1.4 Materials and reagents.

-Self adhesive labels
-Pen
-Water resistant pen
-Big box to keep the samples when dissected
-Field fridge
-Glass bottles
-Recipient for cleaning and rinsing residues
-Latex gloves (only for cleaning the material in the soap, for safety reasons). Not to dissect the fishes.
-Plastic tray
-Vials and taps (sent by IIQAB-CSIC because they are previously cleaned for this purpose)
-Scalpel
-Metal tweezers
-Scissors
-Aluminum foil
-Distilled water
-Lake water
-Distilled water
-Acetone (for trace organic analysis, Merck)
-Soap (Extran MA01, ref. 7555.1000, alkaline, Merck)

1.5 Cleaning.

Correct cleaning of the material is a very important step in the analysis of organic compounds. Plasticizers are present in most recipients and material of common use (*e.g.*, gloves, bottles -distilled water is usually kept in plastic bottles-, ...). The hands and hair can also be a source of organic compounds in the sample. For this reason the cleaning procedure needs to be followed carefully during sampling and analysis.

1. Find a flat horizontal surface to work comfortably.
2. Put the gloves on.
3. Fill the plastic tray with EXTRAN solution (20 g per liter of distilled water).
4. Clean the glass bottle with this solution.
5. Rinse the glass bottle with lake water (3 times)
6. Fill the glass bottle with lake water.
7. Spread an aluminium sheet over the grass.
8. Put in the tray the material to be used and clean it.
9. Once cleaned, rinse it with the lake water stored in (6).

   IMPORTANT: Do not throw the first rinsing water nor the cleaning mixture in the catchment. Store it in a recipient and throw it when being back in the lab. EXTRAN products can be discarded in the inorganic salt recipient for residues of the lab.
10. Rinse again the material with distilled water and later with acetone, to ensure that no contamination of the sample would be obtained.
11. Put the material on the aluminium sheet spread in (7)
12. Cover the clean material with aluminium if not to be used immediately.

1.6 Dissection procedure.

1. Using the cleaned dissection material, the gall bladder should be placed into the 3ml vials, sent to each lab for this purpose. The liver and muscle fillet should be placed and wrapped in a new aluminium sheet.
2. The sample vial and package should be carefully labelled (code number of the fish and tissue) using self-adhesive labels.
   Then, it should be frozen at –20ºC immediately after dissection.

1.7 Transport.

The fish tissues, protocol form and the list of analyses performed to each fish should be placed in a box containing dry ice and sent it to Barcelona. Before mailing contact directly with the responsible persons in our lab.

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1.8 Analyses (extraction).

The tissues are freeze-dried in an oil-free freeze-drier and Soxhlet extracted with (4:1) *n*-hexane-dichloromethane for 18 hours. An aliquot (10%) of the muscle extract is used to measure total extractable lipid weight after evaporation to dryness. Water content is determined by weight difference prior and after freeze-drying.

1.9 Analyses (organochlorine compounds in muscle).

The extract is spiked with TBB and PCB209 in order to assess the analytical recovery. Then it is reduced to 2 ml of *n*-hexane and cleaned up with agitation with sulphuric acid. After vigorous stirring in a Vortex (2 min) the two layers are decanted by centrifugation for removal of the sulphuric acid. The *n*-hexane extract is neutralized by washing (three times) with Milli-Q water for pH neutralization. The *n*-hexane is then concentrated under vacuum to a small volume, *e.g.* 50 µl, for instrumental analysis.

1.10 Analyses (polycyclic aromatic compounds and organochlorine compounds in liver).

The extract is spiked with perdeuterated PAHs compounds in order to assess the analytical recovery. Then it is vacuum evaporated to 0.5 ml and fractionated by column chromatography using columns filled with 5% water deactivated alumina. Three fractions are collected and then evaporated (vacuum rotary evaporation and nitrogen stream) to dryness for analysis.

1.11 Analyses (hydroxilated polycyclic aromatic compounds in bile).

100 µl of bile is treated with 1 ml of 0.4 M acetic acid/sodium acetate buffer, at pH 5.0, containing 2000 units of β-glucuronidase and 50 units of sulfatase and incubated for 2 h at 40ºC. Hydrolyzed metabolites are extracted with 1 ml of ethylacetate (x3); the extracts are recombined and reduced to 100 µl under nitrogen.

1.12 Instrumental analysis of organochlorinated compounds.

A gas chromatograph Hewlett-Packard Model 5890 (Palo Alto, CA, USA) equipped with a ^{63}Ni electron capture detector and a split/splitless injector is used. This equipment is provided with a DB-5 column (5% phenyl-95% methylpolysiloxane, 25 m length, 0.25 mm i.d., 0.25 mm film thickness; J&W Scientific, Folsom, CA, USA). Helium is the carrier gas (30 cm/s). The samples (2 µl) are introduced with an automatic injector Hewlett-Packard Model 7673A in the splitless mode. Injection and detector temperatures are 270 and 310ºC, respectively. Oven temperature is programmed from 60 to 300ºC at 6ºC/min with a final holding time of 10 min. The make up gas is nitrogen (60 cm/s).

1.13 Instrumental analysis of polycyclic aromatic hydrocarbons.

A Carlo Erba GC8000 Series coupled to a mass spectrometer Fisons MD800 is used. This instrument is equipped with a 30 m HP-5 column coated with 5% phenyl methyl silicone. The oven temperature program is from 90 to 310ºC at 4ºC/min, held for 10 min. Injection and transfer line temperatures are 280 and 300ºC, respectively. Helium

is the carrier gas (50 cm/s). Data are acquired in the electron impact mode with an electron energy of 70 eV and the operation is in selected ion monitoring. The injection is in the splitless mode (1 µl injected; hot needle technique), the split valve being closed for 48 s.

1.14 Instrumental analysis of polycyclic aromatic hydrocarbons metabolites.

Quantitative analysis of hydroxilated polycyclic aromatic hydrocarbons is done by thermospray liquid chromatography-mass spectrometry (LC-MS) with a diode array detection as described elsewhere (Koeber, R. *et al*.)

1.15 Quantitation.

Authentic standards of hexachlorobenzene, a-, g- and d-hexachlorocyclohexane, op'-DDE, pp'DDE, op'-DDD, pp'-DDD, op'-DDT, pp'-DDT and the polychlorobiphenyl congeners Nos. 28, 52, 101, 118, 138, 153 and 180 are used. Calibration curves (detector response *vs* amount injected) are performed for each compound. The polycyclic aromatic hydrocarbons are analyzed by reference to standards of fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*k*]fluoranthene, pyrene, benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene and dibenzo[*ah*]anthracene. The range of linearity of the detector is evaluated from the curves generated by representation of detector signal/amount injected *vs* amount injected. All measurements are performed in the ranges of linearity found for each compound. In some cases, re-dilution and re-injection are performed to fit within the linear requirements.

1.16 Compound identification.

Structural identification is confirmed by analysis of selected samples by gas chromatography coupled to mass spectrometry in the chemical ionization mode and record of the negative ions. The chromatographic conditions are the same as described above. A DB-5 column was used. Transfer line and ion source temperatures are 300 and 120ºC, respectively. The reagent gas is methane. Data are acquired by scanning from 50 to 500 mass units at 1 s per decade. The samples selected for GC-MS analysis must allow to elucidate the composition of all the major peaks present in the gas chromatograms obtained with the ECD.

1.17 References:

- **Koeber, R.; Niessner, R. and Bayona, J.M.** 1997. *Fresenius Journal of Analytical Chemistry* 359: 267-273.