Marine
Environment
Laboratories

Mediterranean Action Plan
Barcelona Convention

United Nations
Environment Programme

D. IMAP MONITORING GUIDELINES FOR CI20

D-1. Monitoring Guidelines/Protocols for sampling and determination of contaminants in seafood

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1 Guidelines for seafood sampling and sample preservation

1.1 Introduction

Monitoring seafood (fish, bivalves, cephalopods and crustaceans) for compliance with levels of contaminants set for public health protection is very different from monitoring marine organisms for environmental purposes. Ongoing seafood monitoring programmes for public health reasons generally focus on estimating consumer exposure rather than assessing environmental status. Therefore, in the framework of UNEP/MAP Integrated Monitoring and Assessment Programme Common Indicator 20 (CI20), as well as for the Descriptor 9 of the Marine Strategy Framework Directive (MSFD), sampling plans and procedures, selected tissues analysis and traceability to the location of catching or harvesting of seafood should be redesigned in order to provide required information (JRC, 2010[1]).

Sampling of seafood includes collection of organisms from fishing vessels but also from fish landing harbors and fish markets. This is an important difference from CI17 and CI18 sampling, where sampling site's geographical coordinates are precisely known and recorded. The sampling location of seafood on land is not enough to trace the organism's provenance and it is therefore of paramount importance to also record the original location of the collection of the organisms at sea. The list of seafood to be collected for CI20 extends beyond the sentinel organisms used for CI17 and CI18 (*Mullus barbatus* and *Mytilus galloprovincialis* or *Donax trunculus*) (Annex XXXV). Therefore, each Contracting Party to the Barcelona Convention needs to select the organisms to be sampled and analyzed, based on the commercial importance of species in each country. However, in order to attempt providing a comparison between CI20 and CIs 17 and 18, it is advisable to include the above-mentioned sentinel species in the monitoring programme for CI20.

Sample preservation during transportation to the laboratory for further analysis follows the same procedures described in the relevant Protocols of CI17, with the addition of guidelines for the preservation during transport of cephalopods and crustaceans. In all procedures the important element is to ensure the integrity of the initial sample, avoiding decay and cross contamination during transport. Ice preservation is suggested for a transport of less than 24 hours, while refrigeration (-20 ºC) is the method to follow in case of a transportation period more than 24 hours. Also, sample container's materials should be adequate in order to avoid cross contamination of the samples by metals or organic contaminants.

The aim of monitoring for CI20 is the protection of consumers' health, therefore during dissection, only edible tissues of seafood are to be selected for analysis (the flesh of fish, the whole body of bivalves, the mantle and tentacles of cephalopods and the tail meat without the cehalothorax of crustaceans). Dissection should be carried out by trained personnel in clean conditions and using appropriate tools in order to avoid cross contamination during the process.

The Protocols prepared in the framework of Monitoring Guidance for Sampling and Sample Preservation of Sea Food for IMAP Common Indicator 20, as provided here-below, describe appropriate methodologies for sampling, processing and storage of seafood samples under controlled conditions to ensure the representativeness and the integrity of the biota samples. They are not intended to be analytical training manuals, but guidelines for Mediterranean laboratories, which should be tested and modified in order to validate their final results.

These Protocols aim at streamlining sampling, dissecting and processing of marine organisms in view of assuring comparable quality assurance of the data, as well as comparability between sampling areas and different national monitoring programmes. They provide a step-by-step guidance on the methods to be applied in the Mediterranean area for sampling, sample handling to avoid cross-contamination, as well as the storage conditions in a view of maintaining the sample's integrity during the transfer from the sampling site to the analytical laboratory to ensure the representativeness and the integrity of the samples for analysis. Furthermore, protocols provide guidance on the procedures to dissect the organisms (fish, bivalves, crustaceans and cephalopods) in order to collect the appropriate tissue for analysis, taking care to avoid cross-contamination by metals or organic contaminants, depending on the foreseen analysis.

In order to avoid unnecessary repetitions, reference is also made to the protocols already published and publicly accessible, which can also be used by the Contracting Parties' competent laboratories participating in IMAP implementation. Namely, the here-below elaborated IMAP Protocols build upon previous UNEP/MAP - IAEA Recommended Methods, such as Reference Methods No 6 on sampling of selected marine organisms and sample preparation for trace metal analysis (UNEP/FAO/IOC/IAEA, (1987[2]) (Annex XIX) and Reference Methods No 7 (Rev. 2) on sampling and dissecting marine organisms (UNEP/FAO/IOC/IAEA, (1988[3]) (Annex XX), which were prepared in the framework of the MED POL monitoring programme. They are also streamlined with similar Guidelines for marine biota sampling, sample

[1] JRC (2010). Marine Strategy Framework Directive. Technical Report of Task Group 9: Contaminants in fish and other seafood

[2] UNEP/FAO/IOC/IAEA (1987). Reference methods No 6 (Rev. 1): Guidelines for monitoring chemical contaminants in marine organisms.

[3] UNEP/FAO/IOC/IAEA (1988). Reference methods No 7 (Rev. 2): Sampling of selected marine organisms and sample preparation for trace metal analysis

processing and preservation, which were developed by other Regional Seas Organizations, as follows: HELCOM (2012, Annex XXI: Technical note on biological material sampling and sample handling for the analysis of persistent organic pollutants and metallic trace elements); EC relevant Regulations on marine biota sampling and sample preparation for seafood analysis (Annex XXXVI: EU Commission Regulation (EC) No 1881/2006[4]; Annex XXXVIII: EU Commission Regulation (EC) No 333/2007[5]; Annex XXXIX: EU Commission Regulation (EC) No 836/2011 amending (EC) No 333/2007[6] laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs; Annex XXXX: EU Commission Regulation (EC) No 644/2017[7]). Given the suitability of any of these Guidelines in the context of IMAP, they could be further used by interested IMAP competent Mediterranean laboratories for developing their laboratory specific sampling and sample processing methodologies. The Contracting Parties' laboratories should accommodate and always test and modify each step of the procedures to validate their results.

1.2 Technical note for the sampling of seafood for the determination of heavy metals and organic contaminants

Monitoring seafood (fish, bivalves, cephalopods and crustaceans) for compliance with levels of contaminants set for public health protection is very different from monitoring of marine biota for assessing the quality of the marine environment. Also, ongoing seafood monitoring programmes for the purpose of public health protection generally focus on estimating consumer exposure rather than assessing environmental status. Therefore, in the framework of UNEP/MAP IMAP CI20, as well as for the Descriptor 9 of the MSFD, sampling plans and procedures, selected tissues analysis and traceability to the location of catching or harvesting of seafood should be redesigned in order to provide required information (JRC, 2010).

Under this Technical note on sampling of seafood for the determination of heavy metals and organic contaminants, this Guidelines provides the Protocol for the collection of fish, crustaceans, cephalopods and bivalves for the determination of heavy metal and organic contaminants.

1.2.1 Protocol for the collection of fish, crustaceans, cephalopods and bivalves for the determination of heavy metal and organic contaminants

According to IMAP (UNEP, 2019a[8], UNEP, 2019b[9]) it is proposed "to collect marine organisms mainly commercial species, and similarly to CI17 (where the whole soft tissues or dissected parts are processed to perform analytical measurements of chemical contaminants)". It also underlined that "The sample collection for CI20 could be easily integrated with CI17 in terms of sample monitoring (e.g. from dedicated fishing vessels or from artisanal fleets at port). To be noticed, that in any case, the origin (i.e. area) of the fish captures should be exactly known, including detailed field information (e.g. coordinates)" (UNEP 2019b). Therefore, the FAO fishing area code (origFishAreaCode) should be noted and included in the Reporting Template for CI20.

The sample species for such analysis depends on the commercial marine organisms that are captured in the different Mediterranean areas (locations). Therefore, it is not relevant to propose a specific list of species but rather each Contracting Party will have to define its own list, which may be different from one sub-region to another. A tentative list of commercial species in the Mediterranean basin was prepared by JRC (2010) and is presented in Annex XXXV. Also, a list of available reference species (Code list) for Data Dictionaries and Data Standards related to E09 (CI17 and CI20) within the IMAP (Pilot) Info System is provided in UNEP/MED WG.467/8 (UNEP, 2019b[10]). In order to make monitoring results more comparable between Mediterranean (sub) regions, the Contracting Parties could select a relatively limited number of common target species from the most consumed species of fish and other seafood in the Mediterranean basin, to be monitored during the initial implementation phase of the IMAP programme. It is therefore reasonable to include species that are sampled for biomarkers and general contaminants (such as *Mullus barbatus* and bivalves – *Mytilus galloprovincialis)* in biota analysis as additional information will exist.

Ongoing monitoring programmes aiming at the protection of human health, often rely on retail sampling, at the market. However, in order to use these results for the purpose ofCI20 monitoring, the recording of the exact location of seafood harvesting is of

[4] EU Commission Regulation (EC) No 1881/2006, setting maximum levels for certain contaminants in seafood (Annex II)

[5] EU Commission Regulation (EC) No 333/2007, laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs

[6] EU Commission Regulation (EC) No 836/2011 amending (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs

[7] EU Commission Regulation (EC) No 644/2017), laying down methods of sampling and analysis for the official control of levels of dioxins and dioxin-like PCBs in certain foodstuffs

[8] UNEP/MAP (2019a). UNEP/MED WG.467/5. IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21: New proposal for candidate indicators 26 and 27.

[9] UNEP/MAP (2019b). UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution;

[10] UNEP/MAP (2019c) UNEP/MED WG.467/8. Data Standards and Data Dictionaries for Common Indicators related to pollution and marine litter.

paramount importance. JRC (2010) underlines that "Traceability in the food chain is focused on risk management: unless specific provisions for further traceability exist, the requirement for traceability is limited to ensuring that food business operators are at least able to identify the immediate supplier of the product in question and the immediate subsequent recipient, with the exemption of retailers to final consumers ("one step back – one step forward")." The aim of traceability is to make sure that a direct link is established between the fresh seafood and the specific regions of its capture, in as much detail as possible.

Furthermore, since seafood samples for the protection of human health are often collected at the market, it must be ensured that measured contaminants concentrations in seafood are directly related to the existing environmental conditions at the capture location and that they are not cross-contaminated during treatment, transport and storage. A close cooperation between the samples' providers at the market and the authorities responsible for sampling seafood should be established in order to minimize such cross-contamination.

For contaminants for which regulatory levels have been set provisions regarding sampling procedures are presented in EU Commission Regulations: (EC) No 1881/2006 related to setting maximum levels of contaminants in foodstuffs, (Annex XXXVI), which was amended by (EC) No 835/2011[11] in relation to PAHs maximum levels (Annex XXXVII); (EC) No 333/2007 related to sampling and analysis for lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs, (Annex XXXVIII), which was amended by (EC) No 386/2011 in relation to PAHs methods for sampling and analysis (Annex XXXIX); (EC) No 644/2017 related to sampling and analysis for dioxins and dioxin-like PCBs in foodstuffs; (Annex XXXX). These Regulations, which include sampling plans, sample preparation and analysis may be used as a guidance for seafood sampling and analysis of relevance for UNEP/MAP IMAP mandatory list of contaminants (UNEP/MAP, 2019a), for which regulatory concentrations in seafood have been set by EC. From the list of EC regulated contaminants (EC) No 1881/2006) and (EC) 835/2011, Cd, Hg, Pb, PAHs (Benzo(a)Pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene) and non dioxine-like PCBs are also designated as mandatory contaminants for CI20 monitoring (IMAP Guidance Fact Sheets, UNEP, 2019). Dioxins and dioxin-like PCBs, which are included in the list of EC regulated contaminants for seafood monitoring, are not yet included in the list of IMAP mandatory contaminants for CI20, however the Contracting Parties are encouraged to include all EU regulated contaminants in their monitoring programme for CI20, if possible.

The number of individual organisms to be samples depends on the weight of the "lot" and "sublot". According to the definitions of the Commission Regulations (EC) No 333/2007:

i) "lot" is an identifiable quantity of food delivered at one time and determined by the official to have common characteristics, (such as origin, variety, and in the case of fish (or other biota), also a comparable size;

ii) "sublot" is the designated part of a large lot in order to apply the sampling method on that designated part;

iii) "incremental sample" is a quantity of material taken from a single place in the lot or sublot;

iv) "aggregate sample" is the combined total of all the incremental samples taken from the lot or sublot; aggregate samples shall be considered as representative of the lots or sublots from which they are taken; sublot must be physically separated and identifiable.

Using these definitions, Commission Regulations (EC) No 333/2007 and (EC) 836/2011 suggest the following sampling plans for individual marine organisms (fish, molluscs, cephalopods and crustaceans):

Table 1. Number of packages or units (incremental samples) which shall be taken to form the aggregate sample if the lot or sublot consists of individual packages or units

Number of packages or units in the lot/ sublot	Number of packages or units to be taken
≤ 25	at least 1 package or unit
26-100	about 5 %, at least 2 packages or units
> 100	about 5 %, at maximum 10 packages or units

The aggregate sample shall be at least 1 kg except where it is not possible e.g. when the sample consists of 1 package or unit."

In relation to packaging and transport of samples, Regulation (EC) No 333/2007 underlines that "each sample shall be placed in a clean, inert container offering adequate protection from contamination, from loss of analytes by adsorption to the internal wall of the container and against damage in transit. All necessary precautions shall be taken to avoid any change in composition of the sample which might arise during transportation or storage." Also, "Each sample taken for official use shall be sealed at the place of sampling and identified following the rules of the Member States. A record shall be kept of each sampling, permitting each

[11] EU Commission Regulation (EC) No 835/2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs.

lot or sublot to be identified unambiguously (reference to the lot number shall be given) and giving the date and place of sampling together with any additional information likely to be of assistance to the analyst."

During marine organisms sampling it is important to take into consideration (and record) all biological factors that can influence concentrations of contaminants in fish and other seafood, such as seasonal variation, age, sex. Since the aim of the monitoring is the protection of human health, only the edible portion of the organisms will be analyzed.

Seafood samples should be protected from contamination, which may occur during sampling, sample handling, storage and transfer to the laboratory for further analysis. Seafood samples have to be handled with care to avoid any contact with metals (for heavy metal analysis) or possible sources of organic contaminants (for chlorinated hydrocarbons and PAHs analysis). When seafood transport to the laboratory is done in less than 24 hours, samples can be stored on ice. In case of a transfer longer than 24 hours, samples have to be frozen in -20 °C and transported frozen to the laboratory for further processing and analysis. Each sample should be labelled with the sample's identification number, the type of tissue, and the date and location of sampling.

Guidelines for marine organism collection, preservation and transportation to the laboratory are developed by UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX, HELCOM (2012[12]) (Annex XXI) and US EPA (2000[13]) (Annex XXXXIII).

1.3 Technical Note for the dissection of seafood for the determination of heavy metals and organic contaminants

To collect the edible tissues of seafood for subsequent analysis, the organisms have to be dissected, taking care to avoid any contamination form the dissecting tools and the working environment. Also, dissection has to be undertaken by trained personnel to ensure the removal of the representative undamaged tissues.

For metal determination, the dissection of marine organisms should be made on a metal-free bench, using plastic knives and tweezers for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned with a tissue and rinsed with clean water.

For organic contaminants determination, the dissection of marine organisms should be made on a metallic (stainless steel or aluminum) bench, using stainless steel knives and tweezers for holding tissues during dissection. After each sample has been prepared, all tools and equipment (such as homogenizers) should be cleaned with tissue and rinsed with solvent

After the removal of a tissue sample from the organism, the tools have to be cleaned before being used to remove another organ of the same individual or being used on a different individual

For determination of heavy metals, tools should be:

i) Washed in acetone or alcohol and high purity water.

ii) Washed in HNO_3 diluted (1+1) with high purity water. Tweezers and haemostates in are washed in diluted (1+6) acid.

iii) Rinsed with high purity water.

For determination of organic contaminants, tools should be:

i) Washed in acetone or alcohol and rinsed in high purity water.

The glass/metal/plastic plate used during dissection should be cleaned in the same manner. The tools must be stored dust-free when not in use. Also, the dissection room should be kept clean, and the air should be free from particles. If clean benches are not available on board the ship, the dissection of fish should be carried out in the land-based laboratory under conditions of maximum protection against contamination (HELCOM, 2012).

Under this Technical note on sampling of seafood for the determination of heavy metals and organic contaminants, this Guidelines provides the following four Protocols:

- Protocol for dissection of fish to collect the edible part for analysis;
- Protocol for dissection of bivalves to collect the edible part for analysis;
- Protocol for dissection of crustaceans to collect the edible part for analysis;
- Protocol for dissection of cephalopods to collect the edible part for analysis.

1.3.1 Protocol for dissection of fish to collect the edible part for analysis

Recording biological factors of fish

Guidelines for recording length, weight and sex of fish are presented in UNEP/FAO/IOC/IAEA (1987), UNEP/FAO/IOC/IAEA (1988), and US EPA (2000).

Dissection of fish

Muscle tissues of fish have to be dissected while they are in good condition, otherwise the decay of the tissues will affect the concentration of contaminants. Therefore,

[12] HELCOM (2012). Manual for marine monitoring in the COMBINE programme. Annex B-12, Appendix 1. Technical note on biological material sampling and sample handling for the analysis of persistent organic pollutants (PAHS, PCBS and OCPS) and metallic trace elements

[13] US EPA (2000). Guidance for Assessing Chemical Contaminant Data for Use in Fish Advisories Volume 1 Fish Sampling and Analysis. Third Edition

it is preferable to dissect collected fish the soonest possible, by experienced personnel able to perform the dissection and remove the muscle tissue to be analyzed. Dissection should be done in a clean area free from possible contamination of the sample by metals (for heavy metal analysis) or organic contaminants (for PCBs and PAHs analysis).

According to IMAP requirements, UNEP (2019), the fish tissue to be collected is muscle. Detailed guidelines for the dissection of fish and collection of samples for further analysis is presented in UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX), HELCOM (2012) (Annex XXI) and US EPA (2000) (Annex XXXXIII).

In all procedures, the method requires the removal of the epidermis and the collection of a sample from the dorso-lateral muscle in order to ensure uniformity of samples (Figure 1). It is also suggested to take the entire right dorsal lateral filet as a uniform sample, from which subsamples can be taken after homogenizing for replicate dry weight and contaminant determinations. If the amount of material obtained by this procedure is too large to be easily handled, a specific portion of the dorsal musculature should be chosen for the sample. It is recommended that the portion of the muscle lying directly under the first dorsal fin should be utilized in this case. It is important to obtain the same portion of the muscle tissue for each sample, because both fat and water content vary significantly in the muscle tissue from the anterior to the caudal muscle of the fish (HELCOM, 2012).

In case fish samples are frozen for their transfer from the field to the laboratory, they have to rest until thawed. It is often suggested that the dissection of fish is easiest when the material, at least the surface layers of the muscle tissue, is half frozen. However, for the dissection of other organs, the thawing must proceed further. Extreme care has to be demonstrated during dissection because any loss of liquid or fat due to improper cutting or handling of the tissue makes the determinations of dry weight and fat content less accurate, which is also affecting the accuracy of the reported contaminants' concentrations.

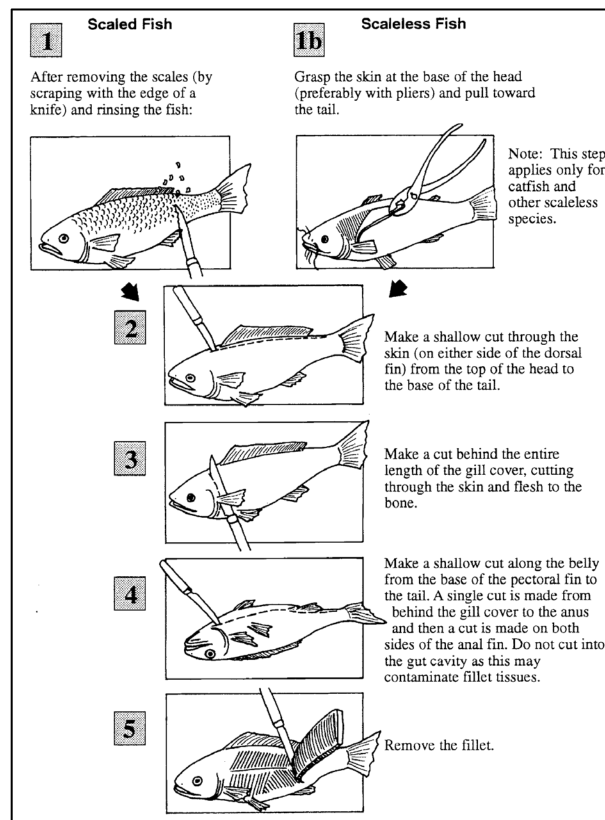

Figure 1. Fish filleting procedure (from US EPA, 2000)

1.3.2 Protocol for dissection of bivalves to collect the edible part for analysis (whole body)

Depuration

Collected bivalves that are alive should be left to void the gut contents and any associated contaminants before dissecting and sample preparation, because gut contents may contain significant quantities of contaminants associated with food and sediment particles which are not truly assimilated into the tissues of the mussels (HELCOM, 2012). Bivalve's depuration over a period of 24 hours is usually sufficient and should be undertaken under controlled conditions and in filtered sea water in the laboratory. The aquarium should be aerated and the temperature and salinity of the water should be similar to that from which the animals were removed.

Recording biological factors of bivalves

Guidelines for recording length and weight of bivalves are presented in UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX), and US EPA (2000) (Annex XXXXIII).

Bivalves' dissection

The whole soft tissue of bivalves is edible therefore, it has to be collected for analysis. Detailed guidelines for the dissection of bivalves and collection of samples for further analysis is presented in UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX), HELCOM (2012) (Annex XXI) and US EPA (2000) (Annex XXXXIII).

In general, foreign materials attached to the outer surface of the shell have to be removed using a clean plastic/stainless steel knife with a strong plastic/metal brush. Handle the mussels as little as possible. For removing the soft tissue for analysis, bivalves should be shucked live and opened with minimal tissue damage by detaching the adductor muscles from the interior of at least one valve (Figure 2). The soft tissues should be removed and homogenized as soon as possible, frozen and kept in plastic containers (for metal analysis) or in metal containers (for organic contaminants' analysis) at –20°C until analysis. Homogenization can be done using stainless steel blades (for organic contaminants analysis) or using an agate mortar, following the drying of the sample.

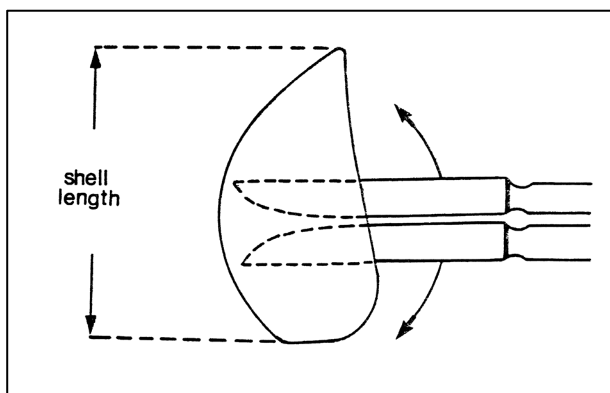

Figure 2. Cutting the abductor muscle

1.3.3 Protocol for dissection of crustaceans to collect the edible part for analysis

Recording biological factors of crustaceans

Guidelines for recording length, weight and sex of crustaceans are presented in UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX), and US EPA (2000) (Annex XXXXIII).

The length of the shrimp is measured from rostrum to uropod (Figure 3) using an appropriate length-measuring device. Weigh the shrimp after placing a clean weighing container (plastic or aluminum foil depending on the analysis to be made) on the balance and note its length and fresh weight.

Crustaceans' dissection

To collect the edible part of shrimps and crayfish the cephalothorax is removed and the tail meat with the section of intestine passing through the tail muscle is retained for analysis (Figure 3). The vein is then removed using a sharp knife. The edible tissue of lobsters typically includes the tail and claw meat. Guidelines for dissection of crustaceans are prepared by UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX), and US EPA (2000) (Annex XXXXIII).

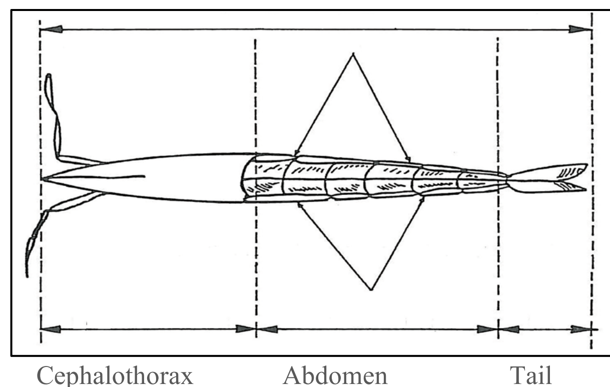

Figure 3. Shrimp

1.3.4 Protocol for dissection of cephalopods to collect the edible part for analysis (mantle and head)

Recording biological factors of cephalopods

For octopus and squid, total length is measured from end of longest arm to posterior end of mantle. Mantle length is measured from midpoint between eyes to the posterior end of mantle.

Dissection of cephalopods

The digestive gland and the internal organs (gills, ink sack, branchial hearts and their appendages, systemic heart and brain) of each cephalopod are totally removed using appropriate tools to avoid contamination. The edible parts of the cephalopod (mantle, head with tentacles) (Figure 4) are stored in clean containers for further analysis (Bustamante et al, 1998[14]).

[14] Bustamante P, Caurant F, Fowler SW, Miramand P. Cephalopods as a vector for the transfer of cadmium to top marine predators in the north-east Atlantic Ocean. Sci. Total Environ. 1998; 220: 71–80.

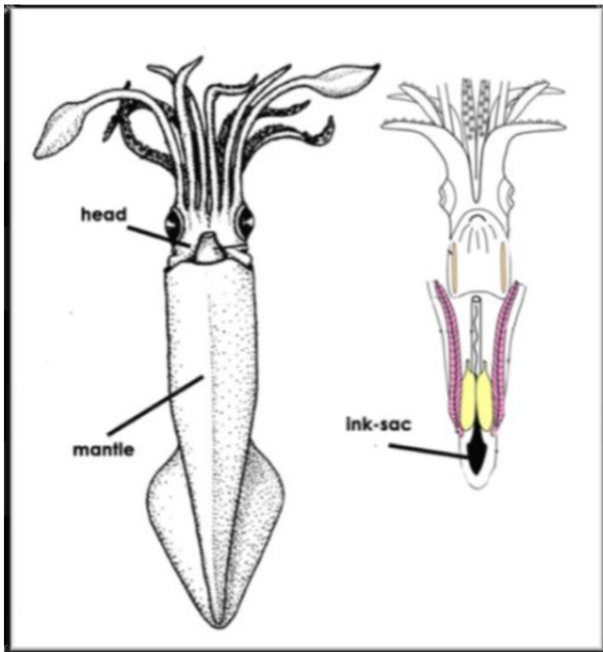

Figure 4. Squid

1.4 Technical note for the sample preservation of seafood for the determination of heavy metals and organic contaminants

Under this Technical note on processing and preservation of marine biota for the determination of heavy metals and organic contaminants, this Guidelines provides the following two Protocols:

- Protocol for the treatment of seafood samples prior to heavy metal determination.
- Protocol for the treatment of biota samples prior to organic contaminants determination.

The Protocols under this Technical Note are similar to the relevant Protocols related to sampling and sample preservation of marine biota samples presented in the framework of CI17 Guideline for biota sampling and samples preservation, for the the determination of of heavy metals and organic contaminants.

1.4.1 Protocol for the treatment of seafood samples prior to heavy metal determination

For contaminants for which regulatory levels have been set, certain provisions regarding sampling procedures and sample preservation are presented in Commission Regulations (EC) No 333/2007 of the European Union (Annex XXXVIII). These methods could be used when determining levels of contaminants in fish and seafood for human consumption in view of monitoring Good Environmental Status of the marine environment. Guidelines for treatment of marine biota samples prior to analysis are proposed by UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX) and HELCOM (2012) (Annex XXI).

a) Storage of wet samples on board/market

Upon collection wet samples have to be stored in such a way as to preserve them from deterioration that will affect the subsequent determination of contaminants. When the fish transport to the laboratory is done in less 24 hours, samples can be stored on ice. However, for longer periods, fish samples have to be frozen (-20 ºC) and transported frozen to the laboratory for further processing. Each sample should be labelled with the sample's identification number, the type of tissue, and the date and location of sampling.

b) Drying of biota tissues

Drying biota tissues is a procedure to establish the wet/dry ratio of the tissues, in order to express metal concentrations accordingly enabling comparisons between different data sets. Dried biota tissues can then be digested for heavy metal analysis, although biota tissues can also be digested wet, without prior drying (HELCOM, 2012).

For metal (except volatile mercury) determination, sediments freeze-drying is the preferable procedure. Alternatively, the biota tissues may be dried at any temperature below 105°C until constant weight. For mercury determination, to minimise losses due to evaporation, a sediment sub sample could be air dried at temperature <50°C (EC, 2010). Frozen biota samples are placed in clean wide-mouth glass or plastic containers suitable for freeze-drying and are freeze-dried for 24 hours taking care to protect them from cross-contamination from particles and vapours. A possible way to protect samples from contamination is to cover the sample containers with a filter paper perforated with a small hole (HELCOM, 2012). Then the containers with the samples are weighted and freeze-dried again for another 24 hours and weighted. If the difference between the 2 weighing is less than 0.5%, drying is completed and the dw/ww ratio can be calculated. Otherwise, the drying cycle can be repeated (24 hours) until the difference between successive weighing is less than 0.5%.

Freeze dried biota tissues are then grinded and homogenized using a metal-free ball mill. Guidelines for processing biota samples for metal determination are provided by ICES/OSPAR (2018a[15]) and HELCOM (2012) (Annex XXI).

c) Storage of dried biota tissues

Freeze-dried tissue samples can be stored in pre-cleaned wide-mouth bottles with a screw cap. Samples intended for the determination of metals can be stored in plastic or glass containers. For mercury determination, samples must be stored in acid-washed borosilicate glass or

[15] ICES/OSPAR (2018a). CEMP Guidelines for Monitoring Contaminants in Biota. Technical Annexes: 1) organic contaminants; 2) metals; 3) parent and alkylated PAHs; 8) chlorobiphenyls

quartz containers, as mercury can move through the walls of plastic containers (EC, 2010[16]).

Containers with biota tissue samples should be archived and kept in storage after the completion of the analysis, in order to be used as a replicate sample in case crosschecking of the results is required or additional determinations are needed in the future. Freeze-dried biota tissues remaining after analyses could be stored in the original sample bottle, closed with an airtight lid to protect against moisture and stored in a cool, dark place. Under these conditions, samples may be archived and stored for 10-15 years. (EC, 2010).

1.4.2 Protocol for the treatment of seafood samples prior to organic contaminants determination

For organic contaminants for which regulatory levels have been set, certain provisions regarding sampling procedures and sample preservation are presented in Commission Regulations (EC) No 333/2007 (PAHs) and (EC) No 644/2017 (PCBs and dioxins) of the European Union (Annex XXXIII and Annex XXXX). These methods could be used when determining levels of contaminants in fish and seafood for human consumption in view of monitoring Good Environmental Status of the marine environment. Guidelines for treatment of marine biota samples prior to contaminants determination are proposed by UNEP/FAO/IOC/IAEA (1987) (Annex XIX), UNEP/FAO/IOC/IAEA (1988) (Annex XX) and HELCOM (2012) (Annex XXI)

a) Storage of wet samples on board/market

Upon collection wet samples have to be stored in such a way as to preserve them from deterioration that will affect the subsequent determination of contaminants. When the fish transport to the laboratory is done in less 24 hours, samples can be stored on ice. However, for longer periods, fish samples have to be frozen (-20 ºC) and transported frozen to the laboratory for further processing. Each sample should be labelled with the sample's identification number, the type of tissue, and the date and location of sampling.

b) Drying of biota tissues

For organic contaminants determination drying procedures depends on the compounds to be analysed. For chlorinated hydrocarbons sediments can be freeze-dried taking care to avoid determinant loss through evaporation by keeping the temperature in the evaporation chamber below 0°C (ICES/OSPAR, 2018). For PAH determination, freeze-drying sediment samples may be a source of contamination due to the back-streaming of oil vapours from the rotary vacuum pumps. Furthermore, drying the samples may result in losses of the lower molecular weight, more volatile PAHs through evaporation. To protect biota samples from cross-contamination from particles and vapours during freeze drying, the sample containers could be covered with a lid or filter paper perforated with a small hole (HELCOM, 2012).

c) Storage of dried biota tissues

Freeze-dried tissue samples can be stored in pre-cleaned wide-mouth bottles with a screw cap. Samples intended for the determination of organic contaminants should be stored in glass containers.

Containers with biota tissue samples should be archived and kept in storage after the completion of the analysis, in order to be used as a replicate sample in case crosschecking of the results are required or additional determinations are needed in the future. Freeze-dried biota tissues remaining after analyses could be stored in the original sample bottle, closed with an airtight lid to protect against moisture and stored in a cool, dark place. Under these conditions, samples may be archived and stored for 10-15 years (EC, 2010).

2 Guidelines for the determination of contaminants in Seafood

2.1 Introduction

Maximum permissible levels for certain contaminants in foodstuffs (including seafood) have been set by FAO/WHO (Codex Alimentarius[17]) and European Commission Regulations (EU Commission Regulations (EC) No 1881/2006 (Annex XXXVI), (EC) No 835/2011 (Annex XXXVII) and EC No 1259/2011[18] (Annex XXXXIV). According to these regulations, maximum permissible concentrations in seafood are set for Cadmium (Cd), Lead (Pb), Mercury (Hg), four PAHs (benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene), dioxins (including furans), dioxin-like PCBs and non-dioxin-lile PCBs.

According to IMAP Guidance Fact Sheets (UNEP/MAP 2019a) the list of contaminants recommended for monitoring under IMAP Common Indicator 20 (CI20) includes Cd, Pb, Hg, the four PAHs and non-dioxin-lile PCBs, while dioxins and dioxin-like PCBs are not yet included in the IMAP list of mandatory contaminants.

Regarding heavy metals, the regulated metals for seafood monitoring in the framework of CI20 (Cd, Pb and Hg) are the same as the mandatory metals for marine biota monitoring in the framework of CI17. Therefore, the analytical methods for the determination of metals in seafood tissues (fish muscle, bivalves' whole body, crustaceans flesh and cephalopods flesh) are identical

[16] EC (2010). Guidance Document No: 25 Guidance on chemical monitoring of sediment and biota under the Water Framework Directive

[17] Codex Alimentarius (FAO/WHO) : http://www.fao.org/fao-who-codexalimentarius

[18] EU Commission Regulation (EC) No 1259/2011, amending Regulation (EC) No 1881/2006 as regards maximum levels for dioxins, dioxin-like PCBs and non-dioxin-like PCBs in foodstuffs

with the relevant analytical Protocols presented in the CI17.

Regarding regulated organic contaminants, PAHs (Benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene), and non-dioxin-like PCBs (PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180) are included in the lists of both regulated contaminants for CI20 and mandatory contaminant for CI17 (biota). Therefore, the analytical methods for their determination in seafood are identical with the relevant analytical Protocols presented in the CI17.

The other EC regulated contaminants (dioxins and dioxin-like PCBs) are not included in the CI17 mandatory contaminants and they require specialized accredited laboratories with appropriate analytical equipment (such as GC-HRMS). Methods for the determination of dioxins and dioxin-like PCBs are presented here-below in the Protocol for the determination of dioxins and dioxin-like PCBs using GC-HRMS. The Contracting Parties to the Barcelona Convention may decide to include the determination of additional, non-regulated heavy metals and organic contaminants in their national monitoring programmes for CI20, although no maximum permissible levels for consumption have been defined yet. Due to the lack of relevant maximum permissible values for the non-regulated contaminants, no adequate Reporting can be provided for these additional contaminants.

The Protocols prepared in the framework of Monitoring Guidance for the Determination of Contaminants in Seafood for IMAP Common Indicator 20, as provided here-below, describe appropriate methodologies for the determination of heavy metals and organic contaminants in seafood samples, in order to ensure quality assured data. They are not intended to be analytical training manuals, but guidelines for Mediterranean laboratories, which should be tested and modified in order to validate their final results.

These Protocols aim at streamlining sample preparation and analysis of marine biota samples in view of assuring comparable quality assurance of the data, as well as comparability between sampling areas and different national monitoring programmes, by providing a step-by-step guidance on the methods to be applied in the Mediterranean.

In order to avoid unnecessary repetitions, reference is also made to the protocols already published and publicly accessible, which can also be used by the Contracting Parties' competent laboratories participating in IMAP implementation. Regarding the determination of heavy metals, here-below elaborated IMAP Protocols build on relevant guidelines developed by UNEP/IAEA (Annex VI: IAEA (2011a). Recommended method on microwave digestion of marine samples for the determination of trace element content; Annex VIII: Recommended method for the determination of selected trace element in samples of marine origin by flame atomic absorption spectrometry; Annex IX: Recommended method for the determination of selected trace element in samples of marine origin by atomic absorption spectrometry using graphite furnace; Annex XI: Recommended method on the determination of Total Mercury in marine samples by thermal decomposition, amalgamation and Atomic Absorption Spectrophotometry; and Annex XII: Recommended method on the determination of Total Hg in samples or marine origin by Cold Vapour Atomic Absorption Spectrometry), HELCOM (COMBINE programme) (Annex XXIII: Technical note on the determination of trace metallic elements in biota; Annex XXV: Technical note on the determination of Total Mercury in marine biota by Cold Vapour Atomic Absorption Spectroscopy) and the US EPA (Annex XXIV: US-EPA Method 200.8: Determination of trace elements in waters and wastes by inductively coupled plasma-mass spectrometry). For organic contaminants determination, here-below elaborated IMAP Protocols build on relevant guidelines developed by UNEP/ IAEA (Annex XIV: Reference Methods for Marine Pollution Studies No 71 for the analysis of selected chlorinated hydrocarbons in the marine environment;); HELCOM (Annex XVI: COMBINE programme, Technical note on the determination of chlorinated biphenyls and organochlorine pesticides in biota; Annex XVI: COMBINE programme, Technical Note on the determination of Polycyclic Aromatic Hydrocarbons in Biota); ICES/OSPAR (Annex XVII: CEMP Guidelines for monitoring contaminants in biota and sediments) and the US EPA (Annex XXXXV: US EPA Method 1613, Tetra- through octachlorinated dioxins and furans by isotope dilution HRGC/HRMS; Annex XXXXVI: US EPA Method 1668, Chlorinated biphenyl congeners in water, soil, sediment, and tissue by HRGC/HRMS). Given the suitability of any of these Guidelines in the context of IMAP, they could be further used by interested IMAP competent Mediterranean laboratories for developing their laboratory specific sampling and sample processing methodologies. The Contracting Parties' laboratories should accommodate and always test and modify each step of the procedures to validate their results.

2.2 Technical Note for the determination of heavy metals in seafood samples

Regulated metals for seafood monitoring in the framework of CI20 are Cd, Pb and Hg. National laboratories may decide to use any validated analytical method they consider appropriate, which meets specific performance criteria (LOD, LOQ, precision, recovery and specificity – EU Regulation (EC) No 836/2011 (Annex XXXIX), amending Regulation (EC) 333/2007). Details on specific requirements for analytical methods, regarding the use of the performance criteria and the "fitness for purpose" approach are also provide in the Regulations.

Performance criteria for methods of analysis for Pb, Cd and Hg as set in (EC) No 836/2011

Parameter	Criterion	
Applicability	Foods specified in Regulation (EC) No 1881/2006	
Specificity	Free from matrix or spectral interferences	
Repeatability (RSD_r)	$HORRAT_r$ less than 2*	
Reproducibility (RSD_R)	$HORRAT_R$ less than 2 *	
Recovery	The provisions of point D.1.2. apply **	
	Maximum Level is < 0.100mg kg^{-1}	Maximum Level is ≥ 0.100mg kg^{-1}
LOD	≤ one fifth of the ML	≤ one tenth of the ML
LOQ	≤ two fifths of the ML	≤ one fifth of the ML

* '$HORRAT_r$' = the observed RSDr divided by the RSDr value estimated from the Horwitz equation using the assumption r = 0,66R. (M. Thompson, Analyst, 2000, 125, 385-386.)

'$HORRAT_R$' = the observed RSDR value divided by the RSDR value calculated from the Horwitz equation.

** D.1.2. Recovery calculations: The result may be reported uncorrected (for metals) if evidence is provided by ideally making use of suitable certified reference material that the certified concentration allowing for the measurement uncertainty is achieved (i.e. high accuracy of the measurement). In case the result is reported uncorrected this shall be mentioned.

In order to assist analytical laboratories of the Contracting Parties, IMAP Protocols have been prepared within this document in order to be used as guidelines for the determination of heavy metals (Cd, Hg and Pb) in seafood samples. The IMAP Protocols are those proposed for marine biota analysis in the framework of CI17. Analytical laboratories should accommodate, test and modify each step of the procedures presented in the Protocols in order to validate their final results. The list of methods and analytical equipment is not exhaustive and laboratories are encouraged to use their own equipment/methods that consider adequate for the required analyses.

The determination of heavy metals in marine biota samples developed for monitoring of CI17, that are also recommended for heavy metals in seafood sample for monitoring of CI 20, include: i) digestion of tissues and ii) analysis of the digested sample for heavy metals using different equipment. They are provided in the following IMAP Protocols:

- Protocol for seafood tissues digestion using nitric acid(microwave assisted digestion in closed systems and digestion on hot plate);
- Protocol for the determination of heavy metals in seafood samples with Flame Atomic Absorption Spectroscopy (F-AAS);
- Protocol for the determination of heavy metals in seafood samples with Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS);
- Protocol for the determination of heavy metals in seafood samples with Inductive Coupled Plasma – Mass Spectroscopy (ICP-MS);
- Protocol for the determination of Total Hg in seafood samples with thermal decomposition, amalgamation and AAS;
- Protocol for the determination of Total Hg in seafood samples with Cold Vapour Atomic Absorption Spectrometry (CV-AAS).

These Protocols are based on Analytical Methods developed by IAEA (Annexes VI, VIII, IX, XI and XII), HELCOM (Annexes XXIII and XXV) and US EPA (Annex XXIV).

Regardless of the analytical method used, heavy metal determination follows some procedures common to all analytical methodologies, such as the calibration of the analytical equipment and the cleaning and handling procedures to avoid the contamination of the samples from the laboratory's environment and the tools and containers used in the analysis.

a) Calibration

Calibration standards prepared from single standard stock solutions or multielement standards, by dilution of the stock solution using dilute acid, as required. All standard solutions have to be stored in polyethylene, borosilicate or quartz volumetric flasks, depending on the best suitability for the respective analytes. Standard solutions with lower concentrations, if prepared correctly and controlled in a QA system (checking of old versus new, and checking with standards from a different source), can be kept for a period no longer than one month

The calibration procedure has to meet some basic criteria in order to give the best estimate of the true element concentration of the sample analyzed (HELCOM, 2012a[19]):

i) The concentrations of standards for the preparation of the calibration curve should cover the range of anticipated concentrations;

[19] HELCOM (2012a). Manual for marine monitoring in the COMBINE programme. Annex B-12, Appendix 4: Technical note on the determination of trace metallic elements in biota

ii) The required analytical precision should be known and achievable throughout the entire range of concentrations;
iii) The measured value at the lower end of the range has to be significantly different from the procedural analytical blank;
iv) The chemical and physical properties of the calibration standards must closely resemble those of the sample under investigation;
v) The analytical instruments should be recalibrated regularly (every 10-20 samples) to correct for instrumental drift and analytical efficiency.

b) Avoiding contamination

To avoid metal contamination in the laboratory all glassware and plastic vessels used should be carefully cleaned. The general cleaning guidelines include:

i) The vessels are allowed to soak overnight in a plastic container in an alkaline surfactant solution (2% in tap or even better distilled water);

ii) Vessels are rinsed thoroughly first with tap or even better distilled water then with ultrapure deionised water (18 MΩ cm, e.g. Milli-Q).

iii) Vessels are left to stand in 10% (v/v) concentrated HNO_3 solution (analytical grade) at room temperature for at least 6 days
iv) Vessels are rinsed thoroughly with ultrapure deionised water (e.g. Milli-Q) (at least 4 times).
v) Vessels are allowed to dry under a laminar flow hood.
vi) Vessels are stored in closed plastic polyethylene bags (e.g. zip-lock variety) to prevent the risk of contamination prior to use.

This procedure should be used for all plastic ware use in the laboratory as tips, cup for autosampler, plastic containers. Leave the vessels to stand in 10% (v/v) concentrated HNO_3 solution (analytical grade) at room temperature for at least 6 days

2.2.1 Protocol for biota tissues digestion using nitric acid

Biota tissues samples have to be digested (wet aching) prior to analysis. The rate of digestion and the efficiency of acid decomposition increase substantially with elevated temperatures and pressure, therefore microwave digestion in closed vessels is the preferred method. However, in case no such equipment is available, sample digestion in open vessels over a hot plate is an alternative method. Biota samples can be digested in wet or dried condition, however regardless of the method applied, it is of paramount importance to secure the complete destruction of all organic material of the sample, as well as to avoid metals losses and the contamination of the sample (HELCOM, 2012a).

The existence of residual dissolved organic carbon compounds in the digested sample would change the viscosity of the solution and therefore may lead to erroneous results when calibration of the AAS instrument is made using aquatic calibration standard solutions. Also, in the GF-AAS, residual organic carbon may undergo secondary reactions with the analyte prior to or during the atomization process causing matrix interferences (Harms, 1985[20]).

a) Microwave acid digestion in closed systems (for heavy metals analysis with AAS, GFAAS and ICP-MS analysis)

Biota tissue digestion can be performed in Teflon, or equal quality vessels of pure material, which are metal free and resistant to strong acids, therefore loss of elements through volatilization and contamination by desorption of impurities from the vessel surface are significantly reduced. Also, since only small quantities of high-purity nitric acid are used, extremely low analytical blanks can be obtained. Microwave systems enable a very fast energy transfer to the sample and a very rapid build-up of high internal vessel temperature and pressure, with the advantage of an enormous reduction in digestion time occurs (HELCOM, 2012a).

Digestion reagents for the analysis of Cd, Pb and other trace elements analysis

i) HNO3 (65%, Suprapur, Merck).
ii) H2O2 (analytical grade) to be kept in the fridge after opening.
iii) Milli-Q deionised water (> 18MΩ cm, Millipore).

According to the IAEA (2011a[21]) recommended method on microwave digestion of marine samples for the determination of trace element content (Annex VI) dried biota tissue samples (approximately 0.2. g) are weighted in the microwave vessel and placed in a laminar hood compatible with acid fume. Approximately 5 ml of nitric acid (HNO_3) are added and each vessel and let to react for at least 1hour (or more if possible). After the room temperature pre-digestion, 2ml of hydrogen peroxide (H_2O_2) are added carefully, the vessels are closed and placed in the microwave apparatus and digestion steps are followed. *Digestion reagents for Mercury analysis*

i) HNO_3 (65%, analytical grade, low in mercury).

ii) Milli-Q deionised water (> 18MΩ cm).

iii) 10% $K_2Cr_2O_7$ (w/v) solution (e.g. 10 g $K_2C_{r2}O_7$ analytical grade diluted into 100 ml with Milli-Q water).

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[20] Harms, U. 1985. Possibilities of improving the determination of extremely low lead concentrations in marine fish by graphite furnace atomic absorption spectrometry. Fresenius Journal of Analytical Chemistry, 322: 53-56.

[21] IAEA (2011a). Recommended method on microwave digestion of marine samples for the determination of trace element content (IAEA/Marine Environmental Studies Laboratory in co-operation with UNEP/MAP MED POL)

Dried biota tissue samples (approximately 0.2. to 1.5.g depending on the expected concentration) are weighted in the microwave vessel and placed in a laminar hood compatible with acid fume. If processing high weight of bivalve (> 1g), add 40 mg of V_2O_5 to each tube (including blanks). Add 5 ml of concentrated Nitric acid (HNO_3) and left to react for at least 1hour. If large amount of sample is used more acid has to be added until the mixture becomes liquid. To control the performance of the digestion procedure, at least 2 blanks should be prepared in a similar manner as the samples for each batch of analysis. Also at least one Certified Reference Material should be used and prepared in duplicate for each digestion batch. These digestions are prepared in a similar manner as the samples. A reference material of similar composition and concentration range should be used. After digestion, the vessels are removed from the microwave apparatus and placed in a ventilated fume hood to cool. When the pressure is adequate, the vessels are opened 1 ml of $K_2Cr_2O_7$ solution is added (final concentration should be 2% v/v) and their content is transferred to a volumetric flask, preferably of Teflon, but glass is also good, and made to a known volume. All reagents should be of analytical grade.

b) Acid digestion in open systems

In case no microwave digestion system is available, it is possible to perform a digestion over a programmable heating plate placed inside a specially designed fume hood, allowing acid treatment. However, for the complete destruction of the organic matter, large quantities of reagents and voluminous apparatus with large surfaces are usually needed and the method is subject to contamination problems (too high blank values) if insufficiently purified acids are used. Also, the rate of reaction and efficiency of acid decomposition in open vessels is lower than in closed vessels under pressure. Therefore, digestion over a hot plate is not a recommended method and should be avoided if possible.

Dried biota tissue samples (approximately 0.2. g) are weighted in the microwave vessel and placed in a laminar flow hood compatible with acid fume. Approximately 5 ml of concentrated Nitric acid (HNO_3) are added to each vessel and left to react at room temperature for at least 1 hour. The tubes are closed and placed in an aluminum block on a hot plate at 90 ºC for 3hrs. The samples are allowed to cool to room temperature, then the tubes are opened carefully, and the samples are transfered in the labeled 50 ml polypropylene graduated tubes or volumetric flask. All reagents are of analytical grade.

A method for biota tissues digestion in open systems, using aqua regia, HNO_3 / $HClO_4$ can be found in Black et al, (2013[22]).

2.2.2 Protocol for the determination of heavy metals in seafood samples with Flame AAS

Flame Atomic Absorption Spectroscopy (AAS) has adequate sensitivity for the determination of a wide range of metals in marine biota tissues. The sample solution is aspirated into a flame and atomized. In case of flame-AAS, a light beam is directed through the flame, into a monochromator, and onto a detector that measures the amount of light absorbed by the element in the flame. Each metal has its own characteristic wavelength so a source hallow cathode lamp composed of that element is used. The amount of energy absorbed at the characteristic wavelength is proportional to the concentration of the element in the sample.

A detailed analytical protocol for the determination of heavy metals in biota tissue samples by flame atomic absorption spectrometry prepared by IAEA (2011b[23]) is presented in the Annex VIII: Recommended method for the determination of selected trace element in samples of marine origin by flame atomic absorption spectrometry.

2.2.3 Protocol for the determination of heavy metals in seafood samples with GF-AAS

In marine biota tissues Cd, Pb, as well as other heavy metals, can be determined by Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS), which has adequate sensitivity for these determinations. For GF-AAS analysis, after the digestion of the biota sample, an aliquot of sample solution (10-50 µl) is introduced into a graphite tube of the GF-AAS and atomized by rapid heating at high temperature. A light beam is directed through the graphite tube, into a monochromator, and onto a detector that measures the amount of light absorbed by the atomized element in the tube. Each metal has its own characteristic wavelength, so a source hollow cathode lamp composed of that element is used. The amount of energy absorbed at the characteristic wavelength is proportional to the concentration of the element in the sample.

The AAS software generally gives typical electrothermal programs for each element for 10 µl of sample in diluted HNO_3 (0.1%) and indications concerning maximum aching and atomization temperatures. More specific information may also be found in the literature, such as recommendations regarding matrix modifiers and the use of partition tubes or tubes with platform. When a program is optimized for the determination of an element in a specific matrix, all information should be reported in the logbook of methods of the laboratory.

For some elements and some matrices, the results obtained are still not satisfactory (e.g. maximum ashing

[22] Black, K., Kalantzi, I., Karakassis, I., Papageorgiou, N., Pergantis, S., Shimmield, T. (2013). Heavy metals, trace elements and sediment geochemistry at four Mediterranean fish farms, Science of the Total Environment. 444, 128–137.

[23] IAEA (2011b) Recommended method for the determination of selected trace element in samples of marine origin by flame atomic absorption spectrometry

temperature is not sufficient to eliminate the background), this procedure should be redone with the addition of a matrix modifier. Different matrix modifiers could be tried before finding the best solution.

A detailed analytical protocol for the determination of heavy metals in sediments by GF AAS prepared by IAEA (2011c[24]) is presented in the Annex IX (Recommended method for the determination of selected trace element in samples of marine origin by atomic absorption spectrometry using graphite furnace).

2.2.4 Protocol for the determination of heavy metals in seafood samples with ICP-MS

Inductive Coupled Plasma – Mass Spectroscopy (ICP-MS) is currently state-of-the-art instrumentation for metal analysis, with the possibility to determine at sub-µg L^{-1} concentrations of a large number of elements in acid digested biota tissue samples. ICP-MS allows a rapid simultaneous determination of a wide range of heavy metals. Most routine instruments utilize a quadrupole mass spectrometer, so mass resolution is not high enough to avoid overlap of double charged elements or multi-element ions (mainly hydrides, oxides and hydroxides) formed in the plasma. The main concern is for the Ar interferences as the plasma is usually an argon plasma, overlapping with As. Some elements are prone to memory effects (particularly Hg) and needs extra precautions to avoid carry over effects. Modern ICP-MS instruments software includes all the tuning and correction formulas needed and described above to perform the analysis (HELCOM 2012a).

A multi-elemental determination of heavy metals by ICP-MS in water and solid samples after acid digestion, is described in the US EPA Method 200.8 (1994[25]). The method was initially intended for inorganic solid samples (soils and sediments) but can also be directly applied to organic samples. According to Enamorado-Baez et al. (2015[26]), for biota tissues the digestion step could use only nitric acid (similar to the US-EPA 3051 method established for sediments, sludge, soils, and oils) but increasing the sample mass to acid volume ratio.

Metal species originating in a liquid are nebulized and the resulting aerosol is transported by argon gas into the plasma torch. The ions produced by high temperatures are entrained in the plasma gas and introduced, by means of an interface, into a mass spectrometer. The ions produced in the plasma are sorted according to their mass-to-charge ratios and quantified with a channel electron multiplier. Interferences must be assessed, and valid corrections applied. Interference correction must include compensation for background ions contributed by the plasma gas, reagents, and constituents of the sample matrix. The US EPA Method 200.8 is presented in Annex XXIV.

2.2.5 Protocol for the determination of Total Mercury in seafood samples with thermal decomposition, amalgamation and Atomic Absorption Spectrophotometry

Total mercury in the marine biota can be analysed by solid Hg analyser, which has adequate sensitivity for this determination. A detailed method describing the protocol for the determination of total mercury (inorganic and organic) in sediment prepared by IAEA (2012a[27]) ("Recommended method on the determination of Total Mercury in marine samples by thermal decomposition, amalgamation and Atomic Absorption Spectrophotometry" Annex VI). With this method, Total Hg is determined without any chemical pre-treatment of the sample, minimising possible contamination and/or additional errors due to sample handling. The method is based on the US EPA 7473 method (US EPA, 2007[28]).

The sample is dried and then chemically decomposed under oxygen in the decomposition furnace. The decomposition products are carried out to the catalytic section of the furnace, where oxidation is completed (halogens and nitrogen/sulfur oxides are trapped). The mercury present in the remaining decomposition products is selectively trapped on an amalgamator. After flushing the system with oxygen, the mercury vapour is released by rapid heating of the amalgamator and carried through the absorbance cell in the light path of a single wavelength atomic absorption spectrophotometer. The absorbance is measured at 253.7 nm as a function of mercury quantity (ng). The typical working range is 0.1–500 ng. The mercury vapour is carried through a long (first) and a short path length absorbance cell. The same quantity of mercury is measured twice with different sensitivity resulting in a dynamic range that spans four orders of magnitude. The typical detection limit is 0.01 ng of mercury

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[24] IAEA (2011c) Recommended method for the determination of selected trace element in samples of marine origin by atomic absorption spectrometry using graphite furnace

[25] US EPA (1994) US-EPA Method 200.8: Determination of trace elements in waters and wastes by inductively coupled plasma-mass spectrometry

[26] Enamorado-Báez, S.M., Abril, JM and Gómez-Guzmán, JM (2013) Determination of 25 Trace Element Concentrations in Biological Reference Materials by ICP-MS following Different Microwave-Assisted Acid Digestion Methods Based on Scaling Masses of Digested Samples. Hindawi Publishing Corporation, ISRN Analytical Chemistry, Volume 2013, Article ID 851713, 14 pages. http://dx.doi.org/10.1155/2013/851713

[27] IAEA (2012a) Recommended method on the determination of Total Hg in marine samples by Thermal Decomposition Amalgamation and Atomic Absorption Spectrometry

[28] US EPA (2007). U.S. Environmental Protection Agency, EPA method 7473, Mercury in solids and solutions by thermal decomposition, amalgamation and atomic absorption spectrophotometry Rev 0. http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/7473.pdf

2.2.6 Protocol for the determination of Total Hg in seafood samples of marine origin by CV-AAS

The method is widely used for the determination of total mercury in biological tissues and it is simple, rapid and applicable to a large number of environmental samples. The typical working range is 0.25–100 ng ml^{-1} for direct injection of cold vapour, using "batch" system (IAEA, 2012b). Cold Vapor Atomic Absorption Spectrometry (CV-AAS) analysis can be performed manually using batch CV-AAS or automatically using flow injection (FIAS) techniques. FIAS is a very efficient approach for introducing and processing liquid samples in atomic absorption spectrometry, reduces sample and reagent consumption, and has a higher tolerance of interferences, lower determination limits and improved precision compared with conventional cold vapour techniques (HELCOM, 2012b[29]).

The biota tissue samples are digested with strong acids and the inorganic mercury is reduced to its elemental form with stannous chloride. The cold mercury vapour is then passed through the quartz absorption cell of an atomic absorption spectrometer (AAS), where its concentration is measured.

In the CV-AAS method, the inorganic mercury is reduced to its elemental form with stannous chloride. The cold mercury vapor is then passed through the quartz absorption cell of an AAS instrument where its concentration is measured. The light beam of Hg hallow cathode lamp is directed through the quartz cell, into a monochromator and onto a detector that measures the amount of light absorbed by the atomized vapor in the cell. The amount of energy absorbed at the characteristic wavelength is proportional to the concentration of the element in the sample.

A recommended method describing the protocol for the determination of total mercury in biota prepared by IAEA (2012b[30]) is presented in Annex XII (Recommended method on the determination of Total Hg in samples or marine origin by Cold Vapour Atomic Absorption Spectrometry). A method for the determination of Total Hg in marine biota using CV-AAS is also suggested by HELCOM (2012b) (Annex XXV) and US EPA (2007b)[31].

2.3 Technical note for the determination of organic contaminants in seafood samples

Regulated organic contaminants include PAHs (Bezo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene) (Regulation (EC) No 835/2011, Annex XXXVII), dioxins, dioxin-like PCBs (Regulation (EC) No 1881/2006) (Annex XXXVI) and non-dioxin like PCBs (PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180) (EU Regulation (EC) No 1259/2011 (Annex XXXXIV**).** Analysis of the four PAHs and the 6 non-dioxin-like PCBs can be done following the relevant IMAP Protocols developed for the determination of PAHs and PCBs in marine biota, in the framework of CI17. However, the Regulation (EC) No 835/2011, which amended Regulation (EC) No 333/2007, doesn't set any maximum level for PAHs in fresh fish, crustaceans or cephalopods, and sets maximum level for benzo(a)pyrene and the sum of four regulated PAHs (Bezo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene) only for bivalves (fresh, chilled or frozen).

Determination of dioxins and dioxin like PCBs, can only be done in a laboratory accredited for such analysis using appropriate methods such as High-Resolution Gas Chromatography/High Resolution Mass Spectrometry (HRGC/HRMS). Sampling and sample preparation for such analysis should follow the requirements presented in EU Regulation (EC) 644/2017 (Annex XXXX).

Analytical methods for non-dioxin like PCBs include Gas Chromatography - Electron Capture Detection (GC-ECD), Gas Chromatography - Low Resolution Mass Spectroscopy (GC-LRMS), Gas Chromatography – Tandem Mass Spectroscopy (GC-MS/MS), Gas Chromatography - High Resolution Mass Spectroscopy (GC-HRMS) or equivalent methods.

National laboratories may decide to use any validated analytical method they consider appropriate, which meets specific performance criteria (LOD, LOQ, precision, recovery and specificity – EU Regulation (EC) No 836/2011) (Annex XXXIX) and EU Regulation (EC) 644/2017 (Annex XXXX).

[29] HELCOM (2012b). COMBINE Annex B-12, Appendix 4, Attachment 1. Technical note on the determination of Total Mercury in marine biota by Cold Vapour Atomic Absorption Spectroscopy

[30] IAEA (2012b). Recommended method on the determination of Total Hg in samples or marine origin by Cold Vapour Atomic Absorption Spectrometry

31 US EPA (2007b). U.S. Environmental Protection Agency, EPA method 7473, Mercury in solids and solutions by thermal decomposition, amalgamation and atomic absorption spectrophotometry Rev 0. http://www.epa.gov/osw/hazard/testmethods/sw846/pdfs/7473.pdf

Performance criteria for methods of analysis for the four regulated PAHs, (EC) No 836/2011

Parameter	Criterion
Applicability	Foods specified in Regulation (EC) No 1881/2006
Specificity	Free from matrix or spectral interferences, verification of positive detection
Repeatability (RSD_r)	$HORRAT_r$ less than 2 *
Reproducibility (RSD_R)	$HORRAT_R$ less than 2 *
Recovery	50 to 120 %
LOD	≤ 0.30 µg kg^{-1} for each of the four substances
LOQ	≤ 0.90 µg kg^{-1} for each of the four substances

* '$HORRAT_r$' = The observed RSDr divided by the RSDr value estimated from the Horwitz equation using the assumption r = 0,66R. (M. Thompson, Analyst, 2000[32], 125, 385-386.)

'$HORRAT_R$' = The observed RSDR value divided by the RSDR value calculated from the Horwitz equation.

Performance criteria to be met in the range of the maximum level for the TEQ value respectively the BEQ value, whether determined as total TEQ (Toxic Equivalents) or total BEQ (as sum of PCDD/F and dioxin-like PCBs) or separately for PCDD/Fs and dioxin-like PCBs, (EC) No 644/2017

	Screening with bioanalytical or physico-chemical methods	Confirmatory methods
False-compliant rate(*)	< 5 %	
Trueness		20 % to + 20 %
Repeatability (RSDr)	< 20 %	
Intermediate precision (RSDR)	< 25 % < 15	< 25 % < 15

(*) With respect to the maximum levels

Performance criteria for the sum of non-dioxin like PCBs, (EC) No 644/2017

	Isotope dilution mass spectrometry(*)	Other techniques
Trueness	– 20 to + 20 %	– 30 to + 30 %
Intermediate precision (RSDR)	≤ 15 %	≤ 20 %
Difference between upper and lower bound calculation	≤ 20 %	≤ 20 %

(*) Use of all six ^{13}C-labelled analogues as internal standards required

The laboratory used for organic trace analysis must be a dedicated facility, isolated from other projects that could be sources of contamination. It must be properly constructed with fume hoods and benches with electric sockets that are safe for use with flammable solvents. The laboratory must have extractors and rotary evaporators cooling water to run the stills. In tropical regions and in dry climates, a refrigerated re-circulating system should be used to reduce temperatures to the required levels and/or to conserve water. Stainless steel or ceramic tiles make good non-contaminating surfaces. If necessary, benches can be coated with a hard epoxy resin and walls can be painted with epoxy paint. A sheet of aluminium foil on the workbench provides a surface which can be cleaned with solvent. A vented storage facility for solvents is essential. Benches must be fitted with frames to hold stills, extractors, etc. The emergency cut-off switch should be accessible from both inside and outside the laboratory. Firefighting equipment should be mounted in obvious places and laboratory personnel trained in their use.

In order to assist analytical laboratories of the Contracting Parties to the Barcelona Convention, IMAP Protocols are proposed for the determination of the four regulated PAHs (Benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene), dioxins, dioxine-like PCBs and non dioxin-like PCBs in seafood samples. Analytical laboratories should accommodate, test and modify each step of the procedures presented in the Protocols in order to validate their final results. The list of methods and analytical equipment is not exhaustive and laboratories are encouraged to use their own equipment/methods that consider adequate for the required analyses.

Under this Technical note, this Guidelines related to sample preparation and analysis of sea food includes the

[32] Thomson, M. (2000). Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst 125, 385-386

following IMAP Protocols for the determination of organic compounds in marine biota samples:

- Protocol for the determination of dioxins and dioxin-like PCBs in seafood samples using Gas Chromatography - High Resolution Mass Spectrometry (GC-HRMS);
- Protocol for the determination of non-dioxin like PCBs in seafood samples using Gas Chromatography-Electron Capture Detector (GC-ECD);
- Protocol for the determination of non-dioxin like PCBs in seafood samples using Gas Chromatography - Mass Spectroscopy (GC-MS);
- Protocol for the determination of PAHs in seafood samples using High Performance Liquid Chromatography – Fluorescence (HPLC-UVF);
- Protocol for the determination of PAHs in seafood samples using Gas Chromatography – Mass Spectrometry (GC-MS).

These protocols are based on analytical methods developed by UNEP/IAEA (Annex XIV: Reference Methods for Marine Pollution Studies No 71 for the analysis of selected chlorinated hydrocarbons in the marine environment), HELCOM COMBINE programme (Annex XXVI:, Technical note on the determination of chlorinated biphenyls and organochlorine pesticides in biota; Annex XXVIII: Technical Note on the determination of Polycyclic Aromatic Hydrocarbons in Biota), ICES/OSPAR (Annex XVII: CEMP Guidelines for monitoring contaminants in biota and sediments: Determination of parent and alkylated PAHs in biological materials) and the US EPA (Annex XXXXV: US EPA 1994b, Method 1613[33], Tetra- through octachlorinated dioxins and furans by isotope dilution HRGC/HRMS; Annex XXXXVI: US EPA (2008) Method 1668[34], Chlorinated biphenyl congeners in water, soil, sediment, and tissue by HRGC/HRMS).

2.3.1 Protocol for the determination of dioxins and dioxin-like PCBs in seafood samples using GC-HRMS

Chlorinated dibenzo-*p*-dioxins (dioxins) and chlorinated dibenzofurans (furans), have similar chemical properties and toxic effects, and are generally determined as a single group. The dioxin-like PCBs are also showing high toxicity and are included in the list of compounds to be determined in seafood for the protection of consumers' health. The most toxic dioxin is the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), while other congeners have different degrees of toxicity (A list with the WHO-toxic equivalent factors for human risk assessment is presented in the EU (EC) Regulation No 1881/2006, Annex XXXVI). Dioxins and dioxin-like PCBs are found in very low concentrations in seafood, therefore analytical methods require LODs as parts-per-trillion (ppt:10^{-12} g 2,3,7,8-TCDD per g of sample) or parts-per-quadrillion (ppq: 10^{-15} g 2,3,7,8-TCDD per g of sample). Therefore, it is very important to efficiently separate these compounds from other organic contaminants, with similar physical and chemical properties before determination.

The determination of dioxins and dioxin-like PCBs in seafood samples involves extraction from the matrix with organic solvents, followed by clean-up and gas chromatographic separation and detection with GC-HRMS (Reiner et al, 2006[35]): Extraction techniques include Soxhlet, liquid/liquid extraction (US EPA 1994b), solid-phase extraction (SPE) (Taylor et al, 1995[36]), or pressurized fluid extraction (Richter et al 1994[37]). Once the extract has been transferred to a suitable solvent, follows a three-stage (silica, alumina and carbon) open-column clean-up. PCB interferences can be eliminated by analyzing extracts on multiple columns (US EPA 2008, Method 1668). A number of analyte-specific columns can be used to reduce both dioxin and PCB interferences and reduce the need for multicolumn analysis.

Dioxins and dioxin-like PCBs are usually determined using High Resolution Gas Chromatography - High Resolution Mass Spectrometry (HRGC-HRMS) employing isotope dilution (Petrovic et al 2002[38], Focant et al, 2005[39]). Methods for the determination of dioxins and dioxin-like PCBs are developed by US EPA (1994b, 2008), ISO Method 18073 (2004[40]), ISO

[33] US EPA (1994). Method 1613, Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS, Office of Water, US Environmental Protection Agency, Washington, DC

[34] US EPA (2008) Method 1668, Revision B: Chlorinated biphenyl congeners in water, soil, sediment, and tissue by HRGC/HRMS, EPA-821-R-08-020. Office of Water, US Environmental Protection Agency, Washington, DC

[35]Reiner, E.J, Clement, R.E, Okey, A.B., Marvin, C.H. (2006). Advances in analytical techniques for polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and dioxin-like PCBs. Anal Bioanal Chem (2006) 386: 791–806.

[36] Taylor KZ, Waddell DS, Reiner EJ, MacPherson KA (1995). Direct Elution of Solid Phase Extraction Disks for the Determination of Polychlorinated Dibenzo-p-dioxins and Polychlorinated Dibenzofurans in Effluent Samples. Analytical Chemistry, 67:1186–1190

[37] Richter B.E, Jones B.A, Ezzell J.L, Porter N.L, Avdalovic N, Pohl C (1996). Accelerated Solvent Extraction: A Technique for Sample Preparation. Analytical Chemistry, 68:1033–1039

[38] Petrovic M., Eljarrat E., Lopezde Alda M.J, Barcelo D. (2002). Recent advances in the mass spectrometric analysis related to endocrine disrupting compounds in aquatic environmental samples. J. Chromatography A 974:23–51.

[39] Focant J.F, Pirard C, Eppe G, DePauw E. (2005). Recent advances in mass spectrometric measurement of dioxins J. Chromatography A. 1067:265–275

[40] ISO (2004) ISO 18073: Water quality—Determination of tetratoocta-chlorinated dioxins and furans—Method using

Method 17585 (2006[41]) and the European Committee for Standardization European Standard EN 1948 (CEN, 1997[42]). An overview of the methodology for sample extraction, cleanup and CG-MS analysis, as well as data quality control and data reporting are presented in the article of Reiner et al (2006): "Advances in analytical techniques for polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and dioxin-like PCBs". Other detection methods for dioxins and furans include Tandem Mass Spectrometry as hybrid/MS (Charles et al 1989[43]) and triple quadrupole MS/MS (Reiner et al, 1990[44], 1991[45]).

Detailed guidelines for the determination of dioxins and dioxin-like PCBs with HRGC-HRMS are proposed by US EPA Method 1613b (1994) (Annex XXXXV) and US EPA Method 1668 (2008) (Annex XXXXVI).

2.3.2 Protocol for the determination of non-dioxin like PCBs in seafood samples using GC-ECD

The determination of non-dioxin like PCBs in seafood samples involves extraction from the matrix with organic solvents, followed by clean-up and gas chromatographic separation with electron capture (GC-ECD) or mass spectrometric (GC-MS) detection. To minimize systematic errors due to insufficiently optimized gas chromatographic conditions, determinant losses (evaporation, unsatisfactory extraction yield), and/or contamination from laboratory ware, reagents and the laboratory environment, it is essential that the sources of systematic errors are identified and eliminated as far as possible (HELCOM, 2012c[46]).

For analysis, the samples are prepared for solvent extraction. To achieve a satisfactory recovery of the chlorinated hydrocarbons, samples are dried by either desiccation with anhydrous sodium sulphate or by freeze-drying. Lipids are then Soxhlet extracted from biota using hexane or petroleum ether. Following initial clean-up treatments (treatment of biota extracts with concentrated sulphuric acid to destroy some interfering lipids), extracts are fractionated using column chromatography.

All reagents, including the distilled water should be of analytical quality. Commercially available solvents like acetone, acetonitrile, dichloromethane, hexane and pentane are invariably contaminated with ECD-active substances; their concentrations vary from batch to batch and with supplier. Reagent quality should be checked by injection of 2 µl of a 100 ml batch of solvent, after concentration to 50 µl in a rotary evaporator.

Quantitative analysis with Electron Capture Detector (ECD) is performed by comparing the detector signal produced by the sample with that of defined standards. Due to incomplete separation, several co-eluting compounds can be present under a single detector signal, therefore, the shape and size of the signal have to be critically examined. The relative retention time and the signal size should be confirmed on columns with different polarity of their stationary phases, or by the use of multi-dimensional GC techniques. The GC should be calibrated before each batch of measurements. Since the ECD has a non-linear response curve, a multilevel calibration is strongly advised. For the purpose of determining recovery rates, an appropriate internal standard should be added to each sample at the beginning of the analytical procedure. (HELCOM, 2012c).

A step-by-step method for the determination of polychlorinated biphenyls in biological samples by GC ECD is prepared by UNEP/IAEA (2011d[47]) (Annex XIV), including the list of reagents, the solvents, standards and examples for the preparation of the stock, intermediate and working solutions. A method for the analysis of PCBs in biota tissues by GC ECD is also proposed by HELCOM (2012c) (Annex XXVI).

2.3.3 Protocol for the determination of non-dioxin like PCBs in seafood samples using GC-MS

The determination of non-dioxin like PCBs in seafood samples involves extraction from the matrix with organic solvents, followed by clean-up (as presented in the Protocol 3.2.), and gas chromatographic separation with mass spectrometric (GC-MS) detection.

Quantitative analysis is performed by comparing the detector signal produced by the sample with that of defined standards, using a mass spectrometer (MS). Often, due to incomplete separation, several co-eluting compounds can be present under a single detector signal. Therefore, the shape and size of the signal have to be critically examined. With a MS detector, either the molecular mass or characteristic mass fragments should

isotope dilution HRGC/HRMS. International Organization for Standardization (ISO), Geneva, Switzerland

[41] ISO (2006) ISO 17585: Water quality—Determination of dioxin-like polychlorinatedbiphenyls—method using gas chromatography and mass spectrometry. International Organization for Standardization (ISO), Geneva, Switzerland

[42] CEN (1997) European Standard EN 1948: Stationary source emissions, determination of the mass concentration of PCDDs/PCDFs. CEN, Brussels, Belgium

[43] Charles M.J, Green B., Tondeur J.R, Hass R. (1989). Optimisation of a hybrid-mass spectrometer method for the analysis of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans. Chemosphere 19, 51–57

[44] Reiner E.J, Schellenberg D.H Taguchi V.Y, Mercer R.S, Townsend J.A, Thompson T.S, Clement R.E (1990). Application of tandem quadrupole mass spectrometry for ultra-trace determination of polychlorinated dibenzo-p-dioxins and dibenzofurans. Chemosphere 20, 1385-1392.

[45] Reiner E.J, Schellenberg D.H, Taguchi V.Y (1991). Environmental applications for the analysis of chlorinated dibenzo-p-dioxins and dibenzofurans using mass-spectrometry. Environ Sci Technol 25:110–117

[46] HELCOM (2012c). Manual for marine monitoring in the COMBINE programme. Annex B-12, Appendix 3. Technical note on the determination of chlorinated biphenyls and organochlorine pesticides in biota.

[47] UNEP/IAEA (2011d). Sample work-up for the analysis of selected chlorinated hydrocarbons in the marine environment. Reference Methods for Marine Pollution Studies No 71.

be recorded for that purpose. The GC should be calibrated before each batch of measurements. Since the MS has a non-linear response curve, a multilevel calibration is advised. For the purpose of determining recovery rates, an appropriate internal standard should be added to each sample at the beginning of the analytical procedure. The ideal internal standard is a PCB which is not present in the sample, and which does not interfere with other PCBs. All 2,4,6-substituted PCB congeners are, in principle, suitable. (HELCOM, 2012c).

A method for extraction, concentration, cleanup and fractionation for the determination of PCBs in biological samples is prepared by UNEP/IAEA (2011d) (Annex XIV). The analysis of PCBs can be done by GC-ECD followed by confirmation using GC-MS. A method for the analysis of PCBs in biota tissues using GC-MS is also proposed by HELCOM (2012c) (Annex XXVI).

2.3.4 Protocol for the determination of PAHs in seafood samples using HPLC – UVF

PAHs emitted from combustion processes are predominantly parent (un-substituted) compounds, while PAHs from petroleum and its by-products contain a range of alkylated compounds in addition to the parent PAHs. High Performance Liquid Chromatography – Fluorescence (HPLC -UVF) has the capacity to determine parent PAHs but has not the required selectivity to be used for alkylated PAHs' determination. However, this is not a limitation for the determination of the four regulated PAHs, which are parent compounds.

PAHs are lipophilic and so are concentrated in the lipids of an organism, therefore they have to be extracted with Soxhlet extraction, or alkaline digestion followed by liquid-liquid extraction with an organic solvent. For Soxhlet extraction, wet tissues should be dried by mixing with a chemical agent (e.g., anhydrous sodium sulphate). Non-polar solvents alone will not effectively extract all the PAHs from tissues when using Soxhlet extraction, and mixtures such as hexane/dichloromethane may be effective. Tissue extracts will always contain many compounds other than PAHs, and a clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. In order to reduce the sample volume to 2 cm^3 solvents are evaporated using a rotary-film evaporator at low temperature (water bath temperature of 30 °C or lower) and under controlled pressure conditions. Evaporation to dryness should be avoided. When reducing the sample to final volume, solvents can be removed by a stream of clean nitrogen gas. Solvents and adsorptive materials must all be checked for the presence of PAHs and other interfering compounds. If such compound are found, then the solvents, reagents, and adsorptive materials must be purified or cleaned using appropriate methods (HELCOM, 2012d[48], Annex XXVIII).

If Soxhlet extraction was used residual lipids have to be removed before the analytical determination, with an additional clean-up stage, using column chromatography with silica and alumina

Guidelines for the determination of PAHs in biological samples using HPLC – UVF are prepared by HELCOM (2012d) (Annex XXVIII) and ICES/OSPAR (2018[49]) (Annex XXIX).

2.3.5 Protocol for the determination of PAHs in seafood samples using GC-MS

GC-MS analytical method has the sufficient selectivity to determine the full range of PAHs including the four regulated contaminants (parent PAH compounds).

The extraction procedure is similar to the procedure described for HPLC-UVF methodology, including Soxhlet extraction, or alkaline digestion, followed by liquid-liquid extraction with an organic solvent. Alternatively, extraction of wet or dry samples of biota may be carried out by pressurized liquid extraction (PLE). Tissue extracts will always contain many compounds other than PAHs, and a clean-up is necessary to remove those compounds which may interfere with the subsequent analysis. The most commonly used clean-up methods involve the use of deactivated alumina or silica adsorption chromatography. When applying fractionation, the elution pattern has to be checked frequently. This should be carried out in the presence of sample matrix, as that can partially deactivate the clean-up column, resulting in earlier elution of the PAHs than in a standard solution (ICES/OSPAR, 2018).

Following cleanup, solvents are evaporated using a rotary-film evaporator at low temperature (water bath temperature of 30 °C or lower) and under controlled pressure conditions, and the sample volume is reduced to approximately 2 cm^3. Evaporation to dryness should be avoided. When reducing the sample to final volume, solvents can be removed by a stream of clean nitrogen gas. Solvents and adsorptive materials must all be checked for the presence of PAHs and other interfering compounds. If such compounds are found, then the solvents, reagents, and adsorptive materials must be purified or cleaned using appropriate methods.

Quantification is done by GC-MS. The two injection modes commonly used are splitless and on-column injection. Automatic sample injection should be used wherever possible to improve the reproducibility of injection and the precision of the overall method. If splitless injection is used, the liner should be of sufficient capacity to contain the injected solvent volume after evaporation. For PAH analysis, the

[48] HELCOM (2012d). Manual for marine monitoring in the COMBINE programme. Annex B-12, Appendix 2. Technical Note on the determination of Polycyclic Aromatic Hydrocarbons in Biota

[49] ICES/OSPAR (2018b). CEMP Guidelines for monitoring contaminants in biota and sediments. Technical Annex 3: Determination of parent and alkylated PAHs in biological materials

cleanliness of the liner is also very important if adsorption effects and discrimination are to be avoided, and the analytical column should not contain active sites to which PAHs can be adsorbed. Helium is the preferred carrier gas, and only capillary columns should be used (HELCOM, 2012d, ICES/OSPAR 2018b).

Detailed methods for the determination of PAHs in biological samples using GC-MS are proposed by HELCOM (2012d) (Annex XXVIII) and ICES/OSPAR (2018b) (Annex XXIX).

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