LIFE APEX

Systematic use of contaminant data from apex predators and their prey in chemicals management

Deliverable B2.4

Guidance on chemical analysis of AP&P

Lead Beneficiary:

Environmental Institute (EI), Slovakia

Deliverable Title	B2.4: Guidance on chemical analysis of AP&P
Related Key Element:	Reviewing and harmonizing quality assurance (QA) for AP&P sampling, sample processing and archiving and for their chemical analysis
Deliverable lead:	Environmental Institute (EI) National and Kapodistrian University of Athens (UoA) Università degli Studi di Firenze (UNIFI) Frauhofer IME (Fh-IME)
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Dissemination level:	Public
Due submission date:	31/08/2019
Grant Agreement :	LIFE17 ENV/SK/000355
Start date of the project:	01/09/2018
Duration of the project:	48 months
Website:	www.lifeapex.eu
Objective	Development of a workflow covering both targeted and NTS analyses will be developed taking into account state-of-the-art practices in Europe, harvesting know-how from the NORMAN network. The guidance will be tested throughout the project duration.

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Executive summary

The deliverable presents the analytical protocols, the standard operating procedures (SOPs) and the quality control and quality assurance for the analysis of APEX predators and their prey. Chapter 1-3 describe the application of generic sample preparation protocols for the analysis of polar and non-polar contaminants of emerging concern in AP&P samples. Chapter 2 is dedicated to the analysis of polar and semi-polar substances by liquid chromatography high-resolution mass spectrometry, whereas Chapter 3 focuses on the determination of non-polar chemicals by gas-chromatography high-resolution mass spectrometry. The generic sample preparation protocols can retain compounds with wide-physicochemical properties and allow the application of wide-scope target screening for quantitative analysis of > 2400 emerging substances. Data processing methods for digital archivation and application of wide-scope suspect screening through NORMAN Digital Sample Freezing Platform (DSFP) are described in Chapter 4. Chapters 5-6 are dedicated to the application of highly-sensitive methods for the determination of persistent organic pollutants such as PCBs, HCB, PBDEs, dioxins, dioxin-like compounds, chlorinated alkanes, novel organophosphorus flame retardants, decloran and the application of GC-MS non-target screening approaches. Finally, Chapter 8 describes the protocol for the determination of mercury in APEX predators and their prey. AP&P samples were subjected for analysis of chemicals described in the deliverable.

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1. Sample treatment in analytical laboratories

In accordance with the LIFE APEX proposal, all LIFE APEX samples collected from Environmental Specimen Banks (ESBs), Natural History Museums (NHMs) and other scientific collections, are sent to the National and Kapodistrian University of Athens (UoA) for their pre-treatment. UoA is the beneficiary responsible for the samples' lyophilization, homogenization and their final distribution to the rest of analytical laboratories for targeted analyses.

The samples' pre-treatment protocol is described below:

- ✓ Receipt of the delivered samples.
- ✓ Check of the sample's documentation as well as the condition of the delivered samples, sending e-mail to the NHM or ESB for briefing.
- ✓ Samples coding based on the "Guidance on assigning unique sample codes to LIFE APEX samples" document.

Specific analysts are responsible for the handling of the samples. The analysts are wearing lab coat, nitrile gloves and masks and their hair is tied back if long.

- ✓ Weighting of the empty petri-dishes (which have the respective code of each sample).
- ✓ Segmentation of the samples and placement into petri-dishes in an isolated room.
- ✓ Weighting of the petri-dishes including the wet samples.
- ✓ Samples were kept refrigerated (-80°C) for at least 5 hours (pre-treatment step before lyophilization).
- ✓ Samples freeze-drying (-55°C, 0.05 mbar, Capacity: 5 kg/24h, Telstar Lyoquest Freeze Dryer) in accordance with the SOP for the lyophilization.
- ✓ Weighting of the petri-dishes including the freeze-dried samples. Calculation of the % water content (the weights and %water content, as long as any other freeze-drying relevant information are registered in a specific excel file).An example of %water content calculation is described below:

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Example of the calculation of the % water content

Before freeze-drying:

Measure the mass of empty Petri Dish (g):13.0607

Measure the mass of Petri Dish containing the wet sample(g):24.5050

Calculate the mass of wet sample (g):11.4443

After freeze-drying

Measure the mass of Petri Dish containing the freeze-dried sample) (g): 16.3629

Calculate the mass mass of freeze-dried sample (g): 3.3022

$$\% \text{ water content} = 100 - \left(\frac{\text{mass of wet sample}}{\text{mass of freeze-dried sample}} * 100\right)$$

%Water content = 100 – [(3.3022/11.4443)*100] = 71%

- ✓ Homogenization of each sample using pestle and mortar or multi in an isolated room (between homogenizations all lab instruments are cleaned up using milli-Q water and acetone).
- ✓ Storing of each freeze-dried sample in amber glass vial, which has the code of the sample, in the freezer (-80°C) in a specific shelf.

Figure 1. Storage of LIFE APEX samples in amber glass vials.

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2. Application of generic sample preparation protocol for analysis of AP&P by liquid chromatography high-resolution mass spectrometry (LC-HRMS)

The simultaneous extraction of analytes with different physicochemical properties from the matrices demands the use of generic sample preparation protocols. The use of generic sample preparation protocols is the most crucial step of the simultaneous determination of emerging contaminants using wide-scope target, suspect as well as non-target methodologies. Furthermore, biota are complex matrices. The lipids and the proteins which are included in biota matrices obstruct the determination of emerging contaminants. Consequently, in the sample preparation protocols, powerful extraction techniques, as well as clean-up steps should be included. Therefore, In accordance with all the aforementioned, validated and generic sample preparation protocols for the determination of LC and GC amenable compounds have been followed in LIFE APEX samples' analysis.

For the simultaneous determination of LC amenable compounds, the technique Accelerated Solvent Extraction (ASE) was used for the extraction of emerging contaminants from the biota matrices, followed by a clean-up step using SPE (in-house mixed mode cartridges). The steps of the sample preparation for the determination of LC amenable compounds are presented in **Figure 4** and described thoroughly in the following steps (bullet points):

- ✓ Weighting of Sample, based on the matrix of analysis (muscle or liver), as described in Table 1.
- ✓ Mixing with Sodium Sulfate (Na_2SO_4), which is used as samples' dispersant, with mortar and pestle.

Table 1. Masses of samples and ratios (dispersant/sample).

	sample weight (g)	***ratio (dispersant/sample)***	***dispersant weight (g)***
Muscle	1.0	4	4.0
Liver	0.2	4	0.8

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- ✓ Spiking with a representative mixof isotopically labelled internal standards from different classes of compounds of UoA target list and with different physicochemical properties (concentration of mix with IS: 1 mg/L in methanol).
- ✓ Accelerated Solvent Extraction (Dionex™ ASE™ 350, Thermo Fisher Scientific, **Figure 2**).

Figure 2. Dionex™ ASE™ 350, Thermo Fisher Scientific

- ✓ Filtering through filter paper if the extract is not transparent.
- ✓ Rotary Evaporation (40°C) till 3-4 mL.
- ✓ Addition of milli-Q water till 15 mL.
- ✓ Addition of 5 mL of n-hexane (defatting), vortexing for 1min, centrifugation 10 min in 4000 rpm, discarding hexane layer.
- ✓ Addition of milli-Q water till 50 mL.
- ✓ Clean-Up Step: Solid Phase Extraction (SPE).

Table 2. ASE Conditions for LC amenable compounds.

Temperature (°C)	50
Pressure (psi)	1500
Heating Time (s)	300
Static Time (s)	420
Number of Static Cycles	3
Flush Volume (%)	60
Purge Time (s)	180
Extraction Solvent	Methanol: Acetonitrile(67:33)

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Sample clean-up is realized by SPE. Layered 'mixed bed' cartridges (**Figure 3**) consisting of Oasis HLB (200 mg) and a mixture of Strata-X-AW (weak anion exchanger), Strata-X-CW (weak cation exchanger) and Isolute ENV+ (300 mg of total mixture) are used. The conditioning of the cartridges is performed with 3 mL of methanol, 3 mL of water. After conditioning, the samples are loaded in the SPE cartridges. The cartridges are dried by passing air through the cartridges for 0.5 to 1 h (using vacuum on the SPE box; cartridges are visually inspected for complete dryness). The elution of the analytes from the adsorbent material is performed by a basic solution (6 mL of ethylacetate/methanol (50/50 v/v) containing 2% ammonia hydroxide (v/v)), followed by an acidic solution (4mL of ethylacetate/methanol (50/50, v/v) containing 1.7% formic acid (v/v)).

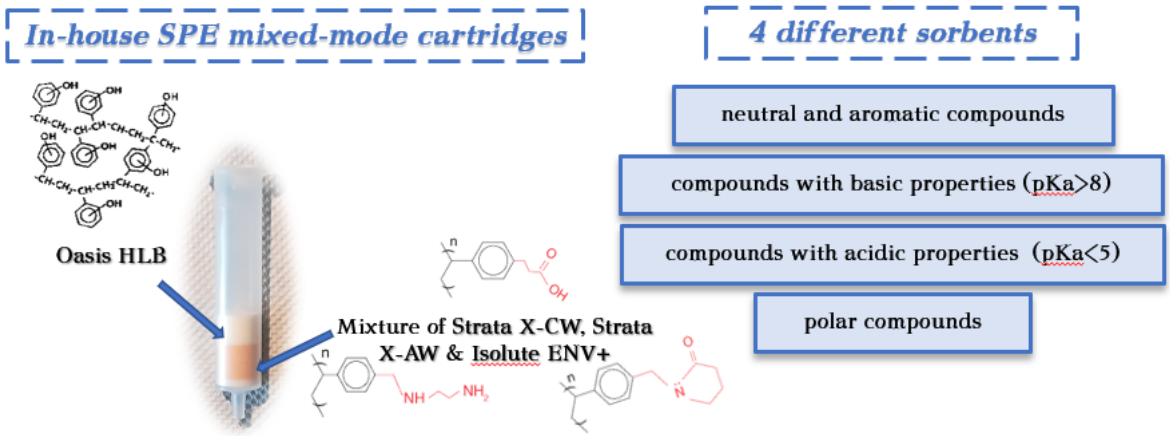

Figure 3. *Mixed-mode SPE cartridges*

- ✓ Evaporation with nitrogen stream at40-45°C, till dryness.
- ✓ Reconstitution with 250 µL (MeOH LC-MS: Milli-Q water) (50:50) and vortex stirring for 1min.
- ✓ Syringe filtering, through Regenerated Cellulose (RC)filter [Chromafil (pore size: 0,2µm, filter diameter: 15mm)] into a 2mL glass vial (an insert is placed inside each vial). Each vial is pre-labeled with the code of the respective sample.

  After the injections in the LC-ESI-QToF MS the vial with the extract is stored, in the freezer (-80°C) in a specific box.

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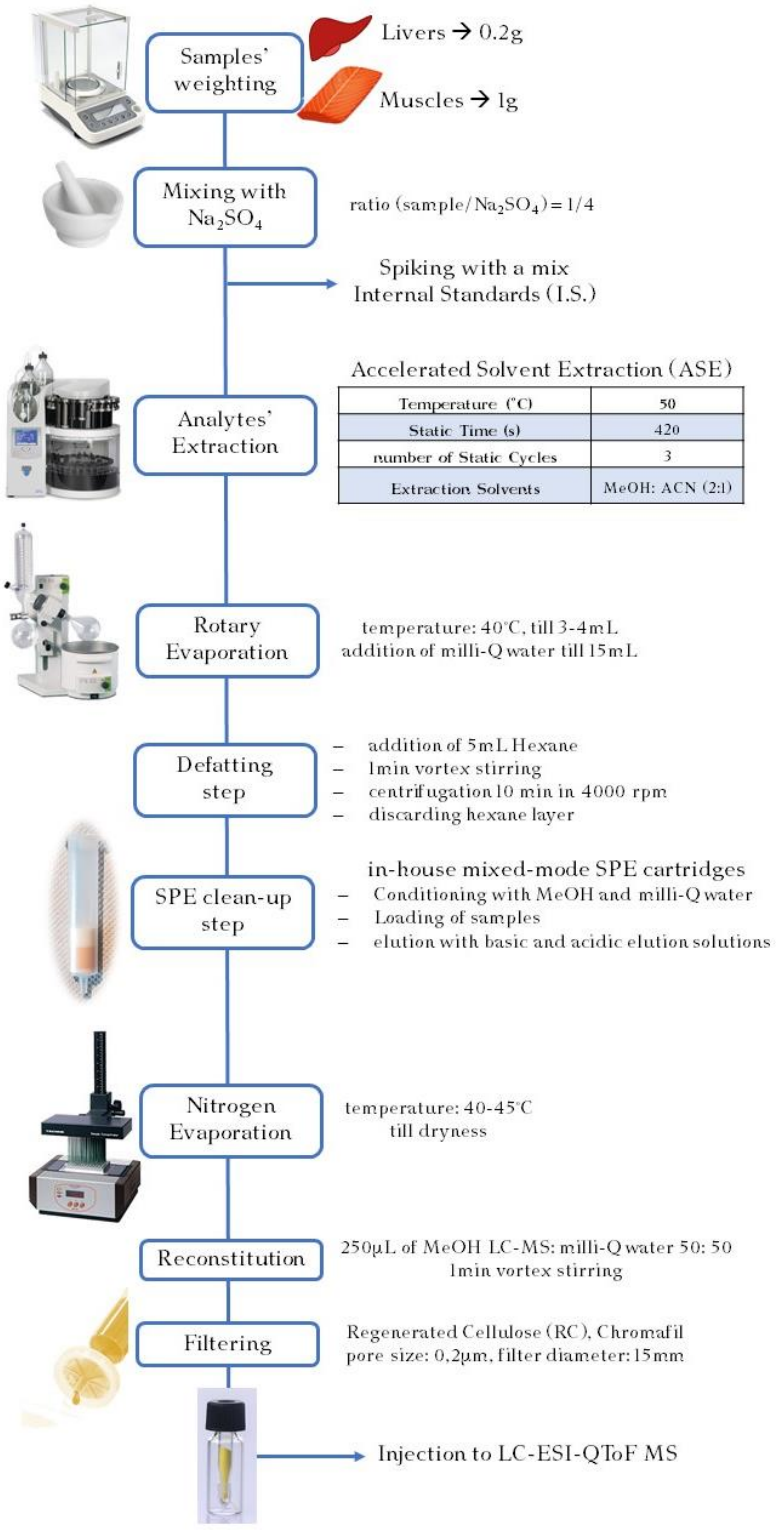

Figure 4. Scheme of the sample preparation protocol for the LC amenable compounds.

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3. Application of generic sample preparation protocol for analysis of AP&P by gas chromatography high-resolution mass spectrometry (GC-HRMS)

The technique of the Accelerated Solvent Extraction (ASE) was also used for the extraction of GC amenable compounds from the biota matrices, followed by a clean-up step using SPE (florisil cartridges). The steps of the sample preparation for the determination of GC amenable compounds are presented in **Figure 5** and described thoroughly in the following steps (bullet points).

- ✓ Weighting of Sample based on the matrix of analysis (muscle or liver), as described in Table 4.
- ✓ Mixing with Sodium Sulfate (Na_2SO_4), which is used as samples' dispersant,with mortar and pestle.

Table 3. Masses of samples and ratios (dispersant/sample).

	sample weight (g)	***ratio (dispersant/sample)***	***dispersant weight (g)***
Muscle	1.0	4	4.0
Liver	0.2	4	0.8

- ✓ Spiking with a representative mixof isotopically labelled internal standards from different classes of compounds of UoA target list and with different physicochemical properties (concentration of mix with IS: 1 mg/L in hexane).
- ✓ Extraction of analytes from matrices using Accelerated Solvent Extraction (Dionex™ ASE™ 350, Thermo Fisher Scientific).

Table 5. ASE Conditions for GC amenable compounds.

Temperature (°C)	100
Pressure (psi)	1500
Heating Time (s)	300
Static Time (s)	300
Number of Static Cycles	3

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Flush Volume (%)	60
Purge Time (s)	180
Extraction Solvent	Hexane: Dichloromethane(67:33)

- ✓ Filtering through filter paper if the extract is not transparent.
- ✓ Addition of 50μL of isooctane as a keeper.
- ✓ Rotary Evaporation (CAUTION: max temperature 30°C) till 10mL.
- ✓ Clean-Up Step: Solid Phase Extraction (SPE).

Sample clean-up is realized by SPE. Strata® FL-PR Florisil [(170 μm, 80 Å), 5 g / 20 mL, Giga Tubes, Phenomenex] cartridges are used. The conditioning of the cartridges is performed using 20mL 10% Isopropanol in Dichloromethane, followed by 30mL of Hexane. After conditioning, the samples are loaded in the SPE cartridges (CAUTION: the elutes are collected during the sample's loading). The elution of theanalytes from the adsorbent material was performed using 20mL ofDichloromethane: Hexane (50: 50), followed by 20mL of Hexane.

- ✓ Addition of 50μL of isooctane as a keeper.
- ✓ Rotary Evaporation (CAUTION: max temperature 30°C) till 10 mL.
- ✓ Addition of 50μL of isooctane as a keeper.
- ✓ Evaporation with nitrogen stream (CAUTION: max temperature 30°C) till dryness.
- ✓ Reconstitution with 250 μL Hexane and vortex stirring for 1min.
- ✓ Syringe filtering, through Regenerated Cellulose (RC) filter [Chromafil (pore size: 0,2μm, filter diameter: 15mm)] into a 2mL glass vial (an insert is placed inside of each vial).Each vial is pre-labeled with the code of the respective sample.
- ✓ After the injections in the GC-APCI-QToF MS the vial with the extract is stored, in the freezer (-80°C) in a specific box.

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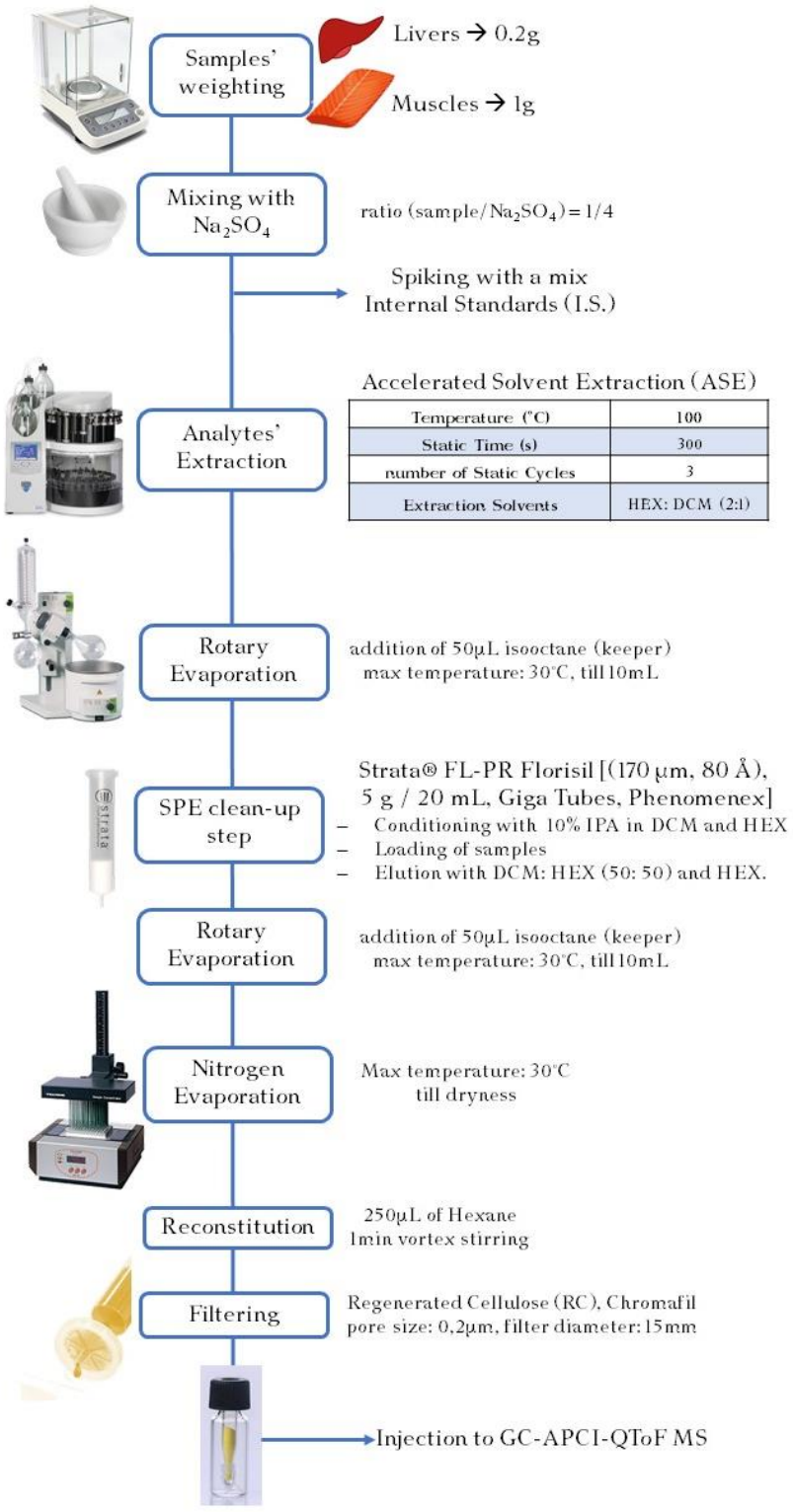

Figure 5. An overall of the sample preparation protocol for the GC amenable compounds.

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4. Data-processing of data acquired using LC and GC coupled to high-resolution mass spectrometry (HRMS)

The chapter demonstrates how to upload LC and GC-HRMS data to DSFP. The first part includes actions to be taken before and during the instrumental analysis (**Figure 6**), whereas the next part explain the actions need to be taken after the instrumental analysis depending on the instrument vendor.

The following actions should be taken before the analysis of the samples:

1. Follow the sample-preparation protocol established by your laboratory, prepare the extracts and reconstitute them according to the selected protocol. Add internal standards to the samples before applying the sample preparation protocol to assure that the extraction was efficient. Prepare field blank or/and procedural blank samples to capture any unintentional contamination. Spike selected samples at the end of the sample preparation procedure (matrix-matched samples) to evaluate the accuracy of the method and obtain recovery values. Follow all needed quality control and quality assurance measures established by your laboratory. All samples intended to be analysed by LC-HRMS should be reconstituted to the vial, containing an organic phase (most commonly methanol) and some water, which is essential for the chromatography to work properly.

2. Add as much water as needed to the RTI mixture in the vial to achieve the same reconstitution proportion as the other extracts (e.g. 50% H_2O and 50% MeOH).

3. Prepare sufficient quantity of filtered mobile phases according to the protocols of your laboratory to run all the samples in one batch.

4. Make sure that column is well-equilibrated, and that ion source is clean.

5. Recalibrate the HRMS before starting the sequence according to the vendor instructions. Calibrant peaks should cover the selected mass range.

The following actions should be taken during the analysis of the samples:

1. Use a reversed-phase chromatographic column and gradient program according to your laboratory protocol. The LC method should re-equilibrate the column for the next injection. The first injection should be regarded as chromatographically uncalibrated and

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should be excluded from subsequent analysis. Make sure that internal standards and spiked compounds are eluted in the expected retention time to verify that the analysis is going as expected.

2. Each extract should be injected in data-independent acquisition (DIA). DIA records all detected masses at low and high collision energy without any prior mass isolation. This, results in complex spectral information suitable for wide-scope suspect screening methods, but less suitable for identification of unknown compounds through non-target screening. Instruments are fast enough and can record one low and one high energy spectra in less than 1 s. Thus, instruments can record low and high collision energy within a single run, either by default or using specific settings. This or any similar approach can be applied as long as the scan rate is not severely affected, i.e. sufficient MS^1 full scan points are required for the DSFP.

3. Depending on the purpose of the experiment, inject the extract in data-dependent acquisition (DDA) as many times as necessary. With this acquisition mode, pre-selected masses are isolated, fragmented and MS/MS spectra are recorded. This mode is ideal for identification of unknown masses of interest. It is recommended to record MS/MS spectra of as many precursors as possible. The DSFP can store these chromatograms as well.

4. Repeat the same injections for the other samples in the sequence

5. In the middle of the sequence, inject the RTI calibrant mixture in full scan mode and record the experimental retention time of the calibrant substances. This will enable you to use retention time index prediction to support the tentatively identified compounds with extra experimental evidence.

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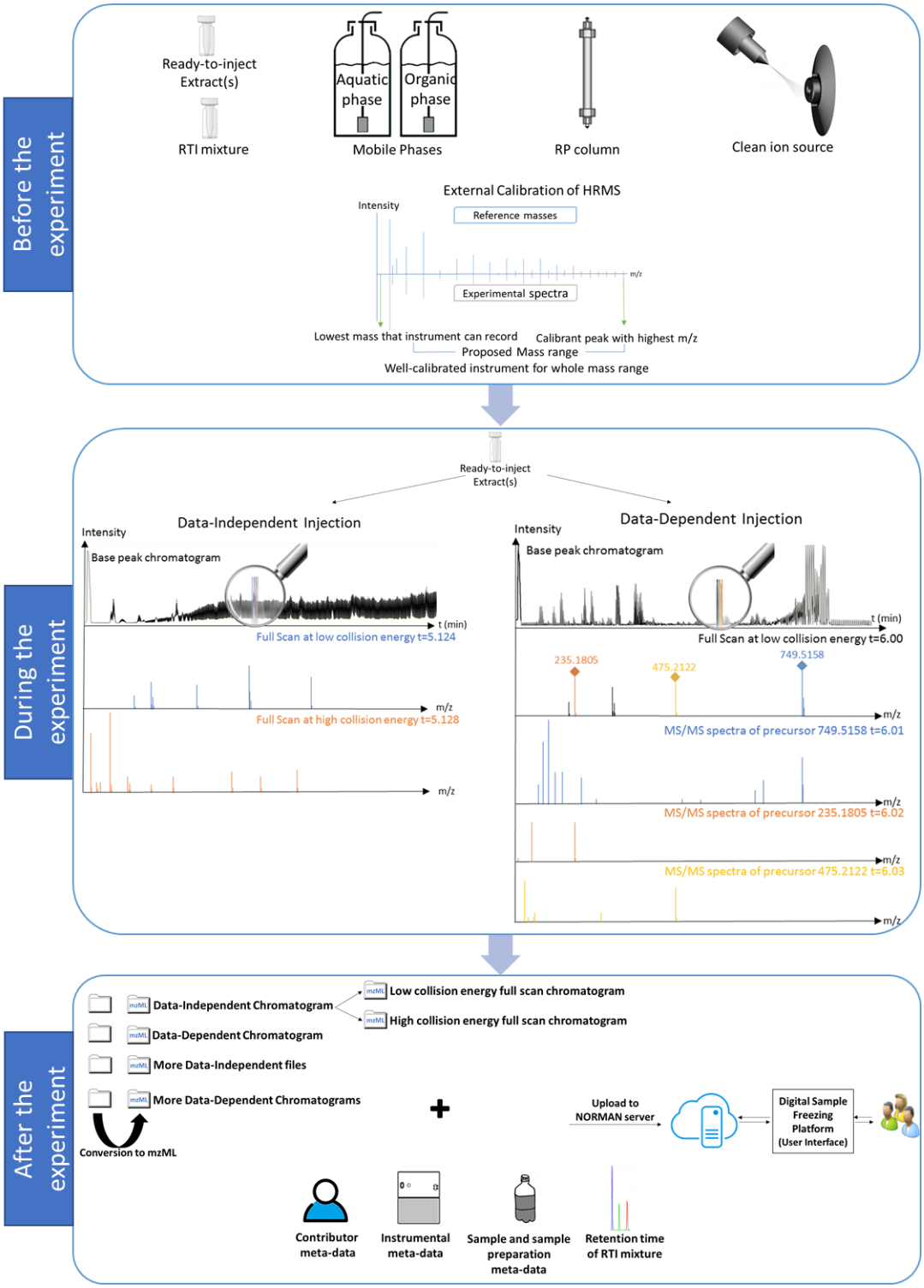

Figure 6. Steps to be followed to contribute LC-HRMS data to Digital Sample Freezing Platform (DSFP). Before starting the experiment, the instrument should be in good condition and well-calibrated. During the experiment, inject the samples in data-independent (DIA) and data-

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dependent acquisition (DDA). Convert the files to mzML and contribute them together with instrumental, sample preparation and RTI information to DSFP

4.1. Post-data acquisition of Bruker files

4.1.1. Conversion of Bruker .d data files to mzML

Bruker files (.d files) can be converted to mzML by Bruker CompassXport (***Figure 7***), which is embedded in DataAnalysis software provided by Bruker. Proteowizard software (**Figure 16**) can also be used for conversion. However, Proteowizard software exports uncalibrated Bruker mzML data files. Therefore, the use of Bruker CompassXport is recommended until the next version of Proteowizard incorporates an update to fix this issue. Most commonly, the calibrant substance is injected in the beginning of each chromatographic run, using a multi-port valve and chromatograms are recalibrated offline based on the experimentally observed and the theoretical *m/z*. Once the files are recalibrated, they can be exported by the following option on the Menu of Bruker DataAnalysis software: **File>Export>Chromatogram Analysis**. The same export approach can be used for files acquired in DDA. The disadvantage of the method described above is that files are processed one by one at a time. Extended tests, however, has shown that this way of recalibration and export, assures the lowest possible mass error and allow reliable conversion. The DDA mzML files can be uploaded to DSFP as they are, while DIA files should be separated as described in the next section.

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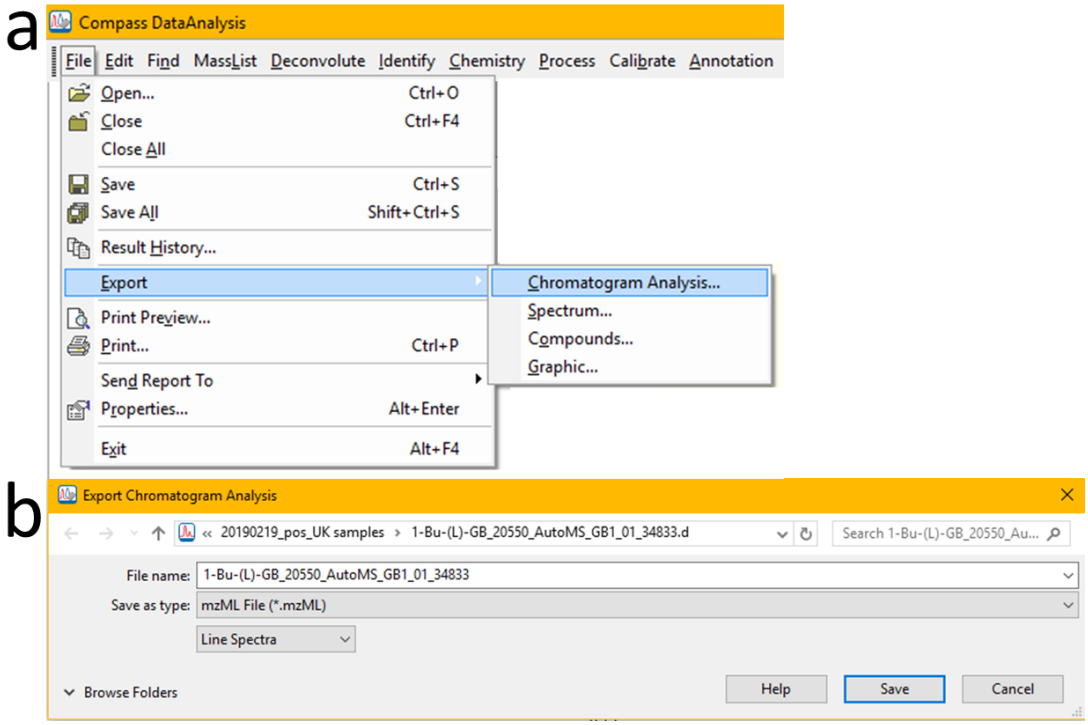

Figure 7. Export of Bruker .d files using Bruker CompassXport v. 3.0.9.2 through Bruker DataAnalysis v.4.3.

4.1.2. Separation of bbCID collision energy channels

Files acquired in DIA (termed as broadband collision induced dissociation (bbCID)) are further processed to separate the two collision energy channels by a web-tool integrated in DSFP. The tool in available in the website under the choice **More tools>Split Data-Independent data** (***Figure 8***). The user can browse the mzML file (**Figure 8b**), insert an intensity cut-off value (**Figure 8d)** and calibrant scan numbers to be removed from the mzML (**Figure *8*e).**

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Figure 8. Split of DIA collision energy layers in Bruker .d files.

"Intensity cut-off" is a numeric value below which spectral peaks are eliminated in all full-scan spectra. Setting an appropriate intensity cut-off value can reduce the size of the produced mzML file drastically and keep intact all the analytical information. The optimum "intensity cut-off" value is the digital noise of the photomultiplier detector. The value is dependent on the instrument vendor and may be different among different models of the same vendor. However, it can be easily determined by the user when the mass spectrometer is "on" and isolated from liquid chromatography. The intensity of the random noise that appears and

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disappears should be used as the “intensity cut-off”. In case, DSFP does not recognize which full-scans belong to which collision energy layer (depends on the information contained in the mzML file), the user should specify the number of the collision energy channels, the collision energy applied (***Figure 8f***) and which full-scan spectra belong to which collision energy layer (**Figure 8g**). It is advised that the same instrumental method is used for analysis of samples. In this case, the field **g (Figure 8g**) will always be the same. DSFP will separate the collision energy channels and appear download buttons, so that the user downloads the mzML files (**Figure 8h** and **Figure 8i**). The converted files (collision layer separated mzML files and the Bruker .d file) contain identical information which is indicated by the base-peak chromatograms for low (**Figure 9a** and **Figure 9b**) and high collision energy channels (**Figure 9c** and **Figure 9d**).

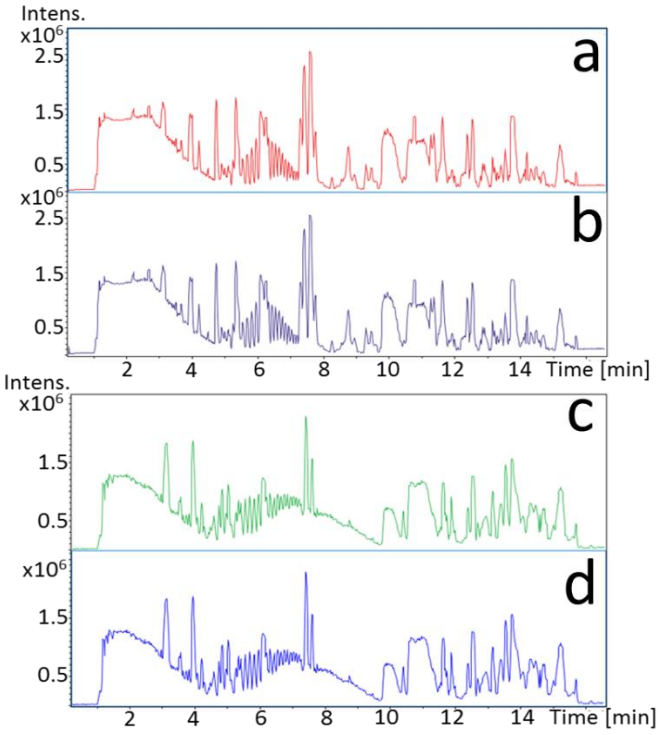

Figure 9. a) Full-scan MS1 (4 eV) base-peak chromatogram (.d file), b) Full-scan MS1 (4 eV) base-peak chromatogram (.mzML), c) Full-scan high collision energy (25 eV) layer base-peak chromatogram (.d file), d)Full-scan high collision energy (25 eV) layer base-peak chromatogram (.mzML file)

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4.1.3. Contribution of HRMS chromatograms to DSFP

Contribution of LC-HRMS chromatograms to DSFP is possible through the option **Contribute** available in the top bar menu (**Figure 10**). In the tab **Basic Information**, the user should specify the institute name, which will auto-fill many fields (e.g. instrumental meta-data, retention time of calibrant substances, etc.). However, basic information for the contributed samples should be specified (**Figure 10b**; instrument type, short name of the sample, title of the project, location of the sample, date of collection and analysis, and enrichment factor).

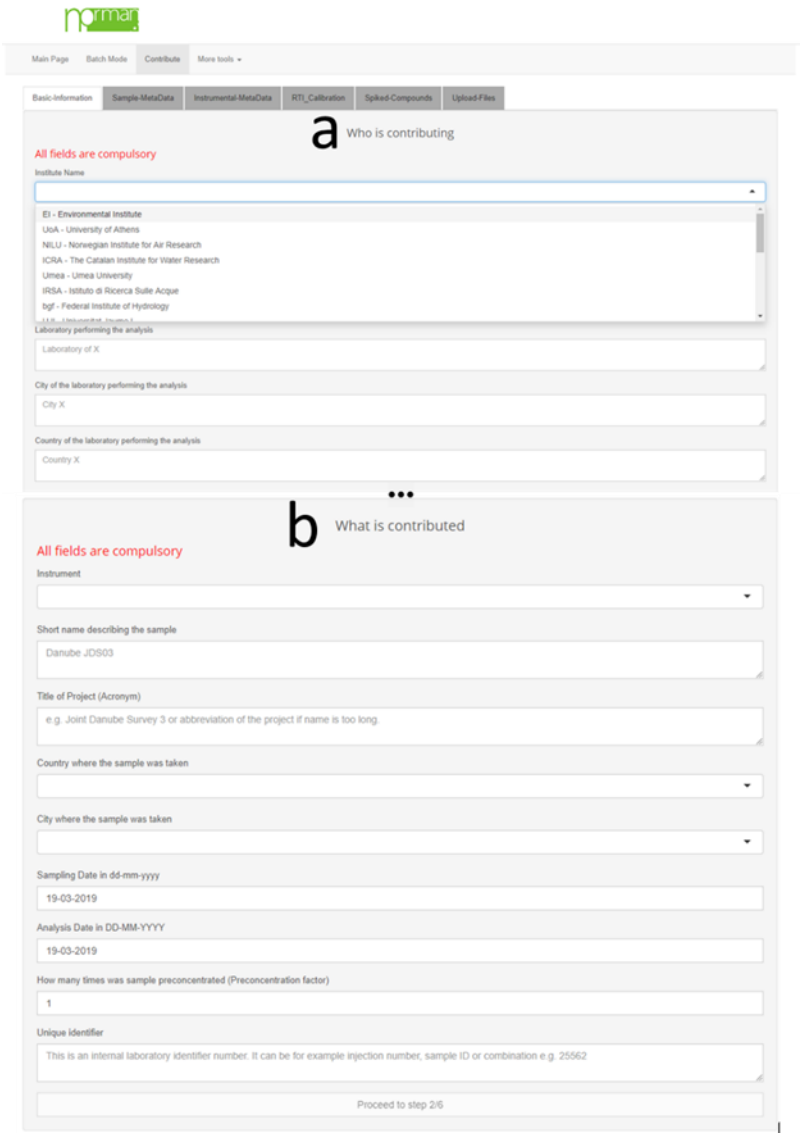

Figure 10. Screenshot from the **Contribute** module of DSFP at step 1 (**Basic Information**).

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The location of the sample can be accurately specified in the next step (**Sample Meta-Data; Figure 11**), if the user inputs the exact coordinates of the sample location. In cases in which the exact location of the sample should not be revealed or is not known, the user can remove the latest digits of the decimal coordinates. Afterwards, the user must specify the type of environmental sample collected and input critical matrix-dependent meta-data information. In the next step (**Figure 12**) the ionisation and the instrumental information are specified.

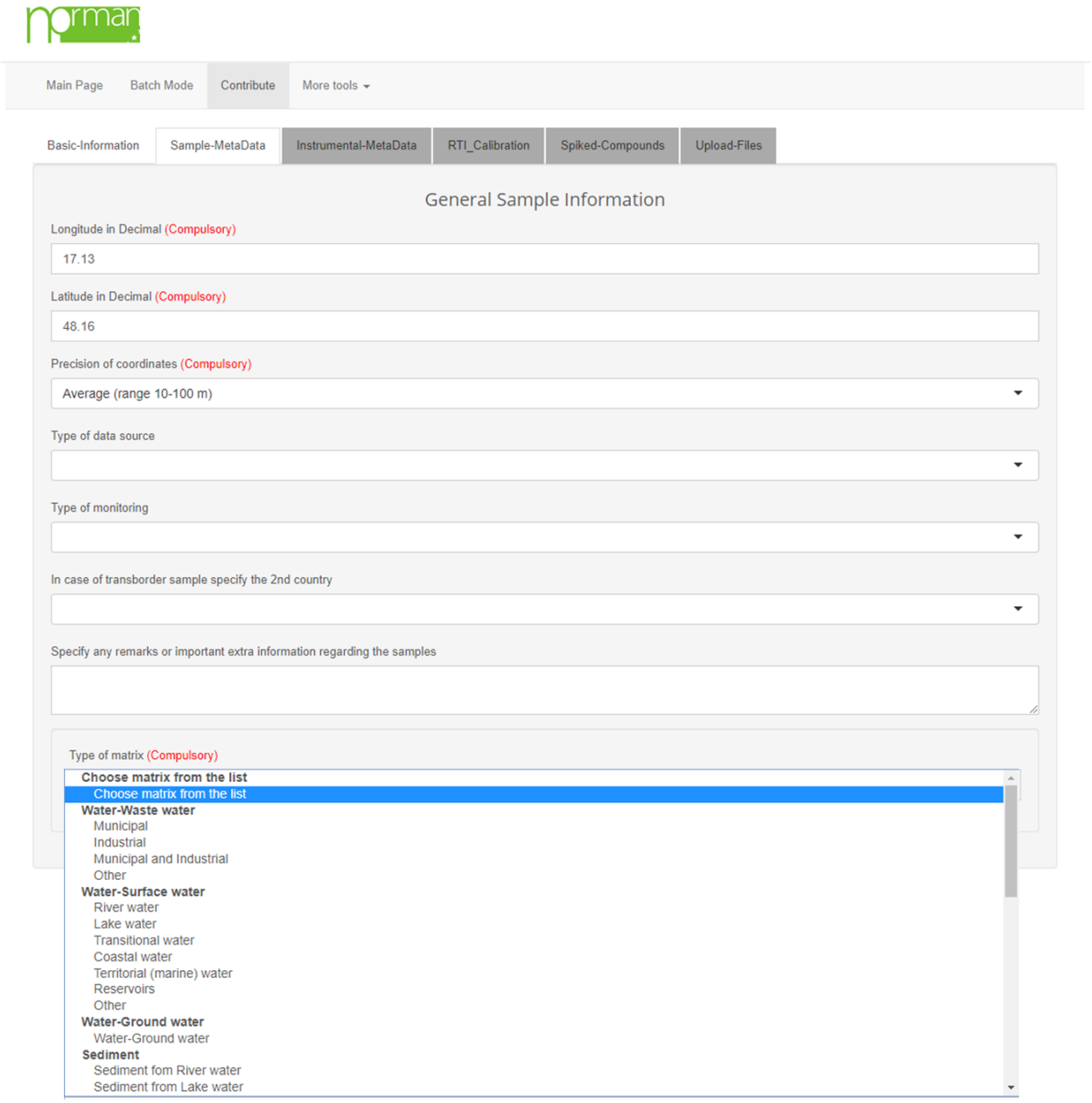

Figure 11. Screenshot from the **Contribute** module of DSFP at step 2 (**Sample Meta-Data**).

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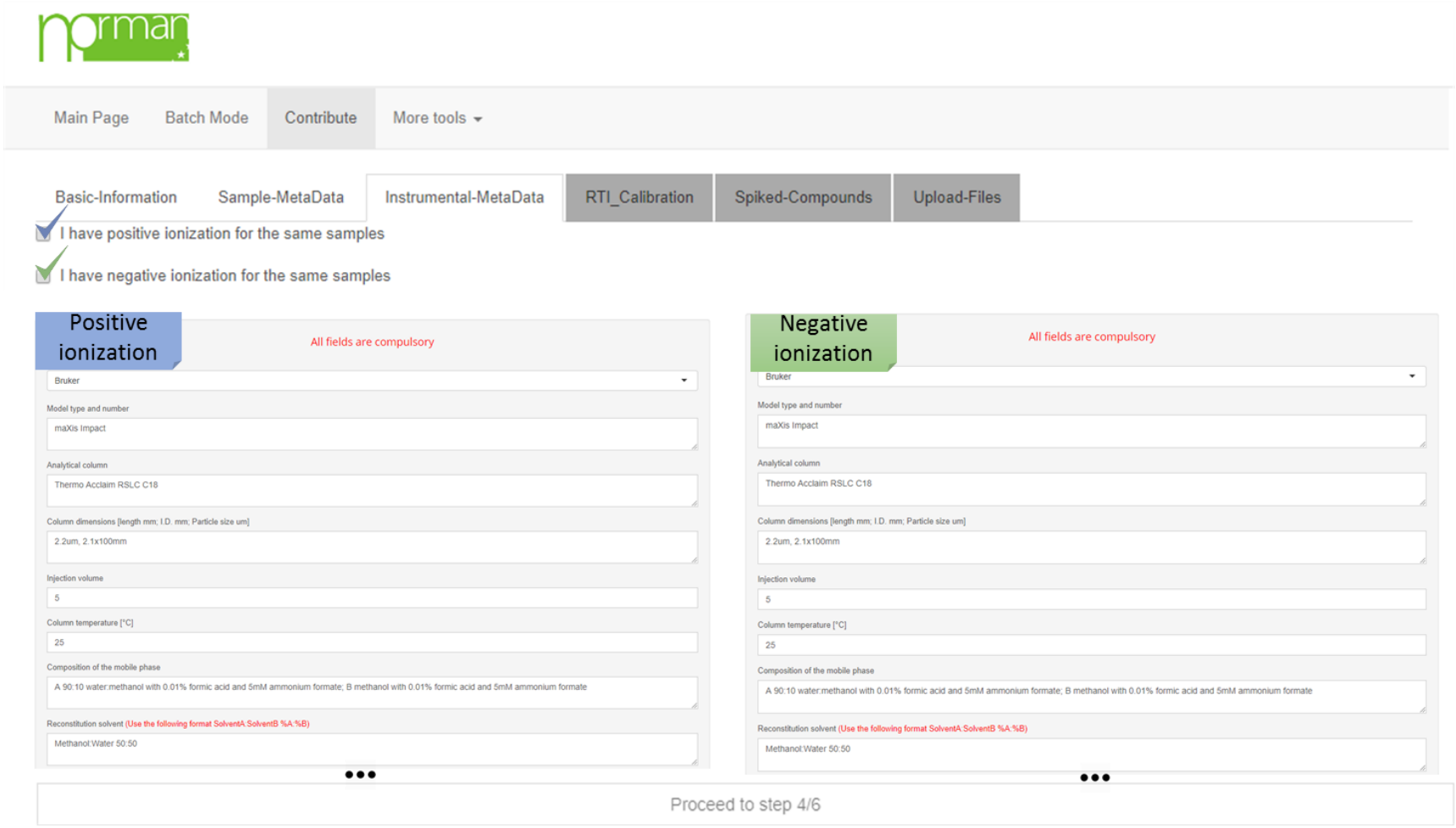

Figure 12. Screenshot from the **Contribute** module of DSFP at step 3 (**Instrumental Meta-Data**).

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Depending on the selected ionization(s) in previous step (**Figure 12**), the respective RTI calibrant table(s) will appear (**Figure 13**). In this step, the user should specify the retention time of the calibrant substances as indicated in the red box of **Figure 13**. Once the user proceeds to the next step, a list of spiked compounds is requested. More specifically, the spiked concentration level and the observed area or intensity must be specified (**Figure 14**). This optional step allows the semi-quantification of the detected suspects during the batch-mode screening process. It is highly recommended to fill in the table. Overall, it is valid that the more information and meta-data is provided to the system, the better results will be acquired during the batch-mode screening process.

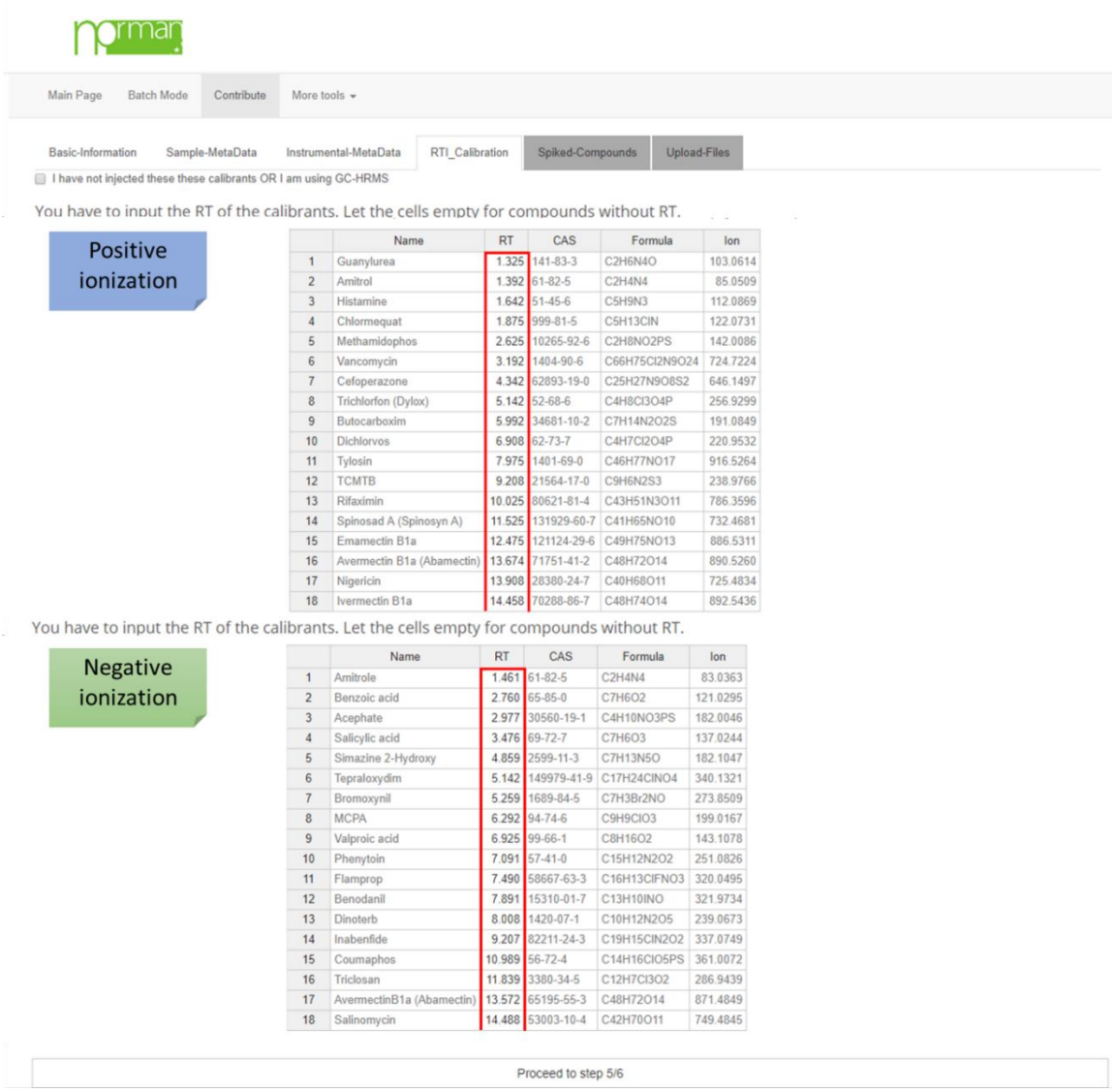

Figure 13. Screenshot from the **Contribute** module of DSFP at step 4 (**RTI calibration**).

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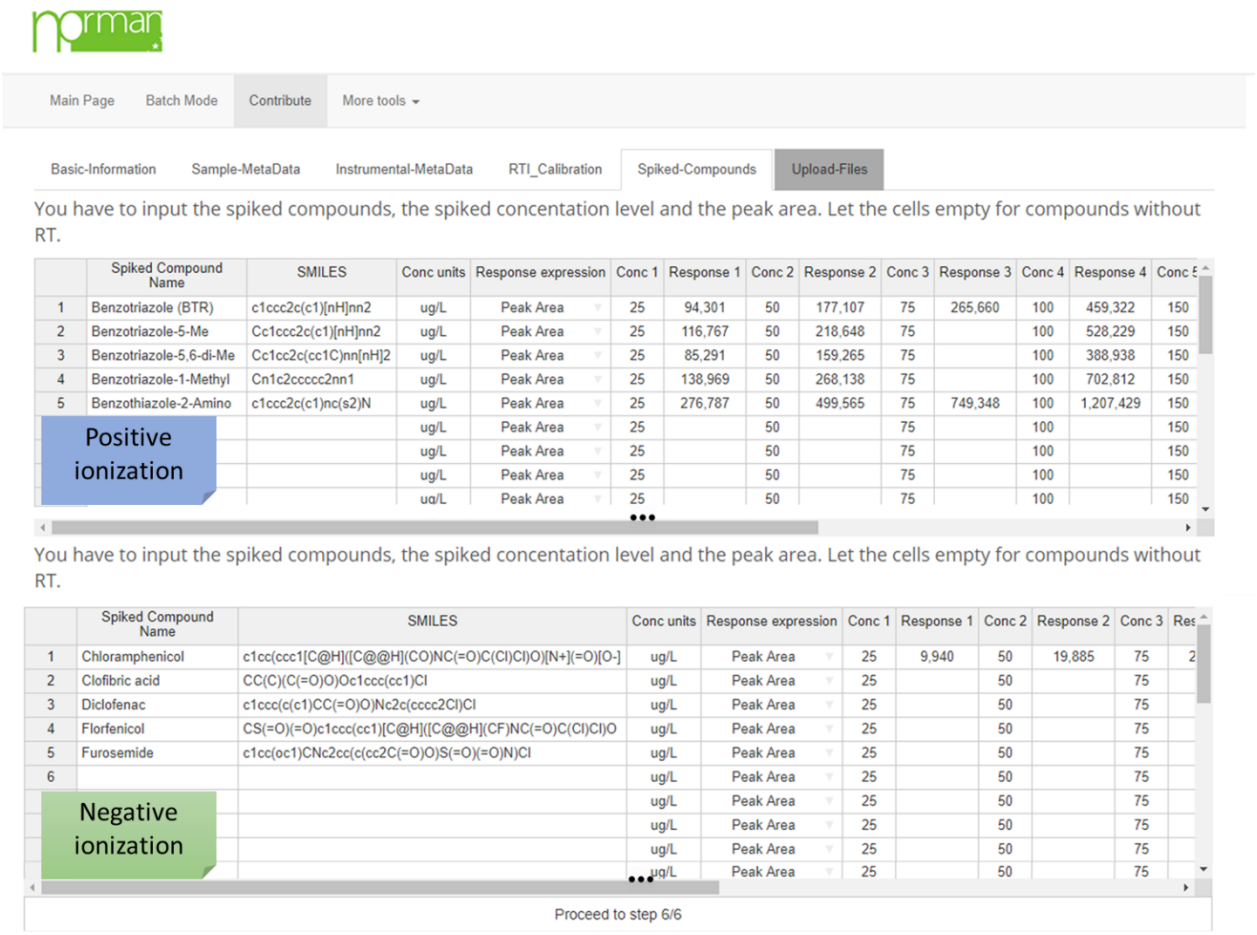

Figure 14. Screenshot from the **Contribute** module of DSFP at step 5 (**Spiked compounds**).

The final step in the procedure is to upload the mzML files (**Figure 15**). The user should select the MS^1 full-scan file (**Figure 15a**). If the user has analysed the sample in DIA, it is requested to specify the number of DIA collision energy channels (**Figure 15b**). Browse buttons (**Figure 15d and Figure 15e**) will appear depending on the number of DIA collision energy channels (input to the field **Figure 15c**). If the sample has been also analysed in DDA method, the user should answer positively (**Figure 15f**) and upload the DDA chromatogram in the respective browse button (**Figure 15g**). If the user has specified in previous steps that chromatograms are available in negative ionisation, then the same fields will also appear for the negative ionisation. Once the user uploads the mzML files, the submit button will be activated. All chromatograms and information provided will be analysed by the DSFP. The progress is indicated by a loading bar. The output of the procedure is reflected in the data collection template (DCT) excel file, which can be downloaded (**Figure 15h**). The next sample can be

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uploaded by clicking the tab "**Basic Information**". All previous details remain unchanged, which makes the procedure of uploading of the second sample faster.

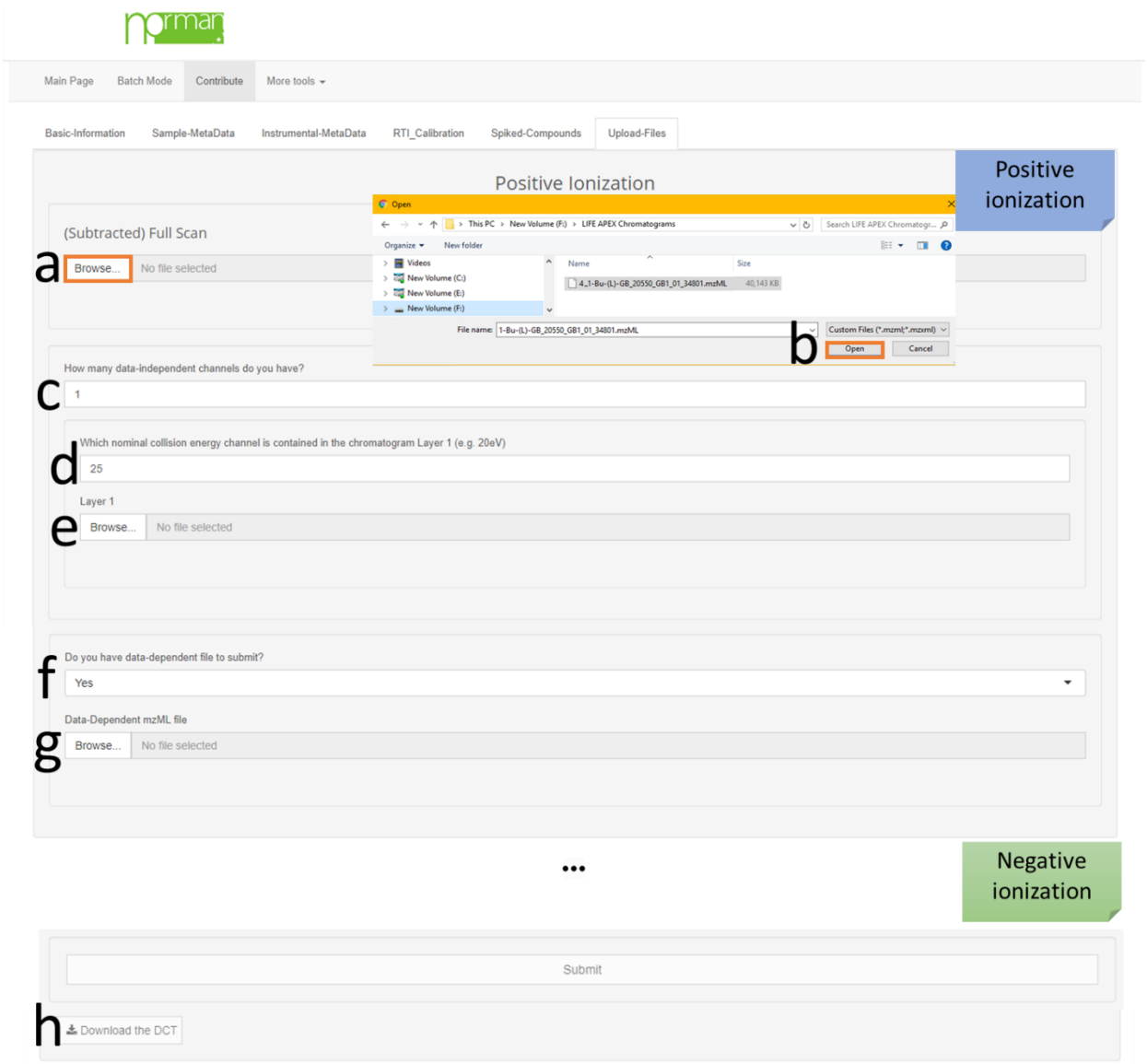

Figure 15. Screenshot from the **Contribute** module of DSFP at step 6 (**Upload Files**).

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4.4 Post-data acquisition of Agilent files

Agilent files can be converted using Proteowizard software (**Figure 16**), which is available to be downloaded in the following link (http://proteowizard.sourceforge.net/downloads.shtml). After installing Proteowizard, use "MSConvert" to convert the files to mzML. Select the files that you want to convert by clicking on browse button. Submitting a conversion request without adding an intensity cut-off may result in mzML files of few Gigabytes. Therefore, it is recommended to enable a filter called "**Threshold Peak Filter**" with "**Count**" as threshold type, "**Most intense**" as orientation and "**Value**" equal to the noise level of the spectral peaks generated by the detector (digital noise). This choice is available under the menu "Filters". The cut-off value should be equal to the digital noise of the photomultiplier detector. If the data is not recorded in centroid mode, enable the filter "**Peak Picking**" (already enabled in **Figure 16**). Afterwards, the user should press add and click the start button. The files will be converted to mzML by default in the same path of the Agilent .D files unless otherwise specified in the output directory field. DDA mzML files are ready to be uploaded to DSFP.

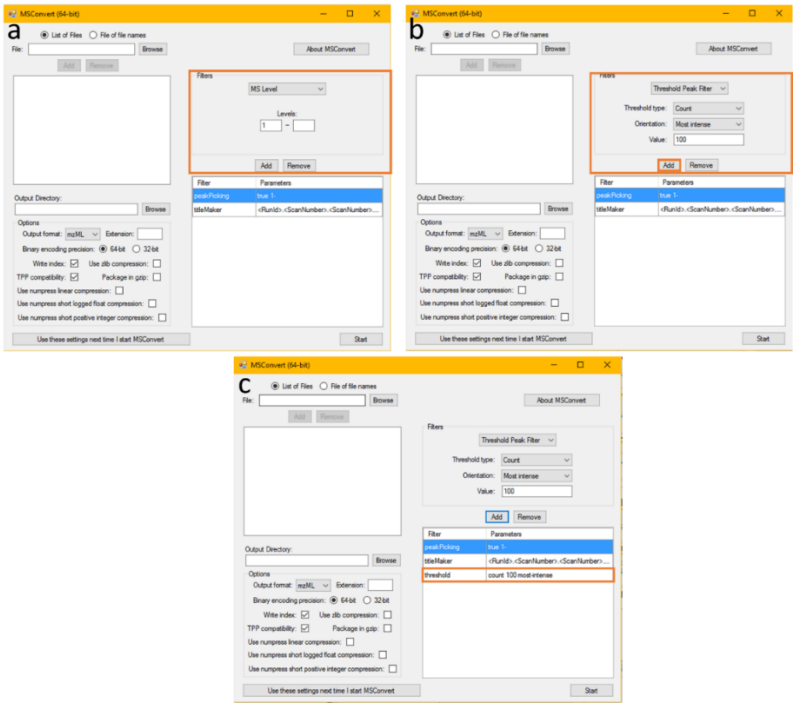

Figure 16. Msconvert.exe from ProteoWizard. The figure demonstrates how to set an intensity cut-off threshold to avoid having large mzML files.

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DIA files contain different collision energy layers which can be split using the tool in DSFP under the option **More tools**>**Split Data-Independent Data** (**Figure 17**). The user can browse the mzML file, set an intensity cut-off value and remove any unwanted scans. If the cut-off value is equal or below the cut-off value set in Proteowizard software, no further data reduction and no further spectral peak removal is applied. The scans of mzML files coming from the conversion of Agilent .D files are automatically recognised. Therefore, the split of the different collision energy layers is straight forward; the user must click on the Submit Processing button, a loading bar will appear and once the split is done, the download buttons will appear.

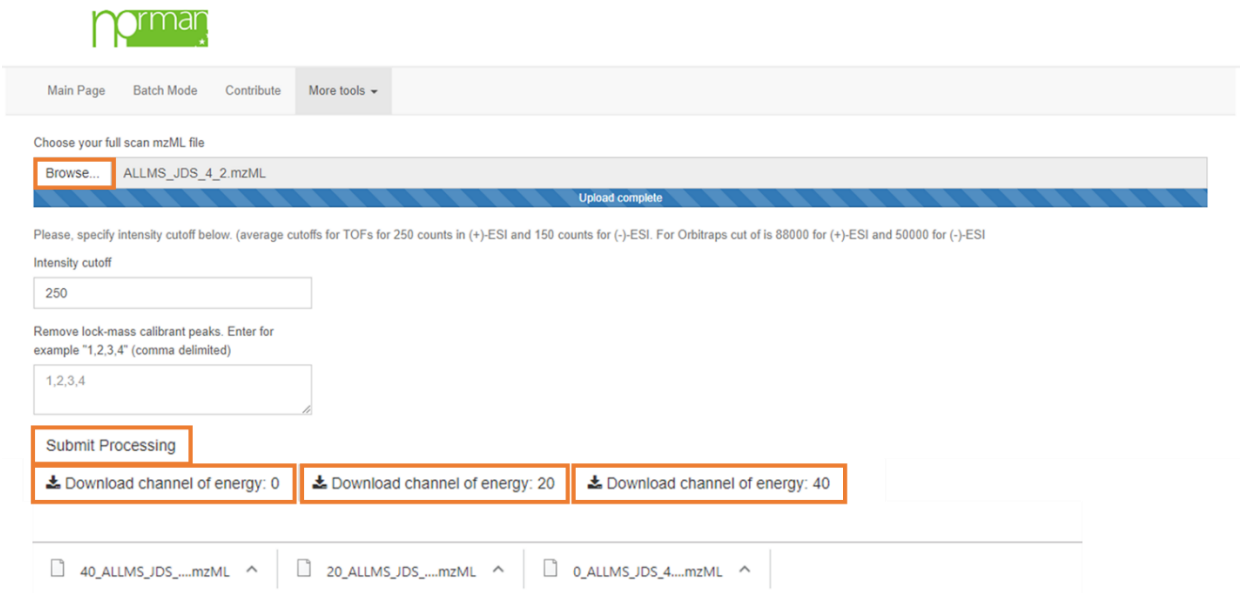

Figure 17. Split of DIA collision energy layers in Agilent .D files.

By default, the DIA method of Agilent HRMS, has two collision energy layers (one at 20 eV and one at 40 eV). Thus, the contribution procedure is the same as the one described in section **04.1.3. Contribution of HRMS chromatograms to DSFP**. The only difference is the number of DIA channels (**Figure 18a**), which should be two. This will add two browse buttons, one for the 20 eV collision energy (**Figure 18b**)channel and another one for the 40 eV collision energy (**Figure 18c**).

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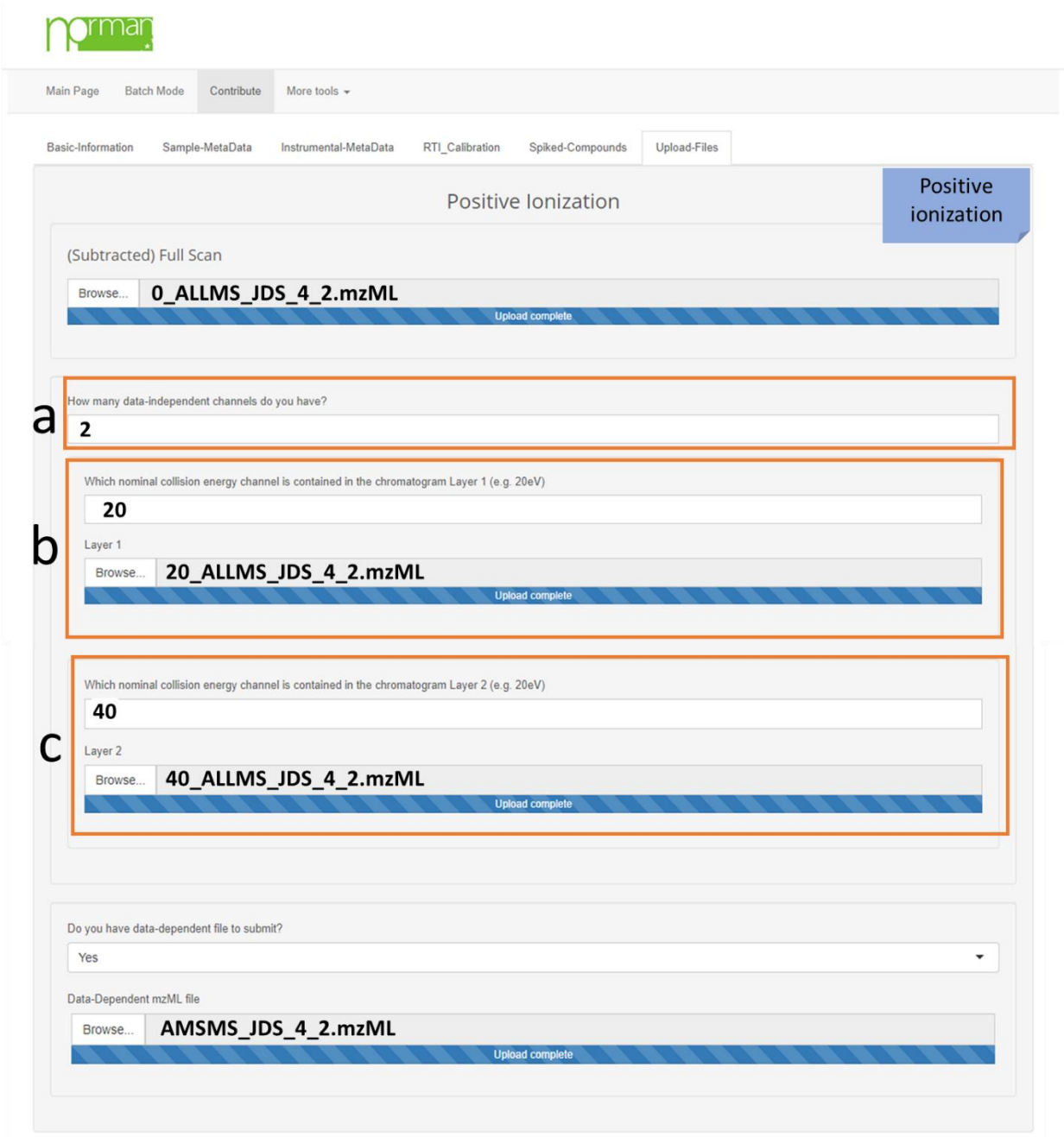

Figure 18. Screenshot from the **Contribute** module of DSFP at step 6 (**Upload Files**) for Agilent files, which have two DIA collision energy channels (20 and 40 eV).

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4.5 Post-data acquisition of Waters files

Waters files can be converted to mzML using either vendor software or Proteowizard (http://proteowizard.sourceforge.net/downloads.shtml). After installing Proteowizard, use "MSConvert" to convert the files to mzML. Select the files that you want to convert by clicking on browse button. Submitting a conversion request without adding an intensity cut-off may result in mzML files of few Gigabytes. Therefore, it is recommended to enable a filter called "**Threshold Peak Filter**" with "**Count**" as threshold type, "**Most intense**" as orientation and "**Value**" equal to the noise level of the spectral peaks generated by the detector (digital noise). For Waters QTOF "Value" parameter should be 350 counts. However, the value should be carefully selected, because this value depends on the detector of each instrument. Data should be recorded in centroid mode, otherwise enable filter "**Peak Picking**". DDA files can be uploaded to DSFP as they are. On the contrary, the DIA mzML files need further processing so that the collision energy layers are separated (example in **Figure 19**). **Figure 19a** represents the base peak chromatogram of a sample. Waters QTOF record sequentially MS^1 and MS^e full scans as other QTOF vendors. The difference is that a calibration lock-mass full-scan occurs at a fixed number of full-scans (e.g. every 56 scans as shown in **Figure 19a**). These lock-mass full-scans should be removed. A tool to separate DIA LC-HRMS data to low and high collision energy channels and remove the lock-mass full scan spectra is integrated in DSFP.

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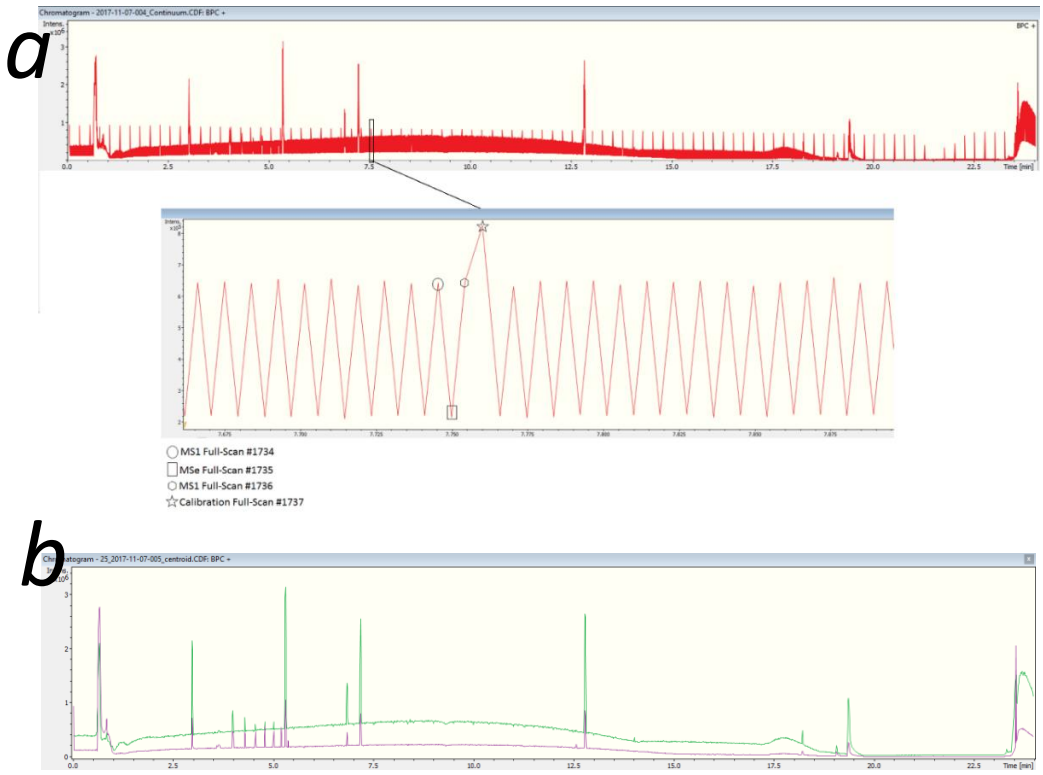

Figure 19a). Example of Waters DIA chromatogram with lock-mass calibration every 56 scans. B). Chromatogram after the separation of the two collision energy layers (MS^1 layer is green and MS^e is purple).

On the top bar menu of DSFP, there is an option **More tools**>**Split Data-Independent Data** to remove the unwanted lock-mass full-scan spectra (**Figure 20a**) and separate the different collision energy layers (**Figure 20b** and**Figure 20c**). Waters vendor software may be used to visualize the scans and specify which scans should be discarded and which not. If the uploaded mzML contains information about the applied collision energies, then the system will request no further information. Otherwise, it will be required to specify the number of collision energy channels, the nominal collision energy of each channel and the scan numbers that correspond to each collision energy channel (**Figure 20**).

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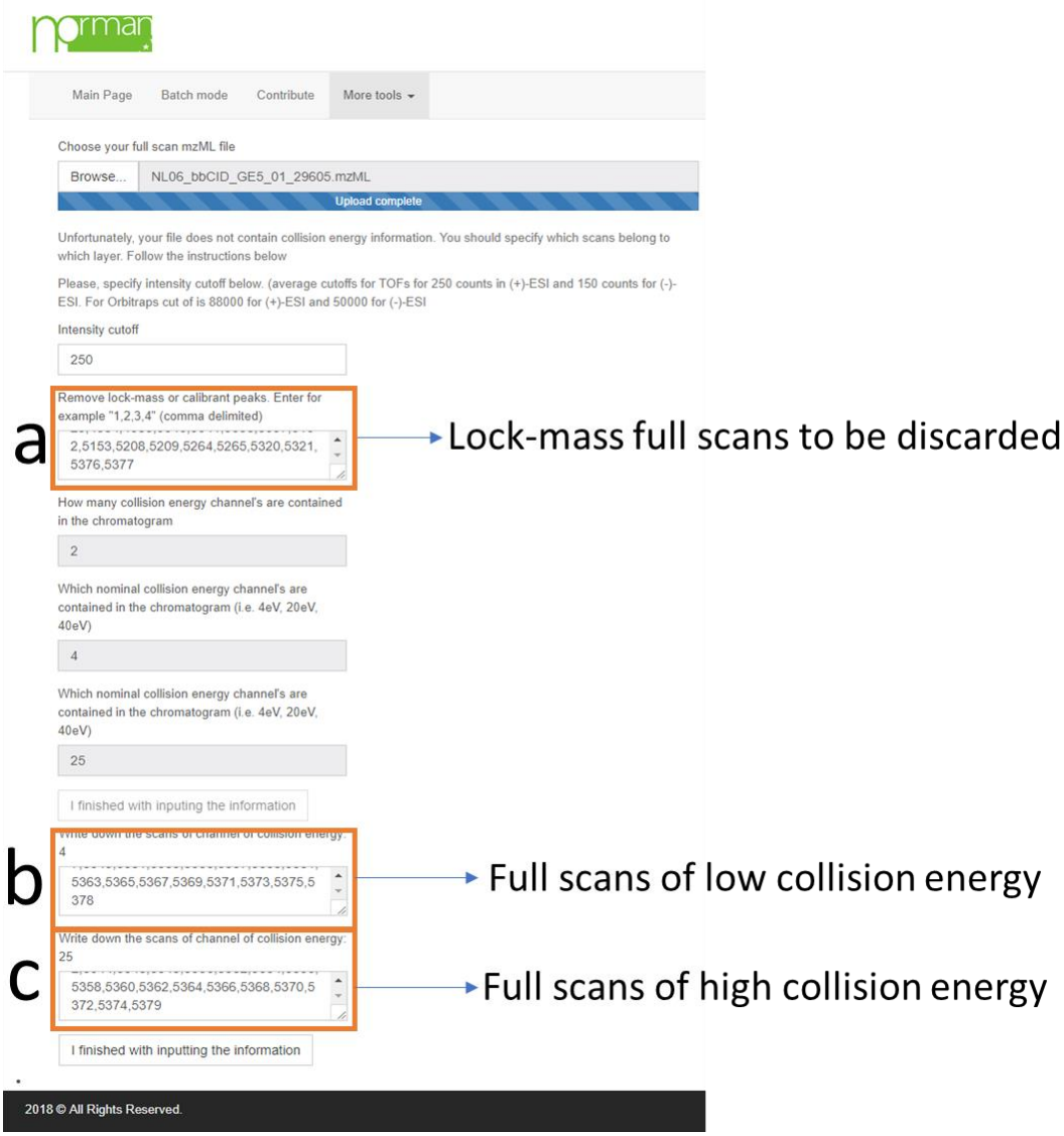

Figure 20. Screenshot from the integrated tool in DSFP, which helps to separate the different layers from DIA LC-HRMS data

Once the separated collision energy channels are saved and the DDA file is saved (If any), select **Contribute** option from the top bar menu and follow the procedure as described in section **4.1.3. Contribution of HRMS chromatograms to DSFP**. DSFP will guide you step-by-step during the upload.

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4.6 Post-data acquisition of Thermo fisher scientific files

Users of Thermo fisher scientific HRMS instruments (hybrid ion-traps-orbitraps and quandrapole-orbitraps) employ in most cases the DDA method. However, it is possible that samples are analysed by DIA method if the isolation window is set as wide as the scan range. The DDA files can be converted to mzML with Proteowizard software (http://proteowizard.sourceforge.net/downloads.shtml) with the same way as previously described (**Figure 16**).

Briefly, use "MSConvert" included in Proteowizard to convert the .raw files to mzML. Simply, select the files that you want to convert by clicking on browse button (**Figure 16**). You may enable a filter called "**Threshold Peak Filter**" with "**Count**" as threshold type, "**Most intense**" as orientation and "**Value**" equal to the noise level of the spectral peaks generated by the detector (digital noise). This choice is available under the menu "Filters". The cut-off value should be equal to the digital noise of the photomultiplier detector. Unlike other vendors, the "**Count**" value is few orders of magnitude higher in Thermo fisher scientific HRMS instruments. This happens, because the detectors in Thermo fisher scientific instruments provide signals with number of counts of many orders of magnitude higher than other vendors. A typical "Count" cut-off value is 80,000 for positive ionization and 50,000 for negative ionization. However, these cut-off values are not valid for all Thermo fisher scientific HRMS instruments. In the newest models of Thermo fisher scientific, the resulting mzML files have reasonable sizes (few MB), even if no "**Threshold Peak Filter**" is applied. In these cases, it is not recommended to apply "**Threshold Peak Filter**", since it is not needed. If the data is not recorded in centroid mode, enable the filter "**Peak Picking**" (already enabled in **Figure 16**). Afterwards, the user should press add and click the start button. The files will be converted to mzML by default in the same path of the .raw files, unless otherwise specified in the output directory field.

DDA mzML files can be uploaded to DSFP without any other action. In case, DIA files are available, conversion of .raw files is performed the same way as DDA. The difference is that the collision energy-layers of DIA files need to be separated. The separation of the collision

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energy layers is straight forward and possible through the tool in DSFP (**More tools**>**Split Data-Independent Data**) described in **Figure 17**.

The mzML files together with the meta-data can be uploaded to DSFP through the "Contribute" option on the top bar menu. The contribution procedure is the same as the one described in section **04.1.3. Contribution of HRMS chromatograms to DSFP**.DSFP will guide you step-by-step during the upload. The field "(Subtracted) Full Scan File" (shown in **Figure 15a**) is mandatory. The DDA mzML file can be uploaded in this field, and also the same file in the field "Data-Dependent mzML file" (shown in **Figure 15g**). If the sample has been analysed in DIA acquisition, then it is recommended to upload the lowest collision energy channel of the DIA run in the field "(Subtracted) Full Scan File" (shown in **Figure 15a**).

4.7 Post-data acquisition of AB Sciex

AB Sciex SWATH DIA acquisition has not been tested yet. However, no significant problems are foreseen with such "fragmented" DIA acquisition method. AB Sciex DDA data can be converted with Proteowizard (http://proteowizard.sourceforge.net/downloads.shtml) as data from other vendors (**Figure 16**). After installing Proteowizard, use "MSConvert" to convert the files to mzML. Select the files that you want to convert by clicking on browse button. It is recommended to enable a filter called "**Threshold Peak Filter**" with "**Count**" as threshold type, "**Most intense**" as orientation and "**Value**" equal to the noise level of the spectral peaks generated by the detector (digital noise). However, for AB Sciex data, very low "**Value**" should be chosen (1 until 10 counts), since intensity values are very low. If the data is not recorded in centroid mode, enable the filter "**Peak Picking**" (already enabled in **Figure 16**). Afterwards, the user should press add and click the start button. The files will be converted to mzML by default in the same path of the AB Sciex .wiff files, unless otherwise specified in the output directory field.

DDA mzML files are ready to be uploaded to DSFP following the steps described in section **04.1.3. Contribution of HRMS chromatograms to DSFP**. DSFP will guide you step-by-step during the upload. The field "(Subtracted) Full Scan File" (shown in **Figure 15a**) is mandatory. The DDA mzML file can be uploaded in this field, and also the same file in the field "Data-Dependent mzML file" (shown in **Figure 15g**).

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5.SOP for the determination of PCBs, HCB and PBDEs in AP&P samples

Adequate cleaning of analytical glassware is an essential lab procedure to avoid interference and determine PCBs, HCB and PBDEs at trace and ultra-trace levels.

All glassware should be warm soaked with an anionic powder detergent (i.e. Alconox) followed by soaking the glassware overnight in a soapy water (i.e. Contrad 2000). After cleaning, rinse the glassware with running tap water, then rinse with distilled water and Milli-Q water more times. It is imperative that all soap, detergents and other cleaning fluids be removed from glassware before use. Rinsed glassware should be placed in an oven to dry. Method results in extremely hot glassware that must be handled carefully with tongs or thick gloves. Then, glassware should be rinsed with Acetone and clean aluminum foil used to cover and provide dust-free storage of clean glassware. In order to avoid any other potential contamination, before using all glassware should be rinsed with a small amount of Acetone, Methanol and/or Dichloromethane (DCM) according to the solvent requested in the analytical procedure. All solvents should be of analytical grade.

5.1. Analytical procedure for determination of PCBs, HCB and PBDEs in lyophilized biota samples.

Samples extraction

Supplies:

- 500 mL flat-bottom flasks, one per sample
- Precleaned boiling chips
- Medium Soxhlet, one per sample
- Acetone, Dichloromethane (DCM) and n-Hexane (Pesticide grade or equivalent)
- Pre-cooled condensers
- Sodium Sulphate (Na_2SO_4) (should be baked overnight at 450°C in a muffle furnace and kept at 150°C in an oven until use. All references to Sodium Sulphate refer to this prepared reagent)
- Teflon tape
- Cellulose thimble
- Surrogate standard solution

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- ✓ Blend about 2 g of lyophilized sample with anhydrous Sodium Sulfate.
- ✓ Place the sample in an extraction cellulose thimble, previously precleaned in Soxhlet with DCM for 12 hours and sonicated with the same solvent for half an hour before use;
- ✓ Place several boiling chips in each flask
- ✓ Fill each flask with 1/3 full of a mixture of DCM: Hexane 3:1 (v/v).
- ✓ Place the Soxhlet into the flask and fill the Soxhlet with the mixture solution almost to the top of the Soxhlet siphon tube.
- ✓ Add a mix of surrogate standards solution onto the sample (100 µL)

Table 4. Example of surrogate and internal standards mixtures for PCBs and PBDEs determination

Surrogate standards mixture	
IUPAC name	**BZ congener number**
2,3',4',5-Tetrachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-70
2,3,3',5,5'-Pentachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-111
2,2',3,4,4',5'-Hexachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-138
2,2',3,3',4,4',5-Heptachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-170
Internal standards mixture	
IUPAC name	**BZ congener number**
2,3,4,4'-Tetrachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-60
3,3',4,5,5'-Pentachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-127
2,3,3',4,5,5'-Hexachloro[$^{13}C_{12}$]biphenyl	$^{13}C_{12}$-PCB-159

- ✓ Connect flask, condenser and Soxhlet and secure in rack above heating mantle.
- ✓ Soxhlet extract the sample for 12 h monitoring until a steady boil, condense and flux pattern has been established.
- ✓ Rotavap DCM/Hexane fraction until 11 mL
- ✓ Use 1 mL of the extract to determine gravimetrically the lipid content
- ✓ Reduce the 10 mL fraction to about 1 mL under a gentle stream of ultrapure nitrogen

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- ✓ Clean-up the extract by a multilayer silica gel column (length 190 mm, I.D. 22 mm) packed from the bottom as follows:
- ✓ glass wool
- ✓ 2 g of activated silica (Sigma Aldrich, high-purity grade, pore size 60 Å, 70-230 mesh)
- ✓ 2 g of acid silica

Figure 21. Clean-up column

- ✓ 2 g of activated silica
- ✓ Anhydrous Na_2SO_4
- ✓ Note: Before use, silica should be Soxhlet extracted with DCM for 12 hours, dried under the fume cupboard and stored in a dark glass bottle; acid silica prepared by adding 12 mL of Sulfuric Acid (96%) to about 26 g of silica, and stored in a dark glass bottle.
- ✓ Wash the column with 100 mL of Hexane. After conditioning, load the sample and elute with 200 mL of Hexane
- ✓ The procedure reported above should be repeated twice after the volume reduction if the lipid removal was not complete and the extract was not limpid.
- ✓ Rotavap the about 5 mL, transfer in a test tube, add a 100 µL of an internal standard mixture and blown down until 100 µL under a gentle stream of ultrapure nitrogen.
- ✓ Lipid content determination
- ✓ The lipid content can be determined gravimetrically.
- ✓ Place 1 mL of the solid/liquid extract into a pre-weighted 5 mL beacker

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- ✓ Samples are evaporated in an oven for 1 h at 70 °C
- ✓ Keep the beacker at room temperature under a fume cupboard for about 30 min before recording the weight on an analytical balance;
- ✓ Repeat the procedure until a constant weight of the beaker is reached.
- ✓ The final result is obtained by five constant consecutive measurements when constant weight was reached.
- ✓ Convert the weight to percent lipids as follows:

$$Lipid\ Content\ (\%) = \frac{Lipid\ Content\ (g)}{1mL} \times \frac{11mL}{Sample\ Weight\ (g)} \times 100$$

where Lipid Content (g) is obtained by the difference between the mean value of five successive measurements when constant weight is reached and the empty beacker weight.

5.2. GC-MS analysis

To maintain mass selective detector (MSD) performance and reduce the frequency of required ion source maintenance, it is fundamental to choose a column phase with the lowest amount of column bleed. For PCBs and HCB analysis, a DB-5MS column (nominal length: 30 m, nominal diameter: 250 µm, nominal film thickness: 0.25 µm) is suggested because it is low bleed and features excellent signal-to-noise ratio and great peak shapes.

A short column 15 m column, with a 5% phenyl, 95% dimethylarylenesiloxane type stationary phase (DB5-MS, nominal length: 15 m, nominal diameter: 250 µm, nominal film thickness: 0.10 µm), is preferred for PBDEs analysis to meet EPA Method 1614 resolution requirements for critical isobariccompounds, such as BDE 49 and 71, and to allow the elution of BDE-209 without on-column thermal degradation that may compromise its qualitative and quantitative determination. It is fundamental, for BDE analysis, to perform a periodic GC column and inlet maintenance because nonvolatile material could persist in sample extracts, deposit onto the front of the column and liner and cause poor transfer of these organics to the GC column, compromising quantification and sensitivity. The presence of the same

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material at the head of the GC column could lead to poor peak shapes and reduced resolution between critical congeners, such as BDEs 49 and 71. In that case, it is suggested to trim about 0.5 m off the front of the column. Remember to properly change the method for the shorter column length to maintain the original separation.

Solvent injections should be repeated more times to check the chromatogram baseline and noise. Make sure that the ion source is clean, check the filament wear and the optics alignment performing autotune/manual tune and tune evaluation procedures.

The inlet septum and liner should be replaced frequently to prevent contamination; it is suggested to replace the gold plate after the fourth liner change. Split-less injection is preferred to perform when analyte concentrations are low.

Remember to control the syringe washing solvents and replace them at least twice a day. Remember also to set an accurate post-run cleaning procedure for the syringe, and clean the syringe periodically sonicating the needle with increasing polarity solvents.

The analysis for determination of PCBs, HCB and PBDEs can be performed with both electron ionization (EI) and negative chemical ionization (NCI) source.

Here, details on GC-NCI-MS quadrupole detector are reported.

The operational conditions of GC-MS for determination of PCBs and HCB were as follows:

Table 5. Operational conditions of GC-MS for determination of PCBs and HCB

Injection method	Splitless
Injection temperature	250 °C
Injection volume	1 µL
Oven initial temperature	100°C
Resolution	Low resolution, 1 unit of mass
Carrier gas	He, constant flow (1 mL/min)
Source Temperature	150 °C
Quadrupole Temperature	150 °C

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Temperature	100 °C, 8 °C / min up to 310 °C (hold for 2 min). Run time: 28.25 min

The operational conditions for the determination of PBDEs are as follows:

Table 6. Operational conditions of GC-MS for determination of PBDEs

Injection method	Splitless
Injection temperature	300 °C
Injection volume	1 µL
Oven initial temperature	90 °C
Resolution	Low resolution, 1 unit of mass
Carrier gas	He, constant flow (1 mL/min)
Source Temperature	150 °C
Quadrupole Temperature	150 °C
Ramp temperature	90 °C for 1 min, then 20 °C / min up to 220 °C, then 10 °C / min up to 300 °C (hold3 for 7 min. Run time: 22.5 min.

The mass spectrometer was operated in NCI mode using methane as chemical ionization and the pressure in the ion source was 1.7 x 10^{-4} Torr.

Mass fragments are monitored by working in SIM (Selected Ion Monitoring) and full scan modes, creating short time windows. Take care to include in each window all the fragments necessary for the target compounds determination. As shown in Table XXXX, characteristic fragment ions at m/z 79 and 81 $[Br]^-$ were monitored for mono-to hepta-BDE and BDE-206 and BDE-207, while m/z 485, 487 and 489 $[C_6Br_5O]^-$ were used for deca-BDE.

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Table 7. Characteristic fragment ions for PCBs in GC-NCI-MS and in GC-EI-MS analysis.

IUPAC name	BZ congener number	TR (min)	NCI-SIM (m/z)	EI-SIM (m/z)	IUPAC name	BZ congener number	TR (min)	NCI-SIM (m/z)	EI-SIM (m/z)
2,4-dibromodiphenyl ether	BDE-7	6.04	79, 81	328, 169	3,3',4,4',5-pentabromodiphenyl ether	BDE-126	10.83	79, 81	404, 406
4,4'-dibromodiphenyl ether	BDE-15	6.41	79, 81	328, 169	2,2',4,4',5,6'-hexabromodiphenyl ether	BDE-154	10.99	79, 81	484, 644
2,2',4-tribromodiphenyl ether	BDE-17	7.32	79, 81	246, 406	2,2',4,4',5,5'- hexabromodiphenyl ether	BDE-153	11.53	79, 81	484, 644
2,4,4'-tribromodiphenyl ether	BDE-28	7.50	79, 81	246, 406	2,2',3,4,4',5'- hexabromodiphenyl ether	BDE-138	12.18	79, 81	484, 644
2,2',4,5-tetrabromodiphenyl ether	BDE-49	8.48	79, 81	326, 486	2,3,3',4,4',5- hexabromodiphenyl ether	BDE-156	12.47	79, 81	484, 644
2,3',4',6-tetrabromodiphenyl ether	BDE-71	8.52	79, 81	326, 486	2,2',3,4,4',5',6-heptabromodiphenyl ether	BDE-184	12.66	79, 81	562, 564
2,2',4,4'-tetrabromodiphenyl ether	BDE-47	8.68	79, 81	326, 486	2,2',3,4,4',5',6-heptabromodiphenyl ether	BDE-183	12.96	79, 81	562, 564
2,3',4,4'- tetrabromodiphenyl ether	BDE-66	8.89	79, 81	326, 486	2,3,3',4,4',5',6-heptabromodiphenyl ether	BDE-191	13.38	79, 81	562, 564
3,3',4,4'-tetrabromodiphenyl ether	BDE-77	9.22	79, 81	326, 486	2,2',3,3',4,4',6,6'-octabromodiphenyl ether	BDE-197	14.67	79, 81	642
2,2',4,4',6-pentabromodiphenyl ether	BDE-100	9.70	79, 81	404, 406	2,2',3,3',4,4',5,6'-octabromodiphenyl ether	BDE-196	15.06	79, 81	642
2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether	BDE-119	9.85	79, 81	404, 406	2,2',3,3',4,4',5,6,6'-nonabromodiphenyl ether	BDE-207	16.83	79, 81, 485, 487	719, 360
2,2',4,4',5-pentabromodiphenyl ether	BDE-99	10.06	79, 81	404, 406	2,2',3,3',4,4',5,5',6-nonabromodiphenyl ether	BDE-206	17.40	79, 81, 485, 487	719, 360
2,2',3,4,4'-pentabromodiphenyl ether	BDE-85	10.66	79, 81	404, 406	2,2',3,3',4,4',5,5',6,6'-decabromodiphenyl ether	BDE-209	20.58	79, 81, 485, 487	799, 332

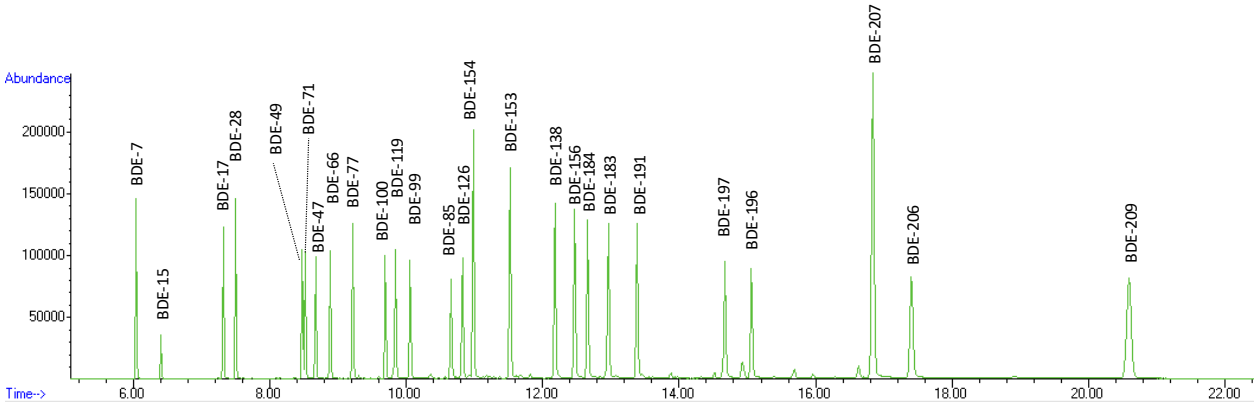

Figure 22. Example of a PBDEs chromatogram

Table 8. Characteristic fragment ions for PCBs in GC-NCI-MS analysis.

IUPAC name	BZ congener number	TR (min)	NCI-SIM (m/z)	IUPAC name	BZ congener number	TR (min)	NCI-SIM (m/z)
Hexachlorobenzene	HCB	10.89	286, 251	2,2',3,4',5,5'-Hexachlorobiphenyl	PCB-146	17.9	360
2,4,4'-Trichlorobiphenyl	PCB -28	13.21	256	2,2',4,4',5,5'-Hexachlorobiphenyl	PCB-153	18.05	360
3,4,4'-Trichlorobiphenyl	PCB -37	13.23	256	2,3,3',4,4'-Pentachlorobiphenyl	PCB-105	18.14	326
2,2',5,5'-Tetrachlorobiphenyl	PCB -52	14.06	290, 292	2,2',3,4,4',5'-Hexachlorobiphenyl	PCB-138	18.65	360
2,2',4,4'-Tetrachlorobiphenyl	PCB -47	14.52	290, 292	3,3',4,4',5-Pentachlorobiphenyl	PCB-126	18.90	326
2,2',3,5',6-Pentachlorobiphenyl	PCB -95	15.54	326	2,2',3,4',5,5',6-Heptachlorobiphenyl	PCB-187	18.98	394
2,2',4,5,5'-Pentachlorobiphenyl	PCB -101	16.11	326	2,2',3,4,4',5',6-Heptachlorobiphenyl	PCB-183	19.10	394
2,2',4,4',5-Pentachlorobiphenyl	PCB -99	16.21	326	2,2',3,3',4,4'-Hexachlorobiphenyl	PCB-128	19.25	360
3,4,4',5-Tetrachlorobiphenyl	PCB -81	16.75	290, 292	2,3',4,4',5,5'-Hexachlorobiphenyl	PCB-167	19.32	360
2,3,3',4',6-Pentachlorobiphenyl	PCB -110	16.93	326	2,2',3,3',4,5',6'-Heptachlorobiphenyl	PCB-177	19.63	394
3,3',4,4'-Tetrachlorobiphenyl	PCB -77	16.99	290, 292	2,3,3',4,4',5-Hexachlorobiphenyl	PCB-156	19.80	360
2,2',3,5,5',6-Hexachlorobiphenyl	PCB -151	17.18	360	2,3,3',4,4',5'-Hexachlorobiphenyl	PCB-157	19.91	360
2,3',4,4',5'-Pentachlorobiphenyl	PCB-123	17.47	326	2,2',3,4,4',5,5'-Heptachlorobiphenyl	PCB-180	20.16	394
2,2',3,4,6,6'-Hexachlorobiphenyl	PCB-145	17.49	326	3,3',4,4',5,5'-Hexachlorobiphenyl	PCB-169	20.65	360
2,3',4,4',5-Pentachlorobiphenyl	PCB-118	17.56	326	2,2',3,3',4,4',5-Heptachlorobiphenyl	PCB-170	20.77	394
2,3,4,4',5-Pentachlorobiphenyl	PCB-114	17.79	326	2,3,3',4,4',5,5'-Heptachlorobiphenyl	PCB-189	21.43	394

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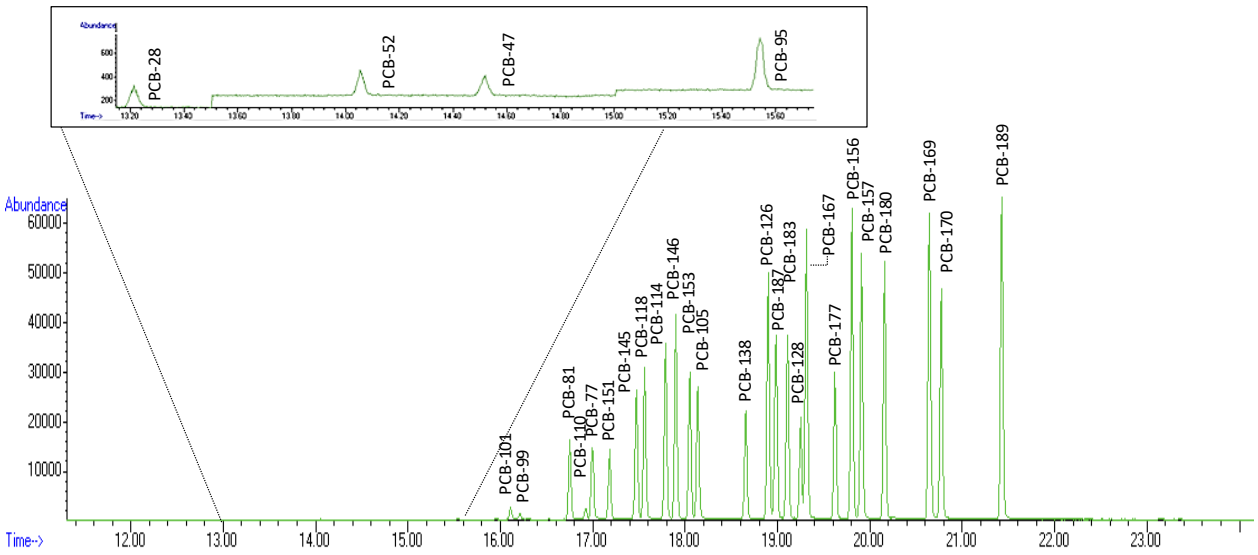

Figure 23. Example of a PCBs chromatogram

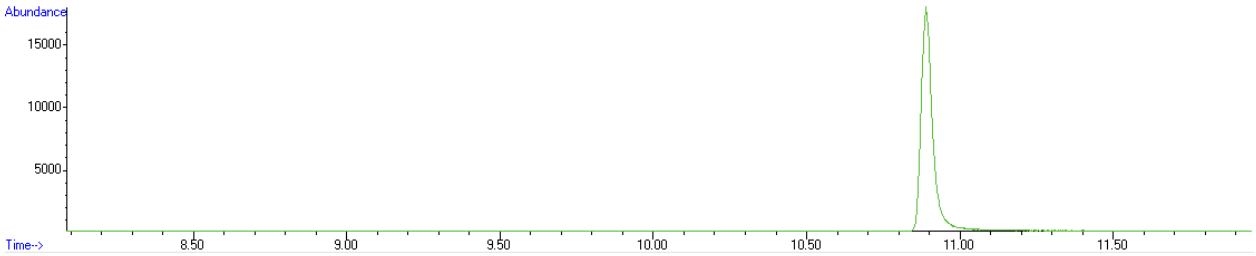

Figure 24. Example of a HCB chromatogram

5.3. Quality assurance and quality control (QA/QC)

- Impurities in solvents and reagents may be checked for artefacts and/or interferences that may compromise the results of sample analysis. All of these materials must be demonstrated to be free from interferences under the conditions of extract preparation and analysis by preparing method blanks with each extraction batch. The same solvents and reagents should be used for the method blank and the associated samples.

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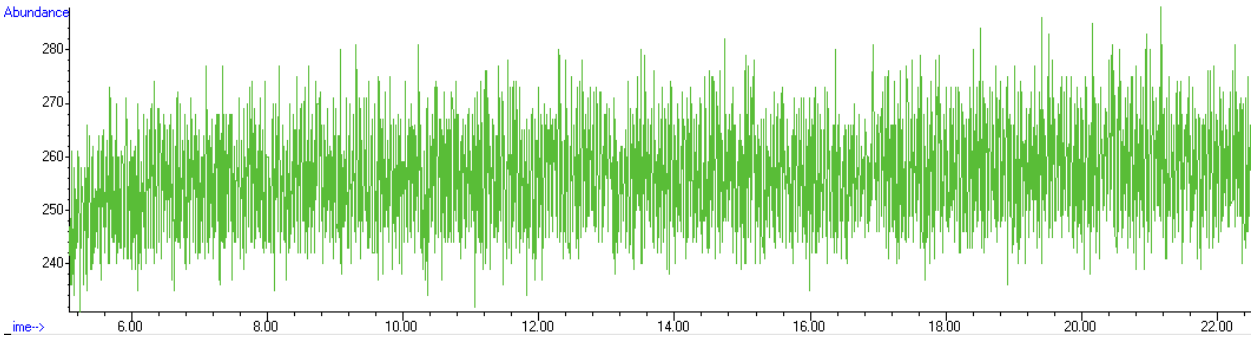

Figure 25. Example of a chromatogram of a blank extract.

- **Blanks (or method blanks)** are measured aliquots of clean matrix (typically consisting in sodium sulphate) that are treated identically to the samples. Surrogates should be added, and the blanks performed through all stages of the sample extraction, concentration, and clean-up procedures. Blanks are important to ensure that there are not systematic contaminations. A blank is extracted with each batch of samples.
- **Matrix spike**, which are samples spiked with a known quantity of the target analyte(s), should be prepared by taking additional sample aliquots, and adding the appropriate amounts of surrogate and spiking solutions. The matrix spike has to be carried through all stages of the sample extraction, concentration, and clean-up procedures. Matrix spike samples serve to measure the **extraction accuracy**, by allowing the comparison of the found amount(s) of target analyte(s) with the spiked amount(s).
- Prior to run a sample sequence, it is suggested to inject the solvent and evaluate the **quality of the baseline**; then an intermediated calibration point must be run at the beginning, in the middle and at the end of the same sequence.
- If target analytes are determined in the blank samples, **LOD** is calculated as the average detected blank concentrations + 3 times the standard deviation and **LOQ** is calculated by adding 10 times the standard deviation and the average of blank samples. In this case samples must be corrected for the blank contribute. For congeners that were not detected, the noise was used to determine LODs.

$$LOD = C_m + 3S_B \quad \text{and} \quad LOQ = C_m + 10S_B$$

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Where $C_m = \frac{\sum C_i}{n}$ and $S_B = \sqrt{\frac{\sum (C_i - C_m)^2}{n-1}}$

- To assess the LOD, a minimum of six replicate injections of the lowest diluted solvent standard solution which a peak area %RSD of 15% should be considered.
- The **precision** of the method should be evaluated in terms of intra-day and inert-day variability. PBDEs, PCBs and HCB in the samples should be analysed successively four times per day during three consecutive days, using the same operating conditions. Intra-day and inter-day precisions should be expressed as the relative standard deviations (RSD) of results from repeated measurements.
- The **linearity** of the calibration curve should be also periodically checked and determined using solvent standards solutions at different concentrations for mono-pentaBDEs, for hexa-octaBDEs and for nona-decaBDEs. All compounds should show excellent linear responses with coefficients of determination r^2 >0.99. A minimum of five concentrations is recommended. The standard solutions should be equally distributed on the range of concentrations and the injection should be repeated to have an average value for each calibration point. The linearity should be evaluated by appropriate statistical methods, the significance of linear regression should be evaluated by analysis of variance (i.e. ANOVA test).
- The **extraction efficiency** should be evaluated for each sample analysed by adding a surrogate standard mixture composed by labelled and/or deuterated target compounds at the beginning of the analytical procedure. It is suggested to use a different labelled/deuterated compound for each group of PCBs and PBDEs.
- Use of reference material and certified reference materials is recommended to estimate the performance of the analytical method and obtaining **exactness** in the analytical measurement. The exactness is quantified by the difference of the found value and the expected value (BIAS). For APEX analysis, two reference materials (freeze-dried fish tissues), one consisting in a naturally fortified salmon, the other in a low-level salmon were purchased from Wellington Laboratories. The detected concentrations for lipid content, HCB, PCBs and PBDEs congeners are then compared to the certified values.

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6. SOP for the determination of Dioxins and dioxin-like compounds (pre-screening by bioassay and confirmation by GC-HRMS), chlorinated alkanes (C10-C13; C14-C17), novel organophosphorus flame retardants, Decloran plus, NTS by GC-MS(EI/PCI/NCI modes) in AP&P samples

6.1. Dioxins and dioxin like compounds (pre-screening by bioassay and confirmation by GC-HRMS) in lyophilized biota samples.

6.1.1. Bioassay

The cell test DR CALUX® for screening of dioxin-like activities can be used to detect not only the 29 regulated dioxin-like substances but also many other persistent organic pollutants with dioxin-like potencies, such as mixed halogenated dioxins/biphenyls.

6.1.2. Analytical procedure

1 g of lyophilized biota sample is divided in two portions in order to perform (i) fat contents determination and (ii) bioassay screening using Dioxin-like compounds (DR CALUX®) All selected samples were selected and analyzed via the dioxin responsive chemically activated luciferase expression bioassay (DR CALUX®) using H4IIE rat liver cells for the persistent dioxin-like compounds (such as PCDD/Fs and dl-PCBs) and their aryl hydrocarbon receptor (AhR) activities (Behnisch, Hosoe, and Sakai 2001, 2003; van Vugt-Lussenburg et al. 2013). As chemical analysis by GC/HRMS (high-resolution mass spectrometry) gives results for the 29 dioxin-like compounds (17 3,4,7,8-chlorinated PCDD/Fs and 12 dl-PCBs), analysis by DR CALUX® was also included in the monitoring program in order to detect further compounds with dioxin-like activity such as halogenated, mixed halogenated (bromine, chlorine, fluorine), methylated, or otherwise substituted PXDD/PXDF/PXBs (X = Br, Cl, F, J), as well as other sulfuric acid stable POPs (such as PBDEs, N-dioxins; Behnisch, Hosoe, and Sakai 2001, 2003).

6.1.3. Sample preparation - QuEChERS

- ASE was used for the extraction of selected compounds from the lyophilized fish samples,

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- Extracts were cleaned up and divided into five fractions using fully automated system (Fluid Management System).

  Extraction:

- 0.5 g of lyophilized sample is placed in 20 ml centrifuge tube
- spike with labelled internal standards at concentration level 50 ng/g
- equilibrate at room temperature for 15 min
- add 10 ml ACN into the centrifuge tube
- vortexing for 1 min
- pour the mixture into another centrifuge tube, containing 4 g anh. $MgSO_4$ and 1 g NaCl
- vortexing, for 1 min, followed by centrifuging at 3250 rpm for 5 min

  Clean up:

- aliquot of upper layer is transferred into centrifuge tube with 50 mg of Z-Sep Plus (sorbent) and 150 mg anh. $MgSO_4$
- vortexing, for 1 min, followed by centrifuging at 3250 rpm for 5 min
- Add 10 ul of labelled injection standard and cleaned extract is injected into GC-MS/MS or GC-MS(NCI) or GCxGC-MS(NCI)

6.1.4. GC-HRMS

samples determined by bioassay testing as positive () are subjected to be confirmed by using isotopic dilution method (ID) coupled with gas chromatography and high-resolution mass spectroscopy (with double focus at ion).

- Dioxins and dioxin-like compounds were determined by using isotopic dilution method (ID) coupled with gas chromatography and high-resolution mass spectroscopy (with double focus at ion),
- ID – employing ^{13}C labelled standards for each compound to be determined,
- ID – requires three types of solutions of standards compounds: (i) at least five calibration solutions that contain 17 congeners of PCDDs and PCDFs, 12 congeners of dl-PCBs, and 6 congeners of ind-PCBs, plus ^{13}C equivalents each of them, (ii) extraction

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standard containing ^{13}C equivalents with well know concentration of each compound to be determined, (iii) injection standard that eliminates the error of injection.

- HRMS – plays a significant role in quantification and qualification of selected compounds,
- Minimum required resolution is 10000, that significantly increasing the selectivity of HRMS against LRMS,
- Mass accuracy is kept at ≤ 2 ppm by injecting the reference compound (PFTBA) to each injection.

6.2. QA/QC

- Method used for the determination of selected compounds fellows these criteria:
- Relative standard deviation (RSD) expressing the accuracy of measurement must be <15%, the relative measurement error must not exceed ± 20% ,
- The laboratory shall take part on the proficiency testing at least one time per year,
- The laboratory shall record the Shewhartove regulation diagrams for the results of particulate reference materials,
- Recovery of ^{13}C labelled extraction standards should be in the following intervals:
- (60–120 % pre PCDD a PCDF a 50–120 % pre PCB),
- Mass resolution must be set at least 10000,
- Isotopic ratio of a quantification and qualification ion must be in interval ± 15% from a theoretical value,
- Deviation of the retention time of whichever analyte and its ^{13}C equivalent must not exceed ± 2 s.

6.3. Determination of C10-C13 and C14-C17 polychlorinated alkanes in lyophilized biota samples – using GC-MS (NCI) as a pre-screening and GCxGC-MS (NCI) confirmation method.

- ISO/DIS 12010 – a method for the quantitative determination of the sum of short chain polychlorinated n-alkanes between the carbon range n-C_{10}-n-C_{17} , in mixtures with chlorine contents between 49% - 67%.

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- Typical GC-MS conditions are presented in **Table 9**, whereas typical retention time ranges for selection ions are presented in **Table 10**.

Table 9. GC-MS conditions for determination of of C10-C13 and C14-C17 polychlorinated alkanes in lyophilized biota samples – using GC-MS (NCI)

Injection method	LVI
Injection temperature	70 °C - 280 °C
Injection volume	20 µL
Oven initial temperature	50°C
Resolution	Low resolution, 1 unit of mass
Carrier gas	H_2, constant flow (2.5 mL/min)
Source Temperature	150 °C
Quadrupole Temperature	150 °C
Temperature	100 °C, 8 °C / min up to 310 °C (hold for 2 min)
Column	DB5 – ms; 15 m x 0.25 mm x 0.25 um

Table 10. Typical retention time ranges for selected ions.

m/z value	Approx. retention time range
70	4.2-7.0
327	5.0-5.7
375	5.4-6.2
409	5.7-6.4
423	5.9-6.8

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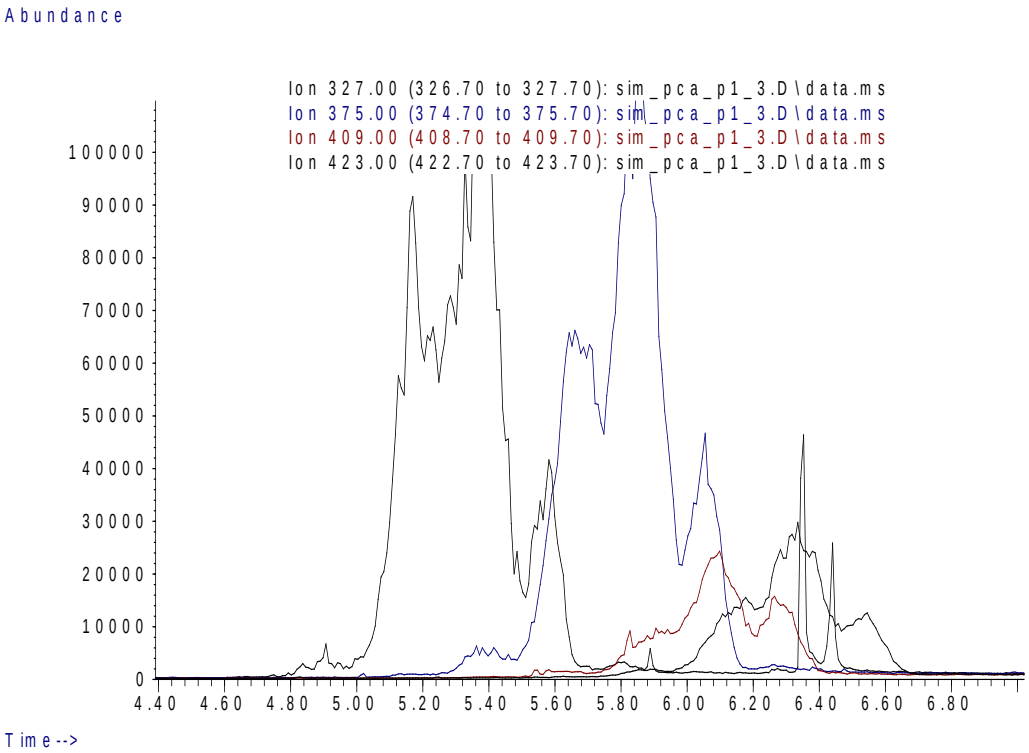

Figure 26. Typical chromatogram showing the overlap of individual m/z values

Typical chromatogram showing the overlap of individual m/z values

- GC-MS(NCI) is used for the selection of positive biota samples and the final confirmation is done by GCxGC-MS (NCI) technique
- Typical GC-MS conditions are presented in **Table 11**.

Table 11. Instrumental parameters for GC-MS analysis

Injection method	LVI
Injection temperature	70 °C - 280 °C
Injection volume	50 µL
Oven	60 ° C (5 min) - 20 ° C/min - 170 ° C - 5 ° C/min - 270 ° C (20 min)
Resolution	Low resolution, 1 unit of mass
Carrier gas	H_2, constant flow_1 (1.0 mL/min), constant flow_2 (18 ml/min)
Source Temperature	140 °C

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Quadrupole Temperature	110 °C
Temperature	100 °C, 8 °C / min up to 310 °C (hold for 2 min)
Column_1	DB5 – ms; 30 m x 0.25 mm x 0.25 um
Column_2	INNOVAX 5 m x 0.25 mm x 0.15 um
GCxGC modulator	modulation period 6 s, sampling time 5.5 s

- internal standards used for quantification:
  - P1 - C_{10}-C_{13} carbon chain length, chlorine contents 51.5 % w/w, Dr. Ehrenstorfer;
  - P2 - C_{10}-C_{13} carbon chain length, chlorine contents 55.5 % w/w, Dr. Ehrenstorfer;
  - P3 - C_{10}-C_{13} carbon chain length, chlorine contents 63.0 % w/w, Dr. Ehrenstorfer;
  - P4 - C_{14}-C_{17} carbon chain length, chlorine contents 51-52 % w/w, technical mixture (NCHZ, Slovakia);
- acquisition rate is set to 30 Hz
- calibration is done by measuring three different composed standard mixtures according to Table 1, Hordalub 17 –s1,Hordalub 80 –s1 and Cereclor 70 –s1 at concentration levels from 10 -100 ng/kg wet weight

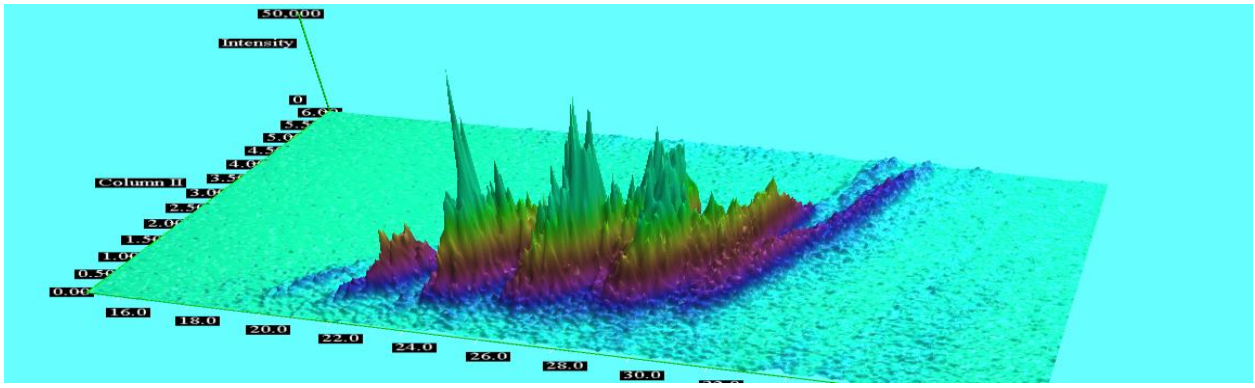

Figure 27. Typical chromatogram of C10-C13 chloroalkanes in 2D and 3D dimension at m/z 70

- All method validation is following the ISO/DIS 12010

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6.4. Determination of novel organophosphorus flame retardants, Decloran plus in lyophilized biota samples – using GC-MS/MS method.

Rapid method for determination of organophosphorus flame retardants and Decloran plus in biota using QuEChERS as a sample preparation in combination with GC-MS/MS. Instrumental GC-MS/MS conditions are shown in **Table 12** and **Table 13**. Typical chromatograms measured in MRM mode are depicted in **Figure 28**.

Table 12. GC-MS/MS instrumental conditions for the determination of organophosphorus flame retardants and Decloran plus

Injection method	Pulsed splitless
Injection temperature	280 °C
Injection volume	2 µL
Oven initial temperature	50°C
Resolution	Low resolution, 1 unit of mass
Carrier gas	H_2, constant flow (1.6 mL/min)
Source Temperature	250 °C
Quadrupole Temperature	150 °C
Temperature	50°C (2min), 20 °C / min up to 240 °C, 8 °C / min up to 315 °C (10 min), 20 °C / min up to 320 °C (5 min)
Column	DB5 – ms; 25 m x 0.25 mm x 0.25 um

Table 13. Average retention times (t_R), multiple reaction monitoring (MRM) transitions, collision energy (CE) for each transition and lowest calibration level (LCL) for each analyte.

Analyte	**tR(min)**	**Quantifier m/z**	**CE (eV)**	**Qualifier m/z**	**CE (eV)**
Tri-isobutyl phosphate	**8.4**	99>81	12	139>99	9
Tri-n-butyl phosphate	**9.2**	99>81	9	155>99	9
Tris(2-chloroethyl) phosphate	**9.8**	249>187	9	249> 125	9

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Tris(monochloropropyl)phosphate	**10.1**	277>125	9	125>99	9
Tris(1,3-dichloro-2-propyl)phosphate	**12.7**	209>99	9	191>74	9
Triphenylphosphate	**13.1**	326>325	9	170>169	9
Ethylhexyldiphenyl phosphate	**13.2**	251>95	9	251>153	9
Tris(2-ethylhexyl)phosphate	**13.4**	99>81	12	113>95	9
Tritolyl phosphate	**14.7**	368>243	12	368>198	9
Tris(2-butoxyethyl) phosphate	**19.7**	357>250	12	357>252	12
Dechlorane Plus Syn	**20.9**	297> 229	9	272>237	12
Dechlorane Plus Anti	**21.4**	297> 229	9	272>237	12
Surrogate standard					
Triphenyl phosphate-d15	**13**	341>339	16	341>241	16

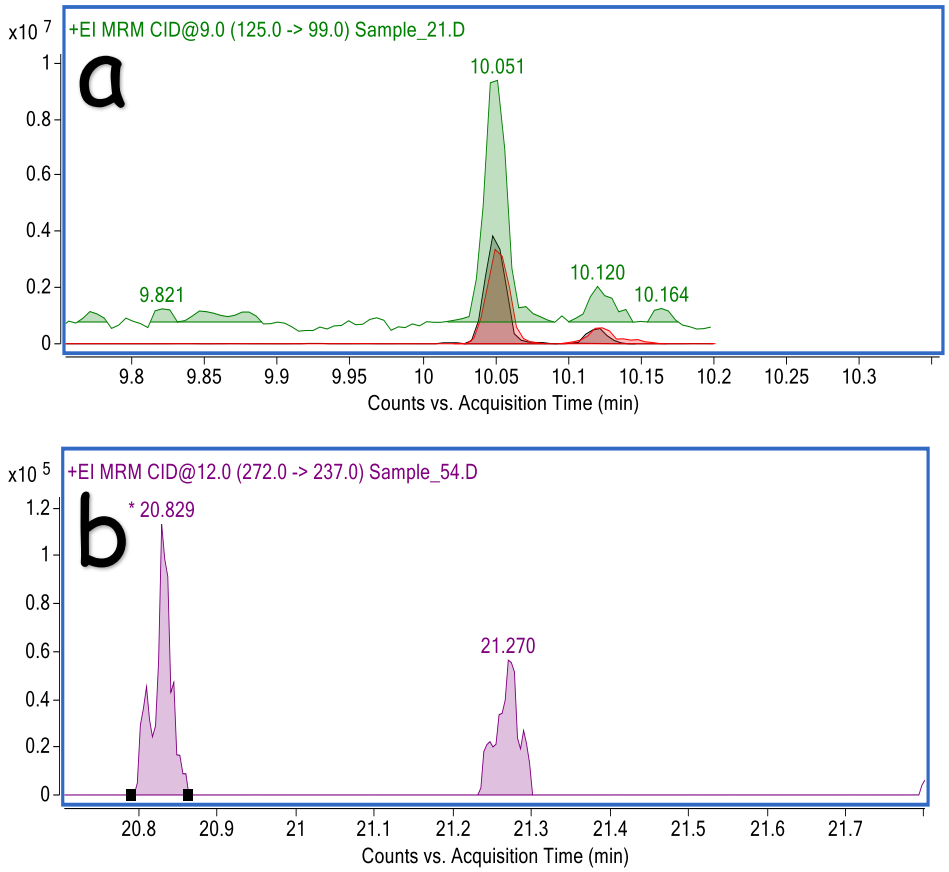

Figure 28. **a)** MRM chromatogram of TCPP isomers in otter sample, **b)** MRM chromatogram of Dechloran plus (anti/syn) isomers in harbour seal sample

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6.5. Non-target screening of in lyophilized biota samples – using GC-LRMS - (EI/PCI/NCI(CH4)-SCAN) and RI approach

The main advantage of using a GC coupled with a single quadrupole mass spectrometer is that there are several available features easily exchangeable for one another and their complementary benefits to each other provide a reasonable higher amount of identified compounds with higher level of confidence. Each sample is subjected to three types of ionization (EI/PCI-CH4/NCI-CH4) in SCAN mode, whilst other conditions are identical as well as a mixture of n-alkanes is measured on to be used for the calculation of retention indices of detected compounds.

EI-SCAN – main benefit is the provision of full spectra of detected compounds with a tentative identification, however, many of them are mixed spectra or spectra with no fragmentation in molecular ion region. The lack of high resolution often results in an incorrect identification even are used an up to date GC-MS libraries.

PCI-SCAN – provides information on the molecular mass of detected compounds.

NCI–SCAN – confirms the presence of compounds containing electronegative atoms, mainly halogens. In the light of its higher sensitivity against EI as well as a different kind of fragmentation is used for the determination of compounds containing atoms such as Br and Cl. Those compounds can be identified by matching with entries in a “home-made” NCI library.

RI – helps to increase level of confidence within some uncertainty (mostly ± 50 iu) in cases if a tentative identification is done and it needs to be decided whether the identification could be correct or not at the retention time of the given compound.

There are three examples of mutual symbiosis of the approaches mentioned above:

a) – molecular ion region with no fragmentation around:

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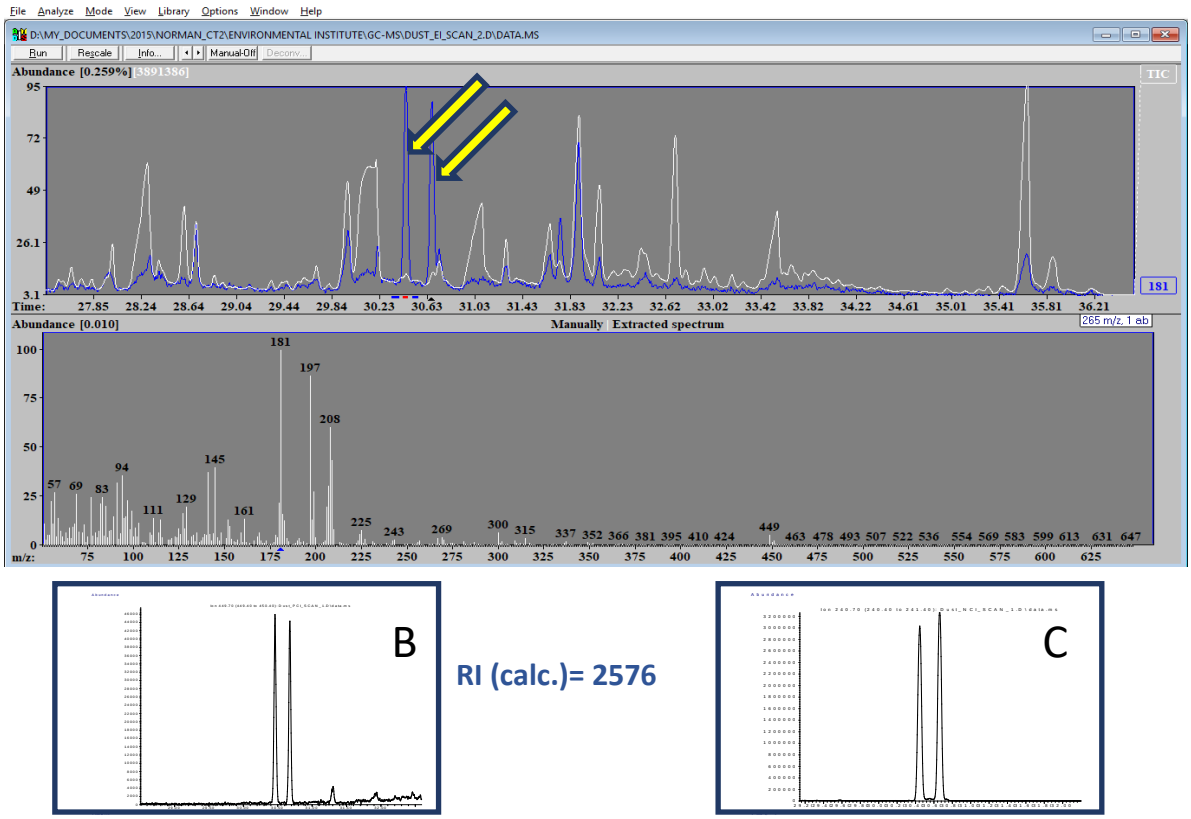

Figure 29. Example of positive identification of two isomers of cyhalothrin (MW=449)

The example of **Figure 29** shows several evidence for the positive identification of two isomers of cyhalothrin (MW=449). Upper EI-SCAN chromatogram displays at m/z 181 two peaks labelled with yellow arrows; their manually extracted spectrum is displayed lower with no fragmentation at molecular ion region. Molecular mass of cyhalothrin isomers is confirmed by the PCI result (window B at m/z 450) and window C demonstrates excellent a signal to noise ration as a result of high sensitivity of NCI because of the contents of halogens (at m/z 240.7). The last proof that confirming the presence of cyhalothrin is the accepatble conformity of calculated RI (2576) with NIST RI (2579).

b) – RI and PCI correction of library false positive identification:

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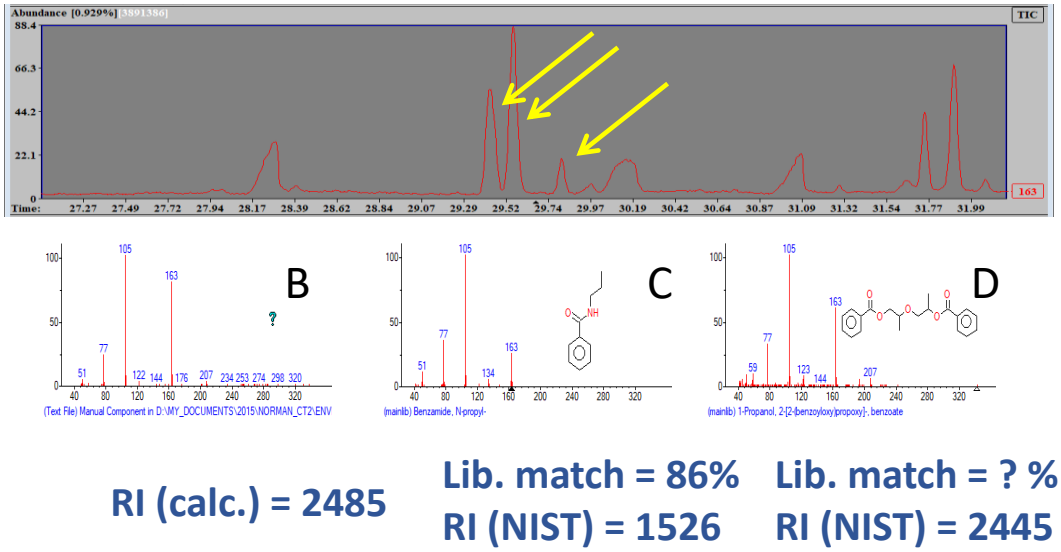

Figure 30. Example of RI and PCI correction of library false positive identification

Upper EI-SCAN chromatogram displays at m/z 163 three peaks labelled with yellow arrows, their manually extracted spectrum is shown in B. C - NIST search offered benzamide, N-propyl- as the best match with score 86%. The mismatch between RI(NIST) and calculated RI shows the false positive of NIST search identification. After applying some constraints there was determined dipropyleneglycoldibenzoate (three isomers) as the best match, as well as confirmed by RI and PCI.

c) – how NCI and RI can extend the number of detected compounds over classical EI results:

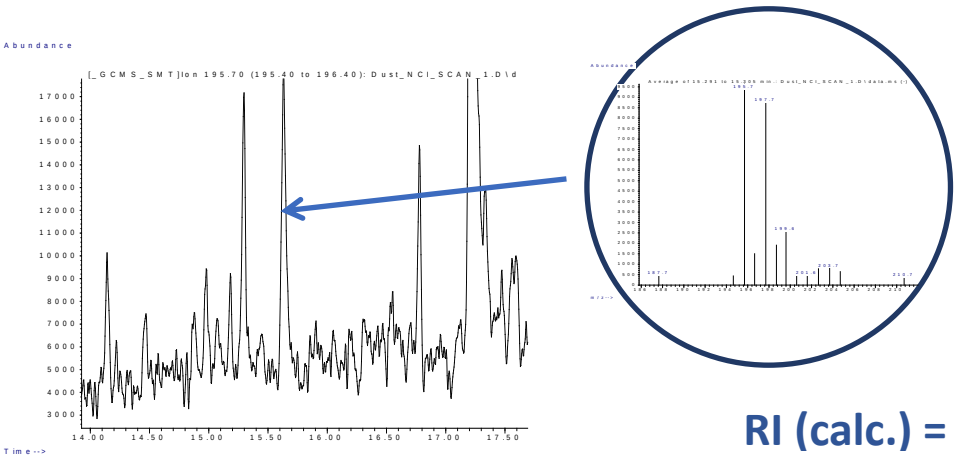

Figure 31. An example of how NCI and RI can extend the number of detected compounds over classical EI results

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An unknown compound is visible on NCI-SCAN at 15.29 min. It should be noted that no evidence of this compound was registered on EI and PCI chromatograms. Chlorine's characteristic isotopic pattern showing the presence of three chlorine atoms, after making a search through a "home-made" NCI library, 1,3,5-trichlorophenol was determined as the best match that it was also confirmed by comparing RI calculated 1371 with RI(NIST) =1335 for 1,3,5-trichlorophenol.

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7. SOP for the determination of Hg in AP&P samples

7.1. General Information

Determination of Hg in AP&P samples in conducted by the German Environmental Speciment Bank. It is in the responsibility of the Federal Ministry for the Environment, Nature Protection and Reactor Safety (BMU) and technically and administratively coordinated by the Federal Environment Agency (Umweltbundesamt). This section describes a method for determining the mercury content in environmental samples by means of direct solid analysis. Previous digestion is not necessary, which means that preliminary sample preparation procedures with their risk of potential contamination of the sample, are kept to a minimum. For the test itself, a special device is required, the direct mercury analyzer (DMA) manufactured by MLS, Leutkirch. This method replaces the cold-vapour atomic- absorption spectrometry method (CV-AAS) described in the procedural guidelines of the German Environmental Specimen Bank (ESB) (UMWELTBUNDESAMT 1996).

7.2. Area of application

This SOP is used for the routine testing of the following types of ESB samples: bladder wrack, mussels, eelpout (muscle tissue), herring gull egg, zebra mussels, bream (muscle tissue), spruce shoots, pine shoots, poplar leaves, beech leaves, earthworm, roe-deer liver, feral pigeon eggs. In principle, the method described in this guideline can also be used for other types of biological sample. If samples are used for which no empirical data are available, a suitable validation of the method for the matrix in question should be carried out. The lower range of application for the process described is approx. 2-3 ng/g for solid samples and approx. 2-3 ng/mL for liquid samples.

7.3. Description of the method

The solid mercury analyser permits interference- free analysis of solid and liquid samples for mercury content. Automatic sample combustion is carried out at approx. 1000°C in a current of oxygen. Following combustion of the sample and catalytic conversion of the combustion gases, elemental mercury is selectively concentrated by amalgam formation and then measured by means of atomic-absorption spectrometry (AAS).

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7.4. Apparatus and Reagents

DMA-80 Direct Mercury Analyser with integrated autosampler, control system lab-TERMINAL 1024 and laboratory balance Precisa XT220 A (complete system from MLS, Leutkirch) was used. For the operation of the apparatus oxygen gas is required (e.g. pressurized oxygen from a cylinder). Additional accessories required for the analysis is catalyst (DMA 8333), amalgamator (DMA 8134) and sample crucible of metal (DMA 8142). Since mercury can also be introduced into the system by the oxygen flow, the gas used should be of 'analysis quality' at least (O_2 > 99.95 %). The safety measures to be observed when working with compressed gases must always be observed. Vessels of glass or plastic (FEP, PFA) may be used. All vessels used for preparing and storing standard solutions or coming into contact with standard solutions, must be free of mercury. The vessels should be rinsed with diluted nitric acid (10%; quality supra or equivalent). After cleaning with acid, the vessels should be rinsed with high- purity water and then dried. As stock solution commercially available solutions with e.g. 1000 mg Hg per litre (as single-element solution) should be used. These solutions have a shelf life of several years. The shelf life stated by the manufacturer should always be observed. Only certified standard solutions should be used. Suitable aqueous calibration solutions (normally in the range of 5 to 1000 µg/L Hg) should be prepared freshly using the mercury stock solution. Standard quality wheat flour is generally used as carrier material for liquid solutions (amount per sample approx. 50 mg). The mercury blank value of the product used should be determined before use (normally less than 0.0010 ng Hg absolute). As an alternative to the standard calibration with solutions generally carried out, a solid material can also be used for the calibration process. Certified reference materials (CRM) are suitable for this purpose, e.g. soil CRMs (e.g. 'light sandy soil BCR 142R') or dogfish liver ('DOLT-3').

7.5. Preparations before measurement

7.5.1. General notes

Before switching on the main unit, the oxygen supply has to be turned on (input pressure 500 kPa). When the main switch at the front is pressed, the control units 'lab-TERMINAL' and the DMA-80 software are started automatically. The automatic sample feeder moves into the starting position and all heating systems are heated up to starting temperature. After

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15-20 minutes, the unit is ready for operation. The accompanying laboratory balance has to be switched on separately.

7.5.2. Check of blank values

To clean the system and check the catalyst and amalgam enrichment, the system blank values must first be verified. This is done by measuring the blank value without the sample crucible. Set the program to '100°C - 0 s - 850°C - 180 s - 60 s' (drying temperature - drying time - incineration temperature - incineration time - flushing time) and then press the start button. When the measuring process is concluded, the result is indicated by the program. The result should be < 0.0010 ng Hg absolute. If this value is not achieved, the measurement of the blank value should be repeated until the result is less than 0.0010 ng Hg absolute. Excessively high blank value readings may be caused by an exhausted catalyst or amalgamator.

7.5.3. Weighing samples

The samples for testing are weighed into special crucibles. The crucibles have a volume of approx. 700 µL corresponding to approx. 500 mg of solid matter (or less depending on the nature of the sample). To optimise the handling of the crucible, 500 µL volume should not be exceeded. To obtain a high degree of accuracy, it is also advisable to weigh liquid samples into the crucible. In this way, evaluation is possible directly using the instrument software. Liquid samples can also be applied to a carrier material with a low blank value (e.g. flour).

7.5.4. Loading the sample feeder

The sample crucibles are loaded into the automatic sample feeder on a turntable with 40 different positions. The turntable can be removed from the analyser for loading. The crucibles (sample boats) are transferred from the balance to the turntable using tweezers. The crucibles have upwardly curving edges to make them easier to grip with the tweezers. The crucibles are positioned with this edge towards the centre of the turntable. After placing the samples

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in the crucible, close the transparent cover of the instrument to prevent contamination with dust or other influences.

7.5.5. Cleaning the sample crucibles

After the test, the ash is removed manually from the crucibles. To clean the crucibles in the DMA-80, the sample changer is loaded with crucibles as for the test, and the empty crucibles cleaned using a similar program. The program is set to '100°C - 0 s - 850°C -180 s - 60 s' ('drying temperature - drying time - incineration temperature - incineration time - rinsing time') and afterwards the start button is pressed. During the program, the crucibles are fed into the system one after another and burned out. When burned-out crucibles are stored in a dust-free location, no further burning out is necessary. Safety note: On conclusion of the cleaning program, the last crucibles processed are still hot. Before sample material is weighed into them, they must be allowed to cool down to room temperature.

7.5.6. Measurement after installing a new catalyst

After installing a new catalyst several blank value tests have to be carried out first. At the start, dust and moisture in the catalyst may produce high blank-value readings. In order to speed up the cleaning process, it is possible to test samples with a high organic content or slightly acidified aqueous samples. In this way, the blank value readings are reduced more quickly. The instrument is ready for use when the absolute blank value is less than 0.0010 ng Hg.

7.6. Measurement procedure

7.6.1. General notes

When testing ESB samples, the ratio of samples being tested to quality-assurance samples is always 2:1. A list of the reference materials used for elemental analysis of ESB samples is given in **Table 14**.

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7.6.2. Calibration

To carry out quantitative tests, the system first must be calibrated by means of suitable standard samples (solid or liquid). The calibration then remains stable for a period of several months. The validity of the calibration must be documented for each test (e.g. by testing certified reference materials). Before starting a new series of analyses, the instrument should be recalibrated if discrepancies appear during the measurement of quality assurance, if maintenance work has been carried out, the location of the instrument has been changed or if more than three months have elapsed since the last calibration. Under normal circumstances the calibration function is linear. The coefficient of correlation r should be greater than 0.995. If r is less than 0.995, and the calibration is to be used in spite of this, the reason must be stated (e.g. calibration in the lowest application range of the method with consequently higher measurement uncertainty). For calibration purposes, aqueous mercury standards of varying concentrations are used which are pipetted onto an organic carrier material (e.g. 50 mg of flour). As a rule, the following concentrations are used (freshly prepared in each case): 1000 µg/L, 500 µg/L, 250 µg/L, 200 µg/L, 100 µg/L, 50 µg/L, 25 µg/L, 10 µg/L, 5 µg/L. Normally 200 µL are used (in addition to 100 µL for the lowest concentrations). Alternatively, the calibration operation can also be carried out with suitably certified reference materials (solids). The differing mercury content for the individual calibration points is determined by means of different weighted quantities.

Standard program for calibration with solids (certified reference materials): Setting: '100°C - 0 s - 850°C - 180 s - 60 s - 12 s-30 s' ('drying temperature - drying time - incineration temperature - incineration time - rinsing time - amalgam heating – measurement time').

Standard program for liquid standards (with or without organic carrier): Setting: '300°C - 300 s - 850°C - 240 s - 60 s - 12 s - 30 s' ('drying temperature - drying time - incineration temperature - incineration time - rinsing time - amalgam heating - measurement time').

7.6.3. Calibration check

When the control program is started, the previous calibration is automatically opened. If a different calibration is required, this must first be activated with the DMA-80 software under CALIBRATION. To verify the calibration, a reference sample of known Hg concentration is first

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tested. The amount of sample material should be selected to obtain an absolute mercury quantity of approx. 10-20 ng Hg. For example, a standard solution with 0,1 mg/kg Hg (weighted quantity of 0,15 g = 150 µL) would be suitable. The sample should be activated in the software as 'reference'. This ensures that the result of the test is compared automatically with the calibration and the deviation indicated. The mercury concentration of the tested reference material should also be entered. It should be ensured that the concentration is set correctly as mg/kg or µg/kg. Following measurement, the difference between the target and the actual values is indicated as sensitivity factor in the column 'Calibration Factor'. The criterion of quality is a correspondence of 100± 10 % between the certified value and the value actually determined

Table 14. Selection of available reference materials

Designation	**Code**	**Certified by**	**Mercury content**
Beech leaves	CRM 100	CRM	260 + 10 ng/g (approx. value)
Spruce needles	CRM 101	BCR	70 ± 2 ng/g (approx. value)
Cod muscle tissue	CRM 422	BCR	559 ± 16 ng/g
Poplar leaves	NCS DC 73350	Institute of Geophysical and Geochemical Exploration	26 ± 3 ng/g
Mussel tissue	NIST 2976	NIST	61 ± 3,6 ng/g
Pig kidney	CRM 186	BCR	1970 ± 40 ng/g
Bovine liver	NIST 1577b	NIST	3 ng/g (approx. value)
Dogfish liver	DOLT-3	National Research Council Canada	3370 ± 140 ng/g
Pine needles	NIST 1575a	NIST	39.9 ± 0.7 ng/g
Sea lettuce (algae)	CRM 279	BCR	51.5 ± 2.9 ng/g
Dogfish muscle tissue	DORM-2	National Research Council Canada	4640 ± 260 ng/g

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7.6.4. Limit of quantification

Following each re-calibration, it is necessary to re-calculate the limit of quantification of the process. The calculation is carried out using the blank value method (estimation of the limit of detection according to the standard DIN 32645, 1994; the limit of quantification is then taken to be three times the limit of detection).

7.6.5. Measurement of blank values

For each test, blank values are also measured (= empty test crucible; see 7.2). In order to compare the sample contents directly, a mass should be entered for the (theoretical) weight of the blank value which is equivalent to the weight of the samples being measured. Otherwise, the absolute Hg contents should be compared. Corrections for blank values are not carried out. If the blank value is too high (> 5 - 10 % of the mercury content of the lowest sample), the system should be checked over (see also 13.1).

7.6.6. Mercury determination

The sample (approx. 10-200 mg or 50-500 µL) is weighted into the crucible on the integrated laboratory balance. The mass of the sample is then transmitted automatically to the test unit by pressing a button. The test process is started after weighing all the samples. The drying, incineration and oxidation times should be adapted to suit the sample material. This also applies for the drying and incineration temperatures as well as the duration of rinsing after incineration. The start button is then pressed to start the test.

Standard program for freeze-dried biotic environmental samples (solids): Settings: '100°C - 0 s - 850°C - 180 s - 60 s - 12 s - 30 s' (drying temperature - drying time - incineration temperature - incineration time - rinsing time - amalgam heating – measurement time).

With dried or freeze-dried solid substances, no drying program is required because there is no risk of splashing caused by delay in boiling. The minimum incineration time is 120 sec. This time is necessary to heat the sample crucible up to the temperature required to release all the mercury. For samples with high organic content, a longer incineration time has to be selected. The general rule is that half the sample weight is equivalent to the incineration time in seconds. Samples with high organic contents often react by spontaneous ignition during incineration in the oxygen stream. It is therefore advisable to distribute these samples well

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over the bottom of the crucible (e.g. press down well to prevent excessive spontaneous reaction).

Standard program for non-dried biological samples: Settings: '300°C - 60 s - 850°C - 180 s - 60 s - 12 s - 30 s' (drying temperature - drying time - incineration temperature - incineration time - rinsing time - amalgam heating – measurement time).

Standard program for liquid samples: Settings: '300°C - 300 s - 850°C - 240 s - 60 s - 12 s - 30 s' (drying temperature - drying time - incineration temperature - incineration time - rinsing time - amalgam heating measurement time).
For liquid samples, a drying operation is essential before the actual incineration operation. This is necessary to prevent the sample from splashing due to rapid rise in temperature and delay in boiling and causing contamination in the system (quartz tube). For samples with a high-water content, the temperature for drying should be set to 300°C. This temperature allows fast drying with- out causing the sample to splash. The length of the drying program depends on the amount of liquid weighted in. As a rule of thumb, the quantity of liquid in mg can be taken as equivalent to the length of the drying operation in seconds. If the sample contains only about 50% moisture, the time for drying is reduced accordingly. If a temperature of less than 300°C is set for water-based samples, the drying operation has to be pro- longed. Even for liquid samples, a subsequent incineration program is essential to fully release all the mercury in the sample. With liquid samples, suitable blank solutions must be tested (e.g. water, with acids, stabiliser etc.).

7.6.7. Evaluation

The data measured are evaluated by the software of the analyser unit. The automatic evaluation is checked for plausibility. For each calibration, a linear regression is carried out to calculate the slope, ordinate intercepts and coefficient of correlation (r) of the linear calibration lines. The concentrations of all the solutions tested (blind values, reference materials, samples) are calculated on the basis of these straight calibration lines.

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7.7. Documentation

The following measurement parameters should be noted as raw data (or entered in a computer file):

- unique sample designation (e.g. ESB code),
- parameters of method (e.g. temperatures and analysis times set),
- calibration used,
- analysis results for mercury for samples and reference materials,
- any observations or comments.

Important note: All deviations from these guidelines must be documented in the raw data of the respective measurement.

7.8. Validation

Before using the DMA instrument for routine testing in the Environmental Specimen Bank program, extensive validation procedures were carried out. This involved testing a number of certified reference materials and ESB reference materials and then comparing the results with the target values. In this way it was possible to establish the suitability of the system for the analysis of ESB samples. For all tests, suitable certified reference materials and (for analyses within the ESB program) suitable ESB reference materials are also tested in order to establish the correctness of the test results and to determine the uncertainty of the analytical data if necessary. The criterion of quality is a correspondence of 100 $\pm$ 15 % (or 100 $\pm$ 20% for concentrations close to the limit of quantification) between the certified value and the value actually determined. For method validation for samples for which no validation data are available, the following process parameters should also be determined:

Selectivity / specificity: these are met if the con- centration measured for the chemical blank value is less than the lowest validated concentration.

Reproducibility: the reproducibility is calculated from the correspondence data of the reference materials via the relative standard deviation (S_{rel}). This condition is fulfilled if the following applies: $S_{rel} < 10$ % ($n \geq 5$).

Limit of detection / limit of quantification: The limit of detection is calculated from blank value analyses (determined according to the standard DIN 32645: blank test method, quick

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estimate). The limit of quantification is produced by multiplying the limit of detection by a factor of 3.

9.9. Control charts

Control charts (as target-value charts) are maintained to document the long-term reproducibility of the process. These are prepared for the various reference materials and for blank values.

7.10. Interferences

7.10.1. High blank values

Excessively high blank values may be caused by contamination in the test system or an exhausted catalyst. If necessary, the system should be cleaned, or the catalyst replaced.

7.10.2. Spontaneous reactions

Large quantities of gas are produced during a spontaneous reaction which may not be collected in the combustion chamber. The resulting pyro- lysis products are then forced too quickly through the catalyst by a pressure wave. This means that the dwell time in the catalyst is too short for complete conversion to take place. These pyrolysis products may cause damage to the subsequent gold carrier and the optical cell. The incompletely converted pyrolysis products may be deposited on the gold carrier and affect the actual mercury signal by additional non-specific absorption.

7.10.3. Statement of results

The results are related to the amount of solid matter used (to the fresh mass or, with freeze-dried samples, to the dry mass). All results for mercury (Hg) should be stated to three significant places.

EXAMPLE: 123 ng/g; 34.5 ng/g; 0.678 µg/g. Measurement results are subject to a degree of uncertainty. In the working range of a process, the measurement uncertainty increases as the concentration in the sample decreases. The degree of uncertainty of a measured value can be determined in a number of ways which are described in 'ISO Guide to the Expression of

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Uncertainty in Measurement (GUM)' (ISO, 1995) and guideline 'Quantifying Uncertainty in Analytical Measurement' (EURACHEM/CITAC, 2000). A practical means of determining uncertainty is the so-called Nordtest process (MAGNUSSON ET AL., 2003; calculation of uncertainty from duplicate measurements of certified reference materials and results of interlaboratory comparisons).

NOTE: For the analysis of Environment Specimen Bank samples, generally six sub-samples from one homogenate are used. The standard deviation of the average value is regarded as the measurement uncertainty of the result. The correctness of the results is verified with the help of certified reference materials. Representative data are given in the appendix

7.11. Analysis report

The following data should be documented in the analysis report:

- reference to this guideline,
- sample identity,
- concentration of mercury with reference to the sample dry weight,
- statement of measurement uncertainty if applicable,
- data on preliminary treatment of sample and digestion,
- any deviations from this guideline.

7.12. Representative analysis results

Representative results of analyses are given in the appendix:

a. Results of the analysis of certified reference materials,
b. Results of the analysis of reference materials from the Environmental Specimen Bank,
c. Results of the analysis of representative samples from the Environmental Specimen Bank

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