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2. Introduction

In the previous additional deliverable AD16.1 “Screening methods inventory”, a primary definition on emerging chemicals as considered within HBM4EU WP16, as well as an overview of the different existing screening approaches were given. A current state-of-the-art was also presented regarding the basic concepts, supporting instrumentation and data processing strategies related to suspect and non-targeted screening. While of already significant and growing maturity in the environmental (e.g., within the NORMAN network) and food safety field of research respectively, this large scale screening issue still remains poorly addressed in human biomonitoring. Possible explanations are (i) lower chemical concentration levels, higher complexity, and/or lower concentration factors that can be achieved for human as compared to environmental and food matrices, meaning an additional level of analytical difficulty, (ii) the presence of often not yet identified biotransformation products (metabolites) rather than pre-identified parent compounds in human matrices, and (iii) the lack of structuration of the necessary HBM networks and laboratories compared to other fields.

Non-targeted analyses (NTA) appear today as a powerful and rapidly developing approach to characterise the human exposome and evaluate its variability over different factors. However, it is still facing a lack of reference methods and procedures as well as harmonised criteria permitting to document method performances and ensure the reliability and comparability of results produced by different laboratories. Indeed, no guidelines were yet proposed to perform NTA from human matrices, while this particular application imposes some specific and significant analytical challenges for which concerted guidelines appear to be necessary.

NTA workflows involve multiple steps of sample preparation, data acquisition, data mining and expert reviewing. The sample preparation step is critical, as a compromise has to be sought between a given concentration factor for enhancing the useful information (signals of interest), a limited selectivity for maximising the range of potentially detected markers, and a sufficient purification or fractionation to limit matrix interferences and related matrix effects. Another important level of necessary consensual guidelines is related to the instrumental analysis, where several options are available for chromatographic separation, ionisation and mass spectrometric acquisition. Finally, the post-acquisition data processing step crucially depends on the availability and performance of bio-informatics tools, which require a high level of collective expertise for reaching a better consolidated QA/QC framework.

In this context, the aim of the present deliverable is to name the crucial methodological questions of NTA workflows, to highlight the necessary compromises and/or to propose guidelines in order to implement NTA in human biomonitoring and environmental health research.

As a global issue before describing these different sections of a NTA workflow, it is of importance to note that the chemical space finally considered by these approaches could not be infinite, but conversely determined by a number of physico-chemical properties of the considered markers and their compatibility with the applied analytical procedure and underlying technology. As illustrated in Figure 1, only chemicals with adequate properties will be finally detected after the extraction, chromatographic separation, and ionisation steps. In the previous deliverable D16.1 “prioritised emerging chemicals”, already existing inventories of emerging chemicals of concern produced at international scale were aggregated, leading to a final global list of more than 70,000 potential markers of interest. Although this extended list remains to be further curated and prioritised (on-going work), it represents a first guiding rail for developing NTA with some indications regarding for instance the relevant range to be considered in terms of molecular weight or polarity (Figure 2), that may contribute to guide the sample preparation and data acquisition processes.

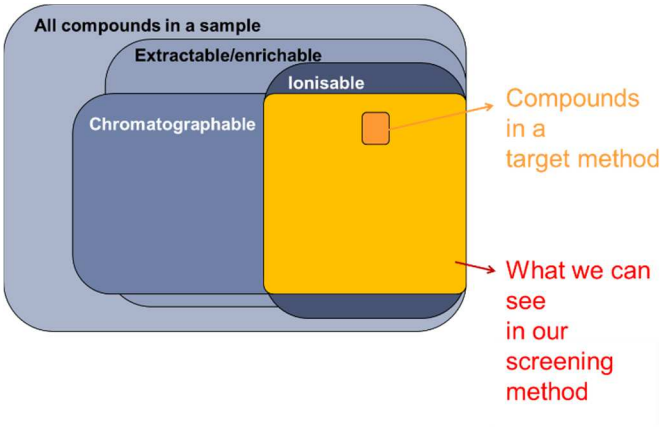

Figure 1: Chemical space finally possibly detected by applying a given methodological NTA workflow.

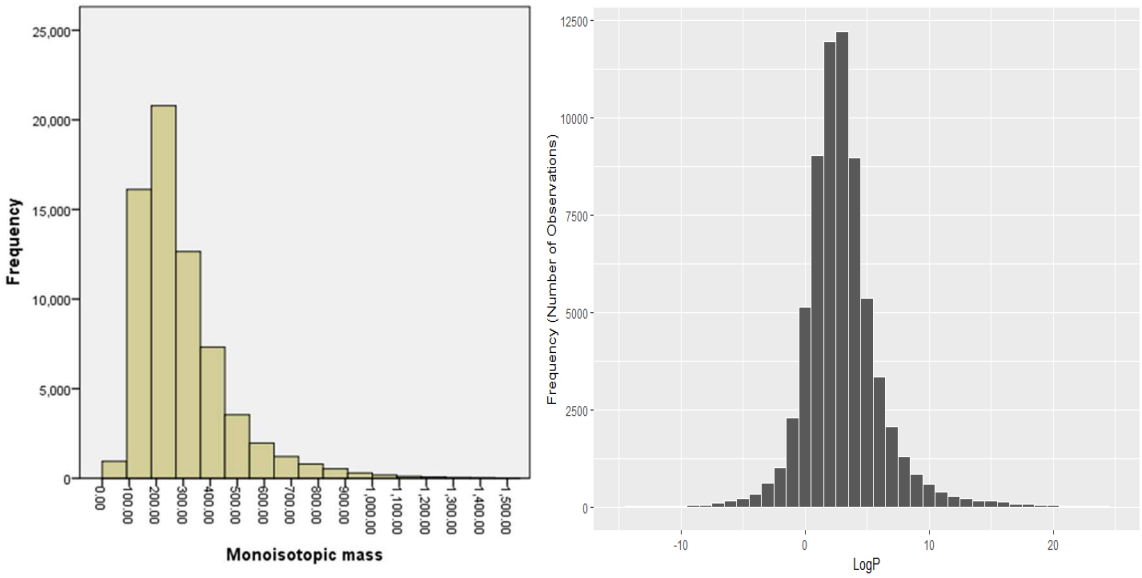

Figure 2: Chemical space (molecular weight and log P) of the exposure markers included in the aggregated list of potential emerging chemicals of interest produced as deliverable D16.1 (HBM4EU_EmergScreenDB database).

3. Sample preparation

Sample preparation is the first step in NTA workflows and a number of challenges have to be overcome to obtain representative results from the analysed sample. In particular, a proper balance between reducing matrix interferences and maximising the range and recovery of as many compounds of interest as possible has to be found. At the present time, no unique sample preparation method is capable to cover such an extended range of exposure markers with diverse properties to be proposed as general reference protocol for NTA. Instead, major sample preparation related issues to be considered in the context of NTA are pointed out in this section, together with suggested guidelines for laboratories aiming to implement these approaches.

3.1 Sample and enrichment

After the pre-analytical phases of sample collection and storage, a first crucial analytical step consists in defining an appropriate starting sample amount for analysis. The sample amount depends on the expected analyte concentration and the detection sensitivity of the applied instrumentation, and it is directly impacting the possible concentration factor of both the compounds of interest and matrix interferences, and the level of efficiency required for the extraction and purification steps. As a general principle, the more important is the sample amount considered for analysis, the more selective should be the purification step in order to exclude matrix interferences and to concentrate compounds of interest. This approach is typically used for targeted analysis, especially for markers present at low concentration levels in complex biological matrices for which sample availability in relatively high quantity is not an issue. Conversely, human biomonitoring matrices are typically available in low amount, leading to limited possibilities of concentration factor, while the concentrations of environmental pollutants or metabolites are typically orders of magnitude lower than concentrations of endogenous compounds, food constituents and drugs[1].

However, developing non-targeted methods based on low sample amounts is in agreement with the small amounts typically available for human matrices. Small sample amounts (typical < g or mL scale) for NTA then appears as a general recommendation, associated to a more limited sample preparation (Figure 3). Importantly, reduced sample amount/volume also permit to limit the associated matrix effect[2] that may impair the final detectability of the signals of interest (Figure 4). This global strategy however requires a highly sensitive and selective detection which is fulfilled to a large extent by the latest generation of HRMS instrumentation. In all cases, method performances in term of sensitivity have to be better documented in the context of NTA (see section 6 below). The reduction of the starting sample amount must also consider the representativeness of the sample, especially for solid matrices (e.g., placenta, adipose tissue).

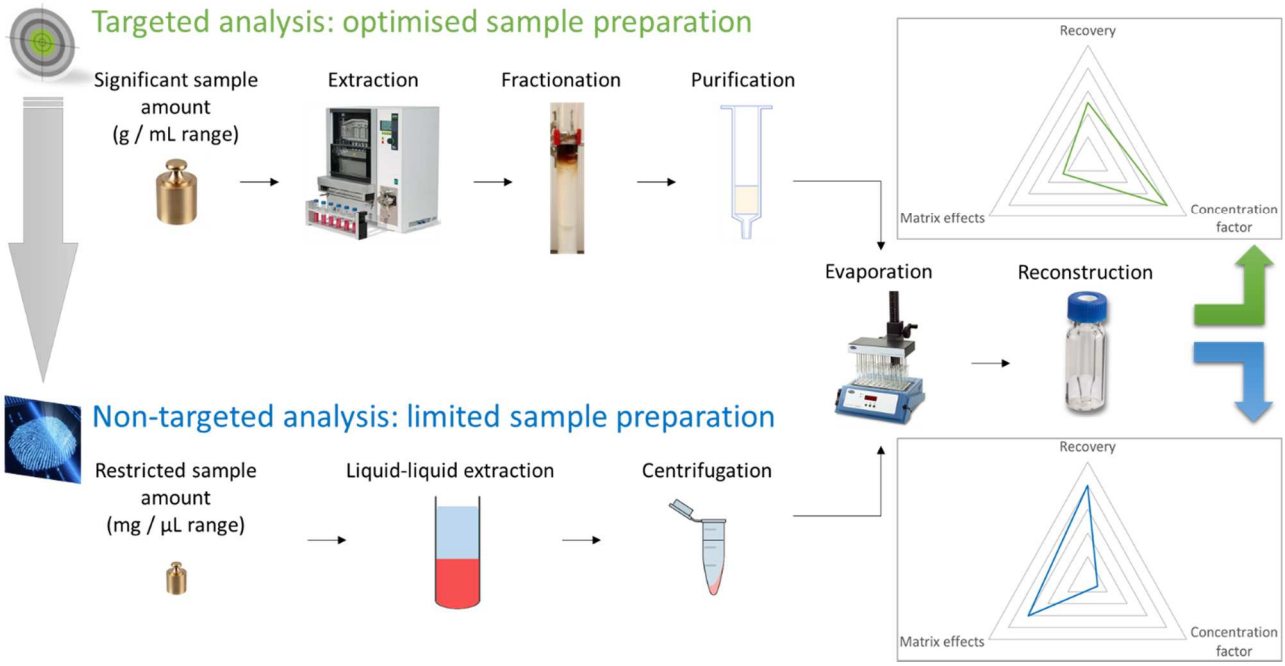

Figure 3: Classical targeted sample preparation approaches (top) usually introduce several steps to isolate the compounds of interest while typical non-targeted sample preparation approaches (bottom) introduce a limited number of steps to preserve sample integrity, so that these two approaches have distinct consequences in term of expected recovery, concentration factor, and matrix effects.

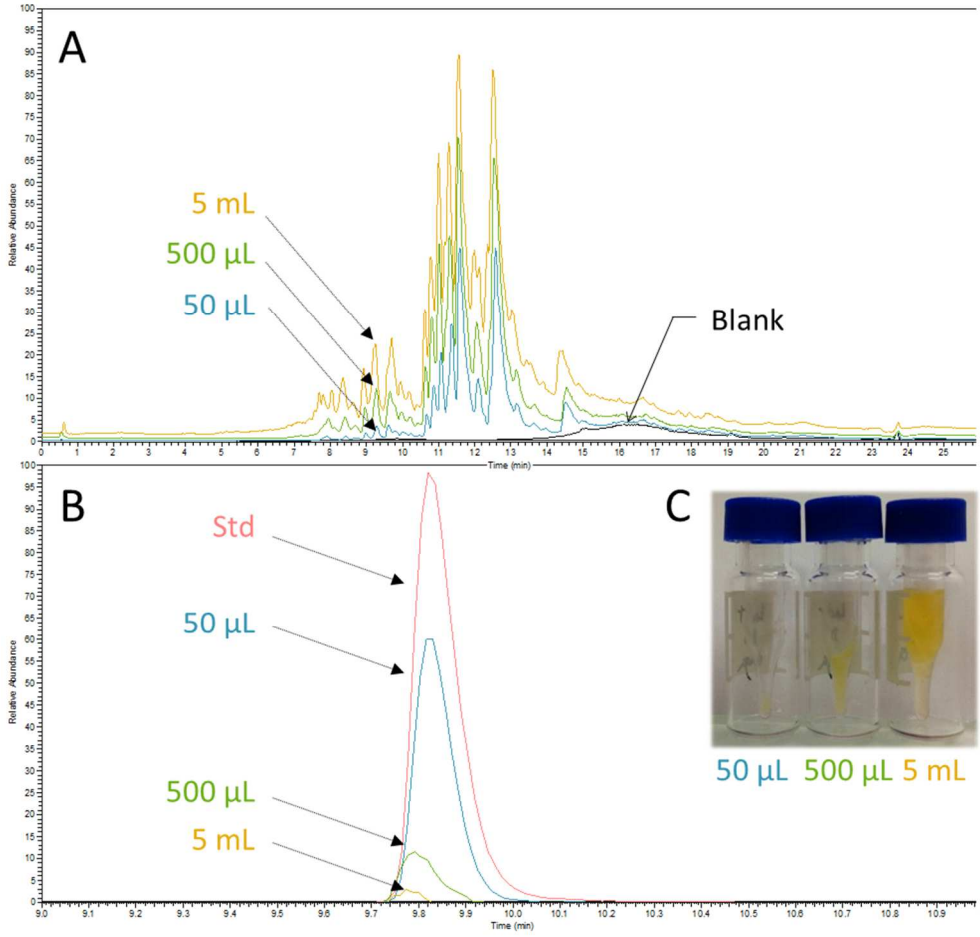

Figure 4: Illustration of matrix effects observed for a breast milk sample prepared according to the same LLE procedure but starting with three different initial sample volumes, i.e. 50 µL (blue), 500 µL (green), and 5 mL (orange). A: Total ion chromatograms (TIC) obtained in LC-HRMS (Thermo Q-Exactive Orbitrap instrument, negative electrospray ionisation mode). B: Extracted ion chromatograms (EIC) of labelled tetrabromobisphenol A (^{13}C-TBBPA, $[M-H]^-$, m/z 554.7860 ± 5 ppm) used as internal standard at identical concentration for the processed samples, as well as in a pure standard at concentration equivalent to 100% recovery (pink). C: Illustration of the observed remaining lipids after treatment of the different considered sample amounts. Higher initial sample amount lead to higher global signal intensity measured on the TIC (A) but to lower particular signal abundance for markers of interest (B) due to higher residual matrix components (C) and subsequent matrix effect and/or lower recovery.

3.2 Extraction, purification and/or fractionation

In the frame of the HBM4EU project, both liquid (urine, blood, breast milk) and solid (placenta, meconium, adipose tissue) matrices will be analysed by NTA. The lipid content of the considered matrix also strongly impacts the selection of the sample preparation process, as matrices with a high lipid content are more challenging than those with low lipid content. To overcome this diversity, several options for sample preparation are possible from the large panel of current techniques available. The main issue in the case of NTA is the definition of an appropriate level of selectivity of the selected protocol. The LC- and GC-HRMS systems used for NTA are not compatible with direct injection of complex liquid (blood, breast milk) or solid matrices. Even when the minimal level of compatibility with injection is reached, matrix interferences remaining in the final extract could impact the detected signal through ion suppression[3]. Therefore, an extraction

followed by a certain level of purification is mandatory. On the other hand, in non-targeted approaches as much compounds as possible should be detected and thus the extraction and purification should be as non-selective as possible. Thus, one of the main challenges associated with NTA is to find a good balance regarding purification selectivity in order to limit matrix interferences and to preserve as much compounds of interest as possible. For any sample preparation technique applied, this issue has to be studied using a mix of appropriate reference standards (see section 6 below for this QA/QC aspect).

A first necessary decision with regard to the sample preparation protocol is related to the inclusion (or not) of a deconjugation[4] step for hydrolysing the glucuronide and sulphate phase II metabolites potentially present for a range of exposure markers. Introducing this deconjugation step permit to finally quantify the total (free + deconjugated) forms of the considered markers through the monitoring of one single diagnostic signal. This strategy then leads to an aggregated indicator of the global internal dose. This approach is typically the one applied with conventional targeted methods especially with regard to a given reference value in a regulatory context. Enzymatic or chemical hydrolysis may be employed for that purpose, but both approaches have advantages and limitations with varying results for the different substances groups or even for different compounds within a same group. Thus, a general recommendation cannot be made. This particular analytical step may also contribute to a global intra- and inter-laboratory variability observed in terms of quantitative results, considering the wide range of possible detailed conditions and the difficulty to reach a maximal and constant deconjugation rate for all markers of interest. Importantly, this step is also susceptible to introduce an external background within the generated global chemical profiles that can somewhat impact the robustness of the results if not appropriately managed. Thus, the definition and application of a single harmonised technical procedure for deconjugation in the context of NTA is probably not realistic. Conversely, a more systematic reporting regarding this issue should be promoted for better documenting the rationale behind the inclusion of such deconjugation step (or not, for instance in case of particular focus on non-hydroxylated markers and/or measurement in tissue or storage biological compartments rather than in urine) and for reliable inter-study comparisons.

Liquid-liquid extraction/partitioning (LLE) appears as a good compromise to extract a large number of compounds with a wide range of physico-chemical properties. LLE is directly applicable to liquid matrices (urine, blood, milk), and could be used under high temperature and pressure for chemicals proven to be stable under these conditions. Solid matrices (adipose tissue, placenta, tissue, meconium…) require a pre-treatment to increase homogeneity and solid-liquid contact surface and thus extraction efficiency. Lyophilisation (freeze-drying) and/or grinding (for instance through a Precellys or similar system) can be strongly recommended for that purpose. For both types of matrices, the choice of the solvent (mixtures) is crucial. In a first approach, a biphasic system can be suggested combining a polar solvent (water, methanol and/or acetonitrile) with one of intermediate polarity (e.g., diethyl ether, chloroform…), and/or one non-polar (e.g., cyclohexane, pentane, toluene…). This basic partitioning permits collecting two complementary fractions from each analysed sample. This could be an efficient way to both divide (so dilute) the whole matrix effect and produce two layers to be possibly characterised by complementary technologies, e.g. LC-HRMS (predominantly hydrophilic compounds) and GC-HRMS (more hydrophobic compounds), respectively. The Bligh and Dyer method[5], originally developed for lipid extraction, is now commonly used for metabolomics analysis, and it seems promising also for NTA, at least for lipid-rich matrices. In brief, this method consists in applying a ternary solvent system with water, methanol and chloroform (Figure 5). This approach indeed appears applicable to various liquid and solid matrices, and may represent a first reliable and well documented basis for implementing these NTA.

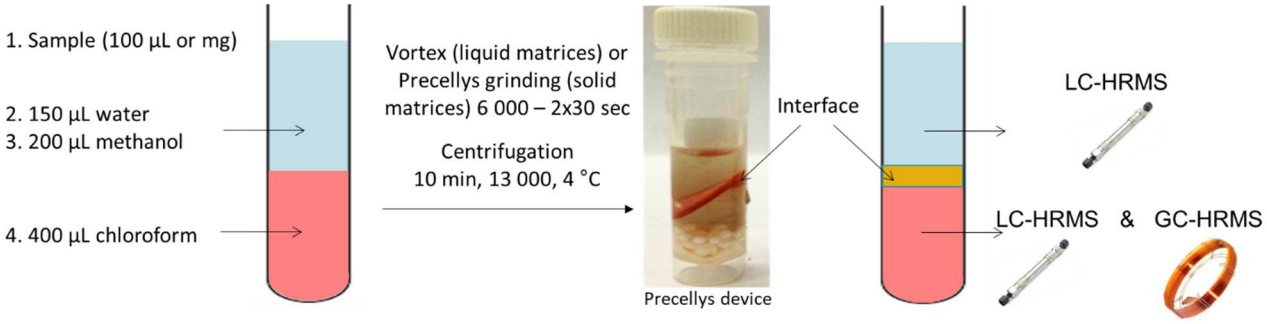

Figure 5: Principle and proposed conditions for Bligh & Dyer liquid-liquid extraction (assisted by Precellys grinding for solid matrices) applied for NTA from human matrices. Two fractions are finally collected, one for highly to relatively polar compounds (to be further analysed in LC-HRMS), and one for relatively to highly non-polar compounds to be further analysed in LC-HRMS and GC-HRMS for complementarity and extended coverage of accessible markers)

Other pre-extraction steps such as protein digestion or hydrolysis, as well as filtration[6] techniques may also be applied. However, these steps may introduce both some variability and some external contribution to the chemical profiles that may complicate the data processing step and affect the final results. Also the option of direct injection for less complex matrices (e.g., (diluted) urine) through TurboFlow[7] injection systems could be evaluated. Based on this initial proposal, many adjustments or alternative conditions may be developed.

Importantly, it should be emphasised that in the emerging field of NTA, no unique reference sample preparation method would appear possible, relevant or even necessary. Considering the extremely wide range of possible exposure markers of interest in a whole NTA context, the combination of several sample preparation methods and resulting extracted fractions appears to be more complementary than problematic. Although well integrated in the WP16 work plan, the objective of harmonisation and standardisation of sample preparation procedures applied to NTA is then likely premature at this stage, since research and development is still required to define adequate methods for to the matrix studied. Conversely, the definition and implementation of appropriate QA/QC procedures permitting a better assessment and documentation of the performance of the proposed methods appears as a priority for methodological evaluation and comparison (see section 6 below).

In parallel to this non-selective strategy basically complying with the objective of non-targeted screening, some selectivity may be introduced towards particular chemical substance groups. This may be justified by specific interests of the developing laboratories considering their own research priorities. This may be also motivated by a pragmatic approach consisting to characterise different fractions of the sample for maximising the (possibly useful) signal-to-noise ratio compared to the more generic approach. To this end, different purification methods can be used with different selectivity. For instance, solid phase extraction (SPE) is largely used for target and non-targeted analyses[8], and a large number of different stationary phases exist which can improve the detectability of specific exposure markers while limiting the number of matrix interferences. In the case of emerging chemical screening, this second strategy may strife to better isolate sample fractions containing particular compound classes such as chloroparaffins, halogenated phenols, benzophenones, pesticides, etc. Thus, a global screening may contain two complementary levels, the first one involving the detection of a broad range of most abundant markers from multiple chemical classes, and the second one involving the detection of markers from given chemical

classes in a more focused way with a better performance, particularly with regard to sensitivity and repeatability.

3.3 Sample extract reconstruction

After the extraction process, the extract is generally concentrated by solvent evaporation (mostly evaporation to dryness). The last step before injection into the analytical instrument thus consists in the reconstitution (dissolution) of the final extract. This non-trivial technical consideration directly impacts the capability of the finally extracted compounds to be correctly re-suspended in an appropriate liquid phase, and to reach the separation and detection system. If the solvent (mixture) used at this step is not properly chosen, not all compounds might be dissolved and could thus not be detected. Because NTA aims to cover a range of various compounds from diverse physico-chemical properties, the use of a mix of solvents with complementary polarities and solubilisation capabilities seems to be a good option to reconstitute the final sample extract. The solvent has also to be compatible with the chromatographic system used for MS analysis. This is particularly true for LC-MS, for which the injection solvent system may greatly influence retention times as well as peak shapes. This also apply to GC-MS in some extend with a more limited panel of possibilities. This issue has to be studied using a mix of reference standards in order to assess the efficiency of the tested solvent for final sample extract reconstruction (see section 6 below). Although no definitive procedure may be given at this stage, the use of a ternary solvent system may be proposed as illustrated in Figure 6.

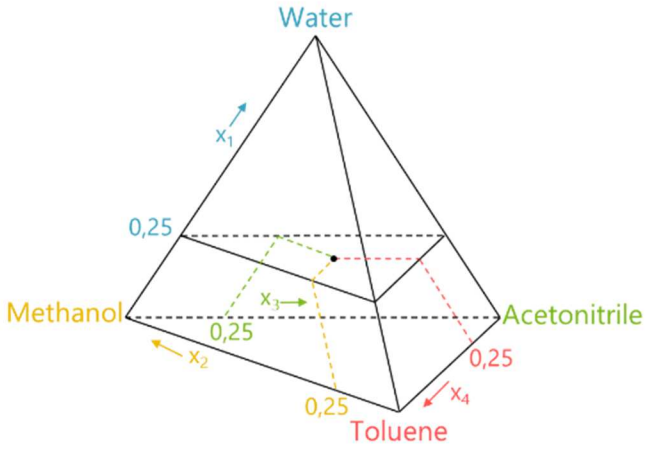

Figure 6: Common solvents possibly used for sample extract reconstruction for LC-MS analysis. Toluene may be employed in small amount (<5%) as keeper[9] for some compounds. Final proportions of these solvents depend on the expected compound polarity and solubility and the LC eluents employed, so that they should be preferably optimised on the basis of a representative mixture of reference compounds during method development.

4. Chromatographic separation and MS data acquisition

The type of chromatographic and spectrometric instrumentation recommended to perform NTA was described in the previous deliverable AD16.1 “screening method inventory”. Briefly, LC-HRMS and GC-HRMS are the technologies of choice, either with Orbitrap or Time-of-Flight (TOF) MS devices. Those two chromatographic separation systems (LC and GC), coupled via various ionisation techniques to mass spectrometry devices are complementary by covering different range of molecular size and polarity (Figure 7). In general, liquid chromatography followed by electrospray ionisation (LC-ESI) appears as the most versatile option for the detection of a large range of markers. This technology is also most commonly implemented in analytical laboratories.

Yet, this first preferred option cannot be considered as the universal one, since gas chromatography followed by electron ionisation (GC-EI) gives access to a fraction of exposure markers not accessible with LC-HRMS. The combination of these two approaches finally allows covering a wide range of compounds in terms of molecular size and polarity. A third option, employing LC-APCI/APPI-HRMS or GC-APCI/APPI-HRMS may be also mentioned. LC-APCI allows to get compounds hard to ionise with ESI, but not amendable to GC (for example some highly brominated flame retardants), while GC-APCI reduces the problem of GC-EI that strong fragmentation occurs and the molecular ion is not often visible.

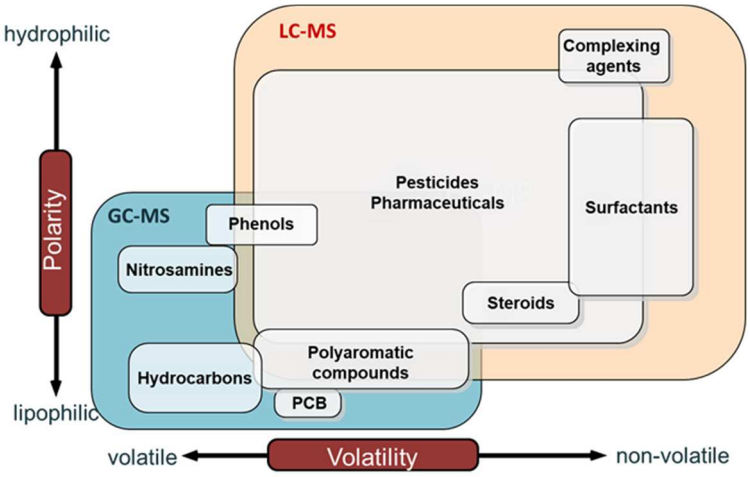

Figure 7: Compound domains coveredby LC-MS and GC-MS based systems in terms of polarity and volatility of the compounds. Adapted from Brack et al 2016[10]

For LC separation, a large diversity of stationary phases, mobile phases and solvent additives can be used. Despite this diversity, reversed phase columns (mainly C_{18}) still remain the first choice due to their efficacy over a wide hydrophobicity range and their widespread use allowing methodological comparisons and harmonisation. Hydrophilic interaction phase (HILIC) and polar embedded reversed phases however are increasingly emerging alternatives for highly hydrophilic compounds and their compatibility with common ionisation sources.

Regarding the mobile phase composition, conventional water/methanol or water/acetonitrile biphasic systems are most commonly used, and both seem to be suitable. A ternary system water/methanol/acetonitrile may also be suggested to take simultaneous benefit of the respective properties of both solvents. The use of a generic elution gradient is recommended rather than a separation under isocratic conditions. An example for suitable LC conditions is given in Figure 8, although no definitive reference protocol should be fixed at this stage. The nature of the solvent modifier employed to promote ionisation is of importance, as it has a direct impact on the obtained

chemical profiles. The use of volatile organic acids such as acetic or formic acids remains the most commonly employed option for the positive ionisation mode, but ammonium acetate or formate buffers are also used. In negative ion mode the same additives (and thus the same chromatographic conditions) may be used as for positive mode, but the use of strong proton accepting additives such as ammonia or fluoride allows an efficient ionisation of phenolic compounds otherwise not covered. Again at this stage, no definitive option can be fixed concerning the obvious efficiency of different approaches. The choice of eluent system impacts the distribution of the adducts formed for the expected exposure markers (e.g., protonated molecules vs. ammonium adducts), thus influencing the annotation. Finally, a rigid and not fully realistic analytical harmonisation should not be imposed at that point to ensure some flexibility for building the MS reference library, permitting to cover the different ions formed.

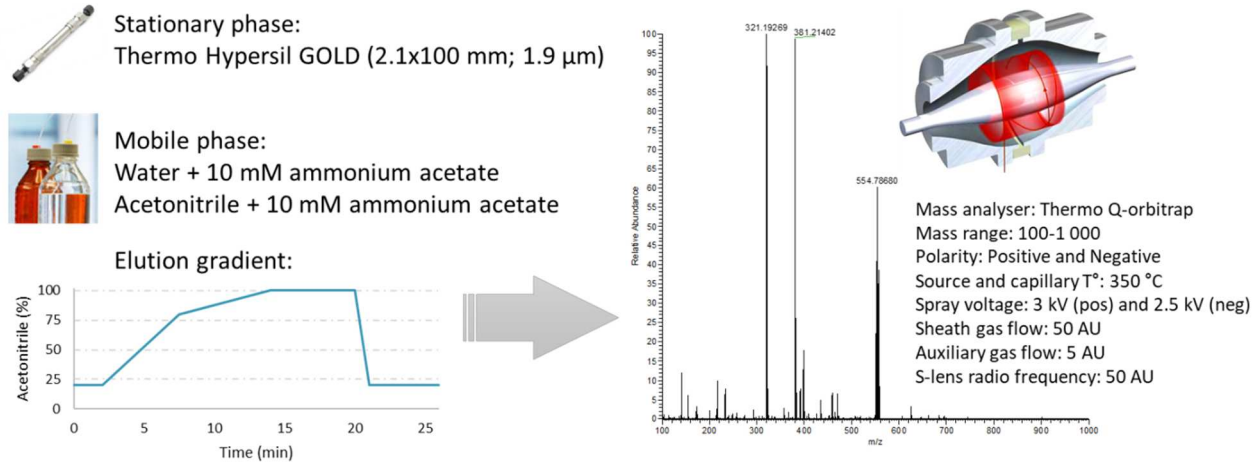

Figure 8: Example of LC-HRMS conditions applied to NTA in the scope of generic screening.

Regarding the MS acquisition conditions, as recommended in the previous deliverable AD16.1, both ionisation modes (positive and negative) should be used to maximise the accessible markers, either through two separate injections or using a single injection in the polarity switching mode for MS devices with sufficiently high scan rates. In term of mass ranges, a first priority should be given to the m/z 50–1000 range basically fitting with the screening of small compounds typically expected in HBM (Figure 2). However, the range from m/z 1000–2000 range may be also possibly informative and optionally covered.

Beside this ionisation polarity and mass range criteria, the full scan mode acquisition is representing the starting recommendation for performing NTA. At this stage ensuring a maximal reliability of the generated data appears crucial especially in terms of resolution and mass accuracy. Appropriate dispositions enabling to control and adjust these two parameters appears necessary, through the requirement of appropriate calibration and lock mass procedures as well as the recurrent analysis of appropriate mixtures of reference compounds (see section 6 below). For state-of-the-art instrumentation, resolution typically exceeds 20,000, and the mass measurement error is below 10 ppm, and for some instruments even below 2 ppm.

To go further, data dependent acquisition (DDA) and data independent acquisition (DIA)[11] are more advanced options that have to be considered for generating structural information in the context of NTA. Those acquisition modes require hybrid MS instruments equipped with fragmentation capabilities. Briefly, DDA is more restrictive than DIA, as only by fragmenting the “n” most intense ions of the MS full scan with an intensity higher than a pre-set threshold are fragmented. In contrast, in DIA all ions are fragmented and data treatment tools are used to assign

the various fragment ions to their respective precursors. Basically, that additional information related to the structure of the compound in addition to its exact mass (so its elemental composition) is the crucial basis of further high confidence level with regard to the unambiguous compound identification.

To demonstrate the principle usability of LC-MS/MS with DDA and subsequent library search, two interlaboratory studies were organised within HBM4EU WP16. The samples analysed represented mixtures of reference compounds. The receiving labs analysed the samples with their respective HRMS instruments employing DDA and DIA techniques for obtaining tandem mass spectral information. Data mining was accomplished in the organising laboratory. Irrespectively of the kind of instrumental settings applied for generating tandem mass spectral information, the true positive rate was exceeding 95%, which indicates that there is already sufficient expertise available within this consortium to enable immediate application of automated fragmentation techniques in NTA. Nevertheless, this advanced data acquisition is currently still a matter of research and development, so that no definitive guidance can be given at this stage, whether DDA and/or DIA should be preferred in the context of NTA. Thus, this issue should be (and is within the present HBM4EU WP16 consortium) a matter of shared discussions aiming to establish the first steps paving the way toward such harmonised practices.

5. Data processing

The final step in non-targeted analysis workflow is data processing, consisting to go from the raw instrumental data to a curated tabulated file (peak list) used for subsequent annotation and statistical analyses. Here a wide range of software tools are available to perform such data processing and extraction of the useful information from the raw data. Some of these tools are integrated solutions from MS vendors (e.g., Metaboscape from Bruker, Progenesis QI from Waters, Sieve and Compound Discoverer from Thermo, Mass Profiler (Professional) from Agilent, MetID from Shimadzu, XCMSplus from Sciex …). Other options are open source software[12], some of them largely implemented in the metabolomics community (e.g., XCMS in the R computational environment, MZmine, Workflow4Metabolomics…), some other being in-house developed solutions (e.g. HaloSeeker[13],…).

Briefly, all these data processing tools aim to detect any signal present in the generated global chemical profiles (peak picking), to align common peaks found in the different samples and report their intensity or area (peak integration). In practice, the settings for the respective algorithms have to be carefully chosen, as they directly impact the obtained information. Even after years of use and experience with these tools, still no consensual guidelines can be provided regarding both a preferable selection from this panel of existing tool or their fine appropriate parameterisation in the context of NTA. As the settings depend also on the analytical configuration (LC and MS settings), a more harmonised procedure is also hard to implement. Thus, it is the priority to establish a common QA/QC protocol permitting to reach a better level of confidence on the produced results and a better comparability between different methodologies despite the diversity of the different data processing approaches used.

Another reasonable approach to overcome the current analytical challenges related to non-targeted data mining strategies seems to be its combination with targeted data mining strategies to a unified data analysis workflow. Initially, suspect screening will be accomplished. Identified targets will be used for calibrating *m/z*-values and retention times, and this will improve reliability of the compound identification steps employed in non-targeted data analysis pipelines. Moreover, identified targets will represent quality controls for feature detection with non-targeted data analysis. Depending on the observed sensitivity, parameters of the algorithms might be changed to increase performance. Peak lists obtained during non-targeted data mining will include already identified targets. Peaks part of such an “inclusion list” do not need to be submitted to the further identification process. Thus, the overall workload for non-targeted data analysis will be reduced. Additionally, targets that were not matched to any feature in a data set during targeted data analysis can be excluded to represent possible outcomes of non-targeted data analysis. Such an “exclusion list” will facilitate and accelerate non-targeted compound identification.

Another crucial issue related to this data processing step is the assessment, control, and management of the external contamination encountered in procedural blank samples. In the case of NTA even more than for any targeted method, it is indeed important to define which part of the generated information is related to the analysed sample and which other part is related to instrument noise or external contamination. In practice there are a number of difficulties, both related to the characterisation of this background and to the way to manage this background through a well-established, reliable and documented blank subtraction process. Several parameters need to be set up with that respect, such as number of procedural blanks introduced in each batch of analyses and the way to average them. Another crucial question is how to establish a reliable limit of reporting for compounds present in the blank and in higher concentration in the sample (this is for instance typically the case for plasticisers). All these points are already requiring significant effort in case of targeted methods, but are clearly coming to a larger extent in the case

of NTA. Even in established workflows, this issue seems still not adequately considered. This issue requires not only strict analytical dispositions, but also new conceptual and computational solutions that are still under debate and a matter of research and development in several groups. This will require at least some months of collaborative work for reaching a reliable protocol to be implemented at the laboratories. In the meantime, an appropriate documentation of the procedures followed for characterising and managing this external procedural contamination in NTA is needed from the concerned labs.

6. Method assessment and QA/QC measures

The analytical chemistry community knows for years how to assess the performance of conventional targeted methods with appropriate quality assurance and quality control (QA/QC) measures. One of the main approaches consists in using one or more reference standard compounds adapted to the (*a priori* known) targeted compounds, to evaluate (then possibly validate) various analytical criteria including efficiency, selectivity, recovery, linearity, limits of detection and quantification, etc... Non-targeted method assessment is facing a more complex situation, where some of the signals of interest are still unknown. However, many concepts from target analysis can be transferred to non-targeted methods. Non-targeted LC-MS workflows will be fit for purpose if they are able to reliably confirm the presence of predefined chemicals being representative for the chemical domain of interest in defined biological material at concentration levels typically observed in humans exposed to those chemicals.

An appropriate set of QA/QC samples should include system suitability test samples, spiked matrix samples as well as zero and blank samples.

Spiked samples represent integral parts of different QA/QC actions. During method validation, they are used to test detection capabilities, reproducibility, as well as reliability of identification. Furthermore, spiked samples are used as QC samples for monitoring performance over a batch of samples (i.e., stability of retention times, chromatographic performance, mass accuracy and resolution, detection sensitivity, stability of signal intensities). It is feasible to select a set of known markers of exposure (within the scope of HBM4EU WP16 in the present case) and covering a large range of physico-chemical properties, to be representative for the expected diversity of marker compounds. These reference compounds may be used as indicators of the method performances at various levels, covering sample preparation (recovery, matrix effect…) but also the data acquisition (chromatographic and mass spectrometric resolution, mass accuracy…) and data processing (peak picking…) steps. In a first attempt to define such set of relevant test compounds for QA/QC purposes in the context of NTA, different lists of test compounds already used by WP16 partners were collected and merged, to finally extract a harmonised common set of markers proposed as consensual test mixture. This proposal could also facilitate further methodological comparisons, including the implementation of inter-laboratory studies. This not definitive proposal of test compounds for methodological assessment is given in Table 1. The corresponding coverage in terms of molecular weight and polarity is illustrated in Figure 9, illustrating a good representativeness of the global attempting list of markers of interest (Figure 2). Based on this list, number and selected concentration level of the markers implemented by each laboratory may be a matter of individual adjustments.

Table 1: Preliminary list of test chemicals proposed as QA/QC for assessing NTA method performance from sample preparation to data acquisition and data processing steps. Additional prioritisation is on-going for defining a subset of test compounds susceptible to be used as a basis for common interlaboratory comparability.

Nb.	InChIKey	Molecular Formula	Monoisotopic Mass	Log P	Usual Name
1	AJPIUNPJBFBUKK-UHFFFAOYSA-N	C8H12N2O	152.095	0.7	2-Isopropyl-4-methyl-6-hydroxypyrimidine
2	BGNTUSKZDOUZCZ-UHFFFAOYSA-N	C18H39O7P	398.243	4.4	Tri(butoxyethyl)phosphate
3	BTJIUGUIPKRLHP-UHFFFAOYSA-N	C6H5NO3	139.027	1.9	p-Nitrophenol
4	DFWFIQKMSFGDCQ-UHFFFAOYSA-N	C6H10ClN5	187.062	1.5	Desethylatrazine
5	DMSMPAJRVJJAGA-UHFFFAOYSA-N	C7H5NOS	151.009	1.3	1,2-Benzisothiazolinone
6	DOIRQSBPFJWKBE-UHFFFAOYSA-N	C16H22O4	278.152	4.7	Dibutylphthalate
7	DQWPFSLDHJDLRL-UHFFFAOYSA-N	C6H15O4P	182.071	0.8	Triethylphosphate
8	DUEPRVBVGDRKAG-UHFFFAOYSA-N	C12H15NO3	221.105	2.3	Carbofuran
9	FHIVAFMUCKRCQO-UHFFFAOYSA-N	C12H21N2O3PS	304.101	3.8	Diazinon
10	FLKPEMZONWLCSK-UHFFFAOYSA-N	C12H14O4	222.089	2.5	Diethylphthalate
11	IISBACLAFKSPIT-UHFFFAOYSA-N	C15H16O2	228.115	3.3	Bisphenol A
12	IOJUPLGTWVMSFF-UHFFFAOYSA-N	C7H5NS	135.014	2	Benzothiazole
13	LRUDIIUSNGCQKF-UHFFFAOYSA-N	C7H7N3	133.064	1.4	5-Methyl-1H-benzotriazole
14	MGWAVDBGNNKXQV-UHFFFAOYSA-N	C16H22O4	278.152	4.1	Diisobutylphthalate
15	MXWJVTOOROXGIU-UHFFFAOYSA-N	C8H14ClN5	215.094	2.6	Atrazine
16	NFMIMWNQWAWNDW-UHFFFAOYSA-N	C8H15N5O	197.128	0.1	Hydroxyatrazine
17	NMGBFVPQUCLJGM-UHFFFAOYSA-N	C10H10O4	194.058	1.8	Monoethylphthalate
18	ODCWYMIRDDJXKW-UHFFFAOYSA-N	C7H12ClN5	201.078	2.2	Simazine
19	PDQAZBWRQCGBEV-UHFFFAOYSA-N	C3H6N2S	102.025	1.1	Ethylenthiourea
20	PSFDQSOCUJVVGF-UHFFFAOYSA-N	C12H10N2	182.084	3.6	Harman
21	PXKLMJQFEQBVLD-UHFFFAOYSA-N	C13H12O2	200.084	2.9	Bisphenol F
22	QHOQHJPRIBSPCY-UHFFFAOYSA-N	C11H20N3O3PS	305.096	4.2	Pirimiphos-methyl
23	QRUDEWIWKLJBPS-UHFFFAOYSA-N	C6H5N3	119.048	1	Benzotriazole
24	RLBIQVVOMOPOHC-UHFFFAOYSA-N	C8H10NO5PS	263.002	2.9	Methyl-Parathion
25	RXZBMPWDPOLZGW-HITVVWEBSA-N	C41H76N2O15	836.525	3.1	Roxithromycin
26	RZJSUWQGFCHNFS-UHFFFAOYSA-N	C12H14O4	222.089	2.5	Mono-iso-butylphthalate
27	SBPBAQFWLVIOKP-UHFFFAOYSA-N	C9H11Cl3NO3PS	348.926	5.3	Chlorpyrifos
28	STCOOQWBFONSKY-UHFFFAOYSA-N	C12H27O4P	266.165	2.9	Tributyl phosphate
29	VPWNQTHUCYMVMZ-UHFFFAOYSA-N	C12H10O4S	250.03	1.9	Bisphenol S
30	VTNQPKFIQCLBDU-UHFFFAOYSA-N	C14H20ClNO2	269.118	3.2	Acetochlor
31	WCYYAQFQZQEUEN-UHFFFAOYSA-N	C5H2Cl3NO	196.92	3.2	3,5,6-Trichloro-2-pyridinol
32	WVQBLGZPHOPPFO-UHFFFAOYSA-N	C15H22ClNO2	283.134	3.1	Metolachlor
33	XZWYZXLIPXDOLR-UHFFFAOYSA-N	C4H11N5	129.101	-1.3	Metformin
34	XZZNDPSIHUTMOC-UHFFFAOYSA-N	C18H15O4P	326.071	4.6	Triphenylphosphate
35	YEDUAINPPJYDJZ-UHFFFAOYSA-N	C7H5NOS	151.009	1.8	2-Hydroxybenzothiazole
36	YZBOVSFWWNVKRJ-UHFFFAOYSA-N	C12H14O4	222.089	3.1	Mono-n-butylphthalate
37	HFZWRUODUSTPEG-UHFFFAOYSA-N	C6H4Cl2O	161.964	3.1	2,4-Dichlorophenol
38	VGVRPFIJEJYOFN-UHFFFAOYSA-N	C6H2Cl4O	229.886	4.5	2,3,4,6-Tetrachlorophenol
39	FAXWFCTVSHEODL-UHFFFAOYSA-N	C6H4Br2O	249.863	3.2	2,4-Dibromophenol
40	CXPJZISGVIVNEL-UHFFFAOYSA-N	C6H2Br4O	405.684	5.4	2,3,4,6-Tetrabromophenol
41	XEFQLINVKFYRCS-UHFFFAOYSA-N	C12H7Cl3O2	287.951	5	Triclosan
42	LDMKXEGTHGJWLG-UHFFFAOYSA-N	C12H4Br6O2	653.531	7.2	6-Hydroxy-2,2,3,4,4,5-hexabromodiphenyl ether
43	VEORPZCZECFIRK-UHFFFAOYSA-N	C15H12Br4O2	539.757	6.8	Tetrabromobisphenol A
44	DEIGXXQKDWULML-MOCCIAMBSA-N	C12H18Br6	635.651	7.1	gamma-Hexabromocyclododecane, (+)-
45	LQDARGUHUSPFNL-UHFFFAOYSA-N	C13H11Cl2F4N3O	371.022	4.4	Tetraconazole

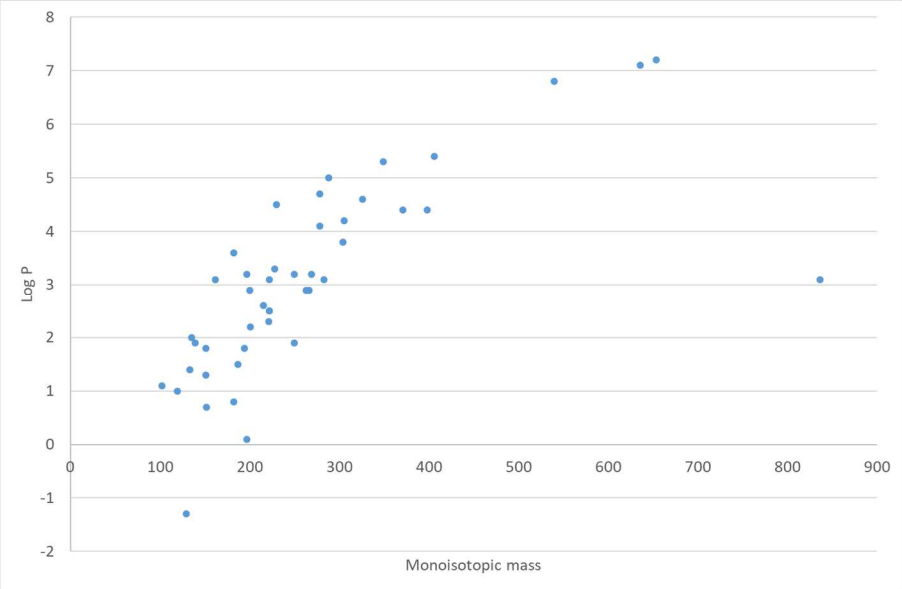

Figure 9: Chemical space (molecular weight and Log Kow) of the exposure markers proposed as test compounds for intra- and inter-laboratory QA/QC dispositions (Table 1).

If sufficient sample volume is available, technical and sample replicates as well as pooled samples will also be considered as QA/QC samples. Other important samples for QA/QC will be blank samples. They will serve for providing an overview on detectable endogenous compounds and for identifying artefacts from the data matrix, in order to identify features arising from contaminants and other components originating from, e.g., tubes, solvent impurities, additives or preservatives.

The evaluation of NTA approaches may also be performed by comparing the NTA results with those obtained by targeted approaches. This recommendation to be implemented during method development represents a valuable way to consolidate the NTA workflow, by accompanying any new analytical option tested with a given “reference” result. Even if conducted for a limited number of markers, this approach is useful to better qualify the NTA performances, including importantly a first evaluation of the false negative and false positive rates.

7. Conclusions

Each step of the NTA workflow requires specific attention. Sample preparation first requires setting a good compromise to find the best balance between a minimal selectivity to comply with the desired diversity of exposure markers of interest and an acceptable purification for limiting matrix interferences and their related impact on the overall method performances. This challenge has to be considered from the starting sample amount to the reconstruction of the final sample extract. The type of adequate MS instrumentation appears well identified, with a proposed combination of both LC-HRMS and GC-HRMS operating both in positive and negative ionisation mode as the optimal option to detect an extended set of markers. But considering the huge number of possible technical choices and parameterisation, the definition of a more definitive guideline is very difficult. Finally, data processing applied to NTA is also requiring computational tools still under development coupled to a common and better shared critical view on the final level of reliability of the produced data. A first focused workshop organised in June 2018 among HBM4EU WP16 partners permitted to start with sharing this experience among laboratories regarding the implementation of NTA applied to human matrices. Within the WP16 2019 workplan, one significant part of the research will address sample preparation and data acquisition components to be adapted in the context of NTA. The further goal is to establish optimised and as far as possible harmonised complementary methods to analyse a large range of markers from various physico-chemical properties. This 2019 AWP will also focus on data processing and more precisely on the blank subtraction and alignment issues. A second working meeting is planned to be organised in May 2019 to progress on this harmonised direction.

Beside the harmonisation issue, the quality of the produced data represents a main concern for our WP16 consortium. The elaboration of unified and harmonised procedures is estimated to be premature considering the current status of many components of these NTA workflow that are still a matter of research and development. Also it has to be emphasised that this new field and its related ambition is justifying to consider and combine different methodological strategies that are supported by the tremendous continuous scientific and technical progress pushing toward more and more relevant available options and tools for facilitating this complex analytical work. Finally, a better assessment and documentation of the method performance through the definition and implementation of appropriate QA/QC measures is required, rather that the elaboration of a too rigid harmonised framework that could impair the creativity and the real potential of these approaches. A reasonable position should be to combine the elaboration of technical and analytical requirements to guide less experienced laboratories that may want to start with NTA, while more advanced laboratories already involved in NTA have interest to maintain a high degree of innovation for further advancing this field, from which the whole community will benefit.

8. References

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