Suspect and non-targeted screening of chemicals of emerging concern for human biomonitoring, environmental health studies and support to risk assessment: From promises to challenges and harmonisation issues

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ABSTRACT

Large-scale suspect and non-targeted screening approaches based on high-resolution mass spectrometry (HRMS) are today available for chemical profiling and holistic characterisation of biological samples. These advanced techniques allow the simultaneous detection of a large number of chemical features, including markers of human chemical exposure. Such markers are of interest for biomonitoring, environmental health studies and support to risk assessment. Furthermore, these screening approaches have the promising capability to detect chemicals of emerging concern (CECs), document the extent of human chemical exposure, generate new research hypotheses and provide early warning support to policy. Whilst of growing importance in the environment and food safety areas, respectively, CECs remain poorly addressed in the field of human biomonitoring. This shortfall is due to several scientific and methodological reasons, including a global lack of harmonisation. In this context, the main aim of this paper is to present an overview of the basic principles, promises and challenges of suspect and non-targeted screening approaches applied to human samples as this specific field introduce major specificities compared to other fields. Focused on liquid chromatography coupled to HRMS-based data acquisition methods, this overview addresses all steps of these new analytical workflows. Beyond this general picture, the main activities carried out on this topic within the particular framework of the European Human Biomonitoring initiative (project HBM4EU, 2017–2021) are described, with an emphasis on harmonisation measures.

1. Introduction

The ongoing expansion of the Exposome concept (Wild, 2005; Dennis et al., 2016; Jones, 2016, Niedzwiecki et al., 2019) and development of related research activities, over the previous decade, reflects the increasing awareness of our environment as a source of human exposure to hundred thousands of chemicals. Integrative measurement of the chemical space that contaminates the environment-

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food-human continuum is supported by technological advances of chemical profiling instrumentation to enable increasingly holistic mass spectral characterisation of biological samples (Andra et al., 2017). This global contamination issue is a growing concern for exposure assessment programmes run by public health authorities, and is a challenge for risk assessment strategies (Louro et al., 2019). Among the myriad of chemical contaminants, major concerns regard *e.g.* persistent organic pollutants (POPs) and endocrine disrupting chemicals (EDCs). However, in recent years, efforts have widened directed at chemicals of emerging concern (CECs).

Despite increasing societal, scientific and policy relevance, CECs are currently a matter of non-consensual definition and various terminology (*e.g.* emerging chemicals, emerging substances, emerging contaminants…). Because the current definitions are not associated with a classification rationale, such as a common mode of action, property, intended use, or regulatory status itmaintains the unclear usage and semantic confusion. As proposed by Sauve and Desrosiers (2014), CECs encompass both new compounds recently detected in the environment-food-human continuum (for instance, newly developed substitutes of banned and/or regulated chemicals) and compounds with known presence, yet for which concerns have recently increased (*e.g.* due to progress of analytical performances, newly identified sources, uses and/or routes of exposure, particularly exposed sub-population, toxicological evidence, evolution of regulatory dispositions…). Importantly, the biotransformation products (named as metabolites below) of CECs are included in this definitionand are of particular relevance to biological matrices, including human samples. Despite this, CEC metabolites are typically less known and studied compared to their parent compounds (Alves et al., 2014).

Besides the definition issue, at present CECs are mostly monitored in environmental matrices and compartments (Dulio et al., 2018; Hollender et al., 2017; Veenaas and Haglund, 2017; Cariou et al., 2016; Hilton et al., 2010); especially water (Newton et al., 2018; Gago-Ferrero et al., 2015; Schymanski et al., 2015; Bourgin et al., 2013) for which several structured initiatives, networks and organizations exist (*e.g.* EPA, EAWAG, NORMAN…). Furthermore, consideration of CECs in the chemical food safety area (Pearce et al., 2019; Knolhoff and Croley, 2016; Cotton et al., 2014; Tengstrand et al., 2013) is becoming more prominent. Conversely, CECs remain less investigated, via suspect or non-targeted screening approaches, in the field of human biomonitoring, except for particular applications focussed on specific classes of compound, such as pesticides (Jamin et al., 2014), pharmaceuticals (Jiang et al., 2016), consumer products (Phillips et al., 2018), or environmental organic acids (Wang et al., 2018). This discrepancy can be explained by (i) the lower abundance of biological material available and/or lower chemical concentration levels typically available/ observed in human samples, compared to environmental and food matrices (resulting in lower possible enrichment factors for the expected markers of exposure), (ii) that in many cases the relevant markers of exposure in humans are not the parent CECs but their metabolites, which may not have been identified yet, and (iii) the level of collaboration and networking among laboratories in human biomonitoring has not reached the same maturity as in other fields (*e.g.* water analysis). This observation induced the elaboration of the present manuscript dealing with the concept of screening chemicals of emerging concern in the specific context of human studies.

From a methodological viewpoint, the topic of CECs is linked to new conceptual frameworks and approaches capitalising on the latest generation of high-level instrumentation that enable more rapid and holistic chemical profiling. Such so-called suspect and non-targeted screening (NTS) approaches, typically based on high-resolution mass spectrometry (HRMS), have been utilised for a while in the metabolomics community for detection of endogenous compounds acting as markers of effect and assess potentially exposure health effect (López-López et al., 2018). Notably, the majority of metabolomics studies are not longitudinal, compared to biomonitoring which inherently requires long-term data to interpret and assess impact and changes over time. Furthermore, investigation of population exposure often lacks true control groups, due to the ubiquitous nature of many contaminants. Effects can be present/absent with a degree of severity whereas exposure is, by nature, a gradient that can be more complex to discern. Although similar analytical workflows can be applied in the present exposomics context to assess exposure focused on exogenous chemicals and related markers of exposure, there are key specificities that distinguish these two areas (Fig. S1). (a) The associated contextual scientific information and knowledge differs between metabolomics (physiology and biology) and exposomics (chemistry and risk assessment). (b) The underlying physicochemical processes are different for endogenous markers of effect as for metabolomics (molecular biochemistry) to markers of chemical exposure (modalities of transfer and fate through the environment-food-human continuum), each requiring distinct contextual and scientific knowledge. (c) The breadth of supporting annotation databases, permitting to assign a detected signal to an identified chemical with a given confidence level differ in the two areas: whilst extensive databases focused on endogenous markers are widely used in the metabolomics community (*e.g.* HMDB database (Wishart et al., 2018)), extended and reliable tools to annotate exogenous exposure markers are lacking for exposomics investigations, especially at European level. For instance, there is not US EPA Comptox Dashboard equivalent in Europe to assess the European exposome. (d) Lastly, suspect and non-targeted screening of markers of chemical exposure in human matrices lacks comparable and reproducible methods and procedures, as well as harmonised criteria for documenting method performance and ensure the reliability and comparability of results produced by different laboratories (Rochat, 2017). Though harmonisation proposals have been elaborated in the metabolomics field (Viant et al., 2019; Broadhurst et al., 2018; Dudzik et al., 2018; Rocca-Serra et al., 2016); uptake in the research community remains low (Spicer et al., 2017a) Notably, and of particular relevance to exposomics research, few proposals include risk assessment. Therefore, despite being touted as having potential, the practical application of exposomic screening and NTS in a regulatory and support to policy context needs greater development (ECHA, 2016).

The Human Biomonitoring for Europe initiative (HBM4EU) aims to coordinate and advance human biomonitoring (HBM) in Europe by generating evidence of the actual exposure of citizens to chemicals and possible related health effects, in order to support policy making (Ganzleben et al., 2017). Specific actions carried out in the HBM4EU project are particularly dedicated to the research and elucidation of CECs in various human sample types, inclusive of conventional HBM matrices (urine, blood) and alternative ones (*e.g.* breast milk, placenta, meconium…). One component of HBM4EU aims at the development and implementation of large scale suspect and non-targeted screening methods dedicated to the detection of markers of internal chemical exposure for HBM, environmental health studies and support to risk assessment purposes. In this context, the main aim of the present paper is to provide an overview of the basic principles, promises and challenges of suspect and non-targeted screening approaches specifically applied on human samples. Centred on liquid chromatography (LC) coupled to HRMS-based data acquisition methods considering the current wider implementation of this technique in laboratories, this overview addresses all steps of these new analytical workflows. The main activities conducted in the particular framework of the HBM4EU initiative are described with an emphasis on harmonisation measures.

2. Scene-setting definitions

Depending on the level of pre-existing knowledge associated to the considered markers of exposure, three related methodological approaches can be used to stratify the human chemical exposome, namely (i) targeted methods for known compounds, (ii) suspect screening for known unknowns and (iii) non-targeted screening for unknown

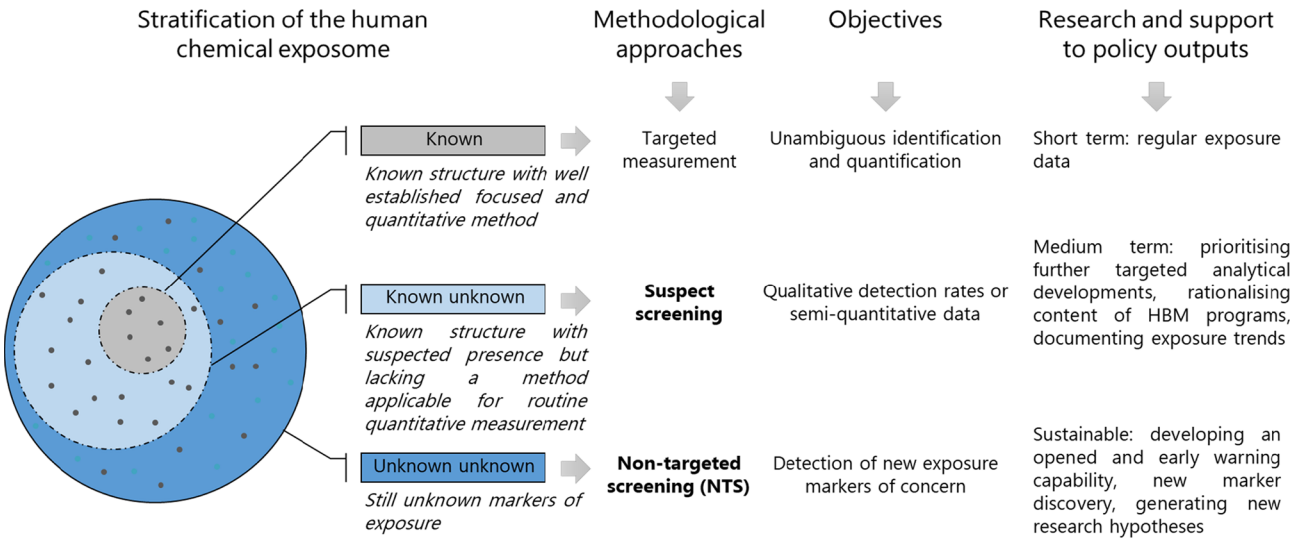


Fig. 1. Conceptual view of the human chemical exposome, related methodological approaches, and associated objectives as considered within the HBM4EU project.

unknowns (Fig. 1). Suspects can be "converted" into targets by collecting comprehensive mass spectrometric reference data that enables unequivocal identification of the suspect compound (usually reliant on the availability of reference standard compounds). The remaining signals in the sample are generally termed "non-targets" or "unknown-unknowns", for which no identity can be readily assigned, requiring further structural elucidation using non-targeted approaches.

2.1. Targeted screening

"Targets" are compounds of known chemical name and structure, for which quantitative targeted methods are available, alongside some exposure and risk assessment data (Smolders et al., 2009). Highly selective sample preparation is typically undertaken in order to isolate these targeted compounds with maximal removal of matrix interferences (Yusa et al., 2012). Detection and quantification is often conducted using low-resolution mass spectrometers (*e.g.* triple quadrupole – QqQ), usually operated in selected reaction monitoring (SRM) acquisition mode, to provide both high specificity and sensitivity. Identification is supported by comparison with reference data acquired from certified standards (chromatographic retention time, MS and MS/MS spectra) used to validate compound identity prior to analysis. Quantification, is preferably performed using the isotopic dilution method, permitting to reach maximal performances with reduced uncertainty. A number of guidelines already exist to harmonise method performances assessment (*e.g.* 2002/657/EU for food). To some extent, targeted screening can be also conducted using high resolution instrumentation (*e.g.* Orbitrap or time of flight – ToF), opening the door to simultaneous targeted analyses with suspect and non-targeted ones (as described below) altough some limitations may be encountered in this case compared to fully designed targeted methods based on tandem mass spectrometry (Cajka and Fiehn, 2016).

2.2. Suspect screening

"Suspects" are known compounds ("known unknowns") in terms of chemical name and structure which are expected ("suspected") to be present in a sample. The typical approach applied in this case is large-scale suspect screening aiming to generate semi-quantitative data and contribute to better prioritisation for further targeted developments (Cortéjade et al., 2016). The same approaches are also helpful to elucidate the composition of complex mixtures by simultaneously generating exposure data for a wide range of markers from each individual sample. In most cases, analytical standards are not readily available and therefore, relevant analytical methods are not validated and compound identities not definitive. To some extent, suspect screening can be considered an extension of multi-class/multi-residue analysis, whereby some markers may be unambiguously identified ans possibly quantified as per a targeted method, while others are mostly qualitatively measured. This qualitative annotation step refers to the assignment of a given compound identity to a signal detected by suspect or non-targeted approaches and relies on the elaboration and implementation of reference libraries to match the generated experimental data with structural descriptors indexed from a list of *a priori* defined chemical compounds (Fig. 2).

2.3. Non-targeted screening

Non-targeted screening aims to detect "unknown unknowns" compounds without any *a priori* criteria (Fig. 2), to identify potential new markers of exposure and toxicological concern (Sobus et al., 2018). Generally, sample preparation and data acquisition are similar for suspect and non-targeted screening whereas data analysis/mining are different. Although highly challenging, this approach represents the most promising strategy to advance our knowledge of the human chemical exposome. In addition, it will enable better anticipation of future health threats and related risk assessment and regulatory dispositions. The development and implementation of NTS requires advanced capabilities and good integration of new front-of-science data management aspects (advanced data acquisition and processing facilities, bioinformatics and modelling tools). A solid basic knowledge of chemistry (MS, NMR, chemical synthesis) and biochemistry is essential to allow the unambiguous structural elucidation and relevant interpretation and contextualisation of compounds besides the revealed signals. NTS is then coming with new paradigm modifying the conventional hypothesis-driven research approach to a data generating hypothesis-driven approach, as a really open way to characterise biological samples.

2.4. Harmonisation

Before describing the different components of suspect and NTS workflows, it is important to note that the coverage of chemical space is directed by the physicochemical properties of the considered exposure markers, their compatibility with the applied analytical procedure and underlying technology. As illustrated in Fig. S2, only chemicals with compatible properties will be detected after the applied sample preparation, chromatographic separation, ionisation and mass spectral detection steps detailed in the following sections. Therefore, global methodological harmonisation in this field would lead to a loss of useful

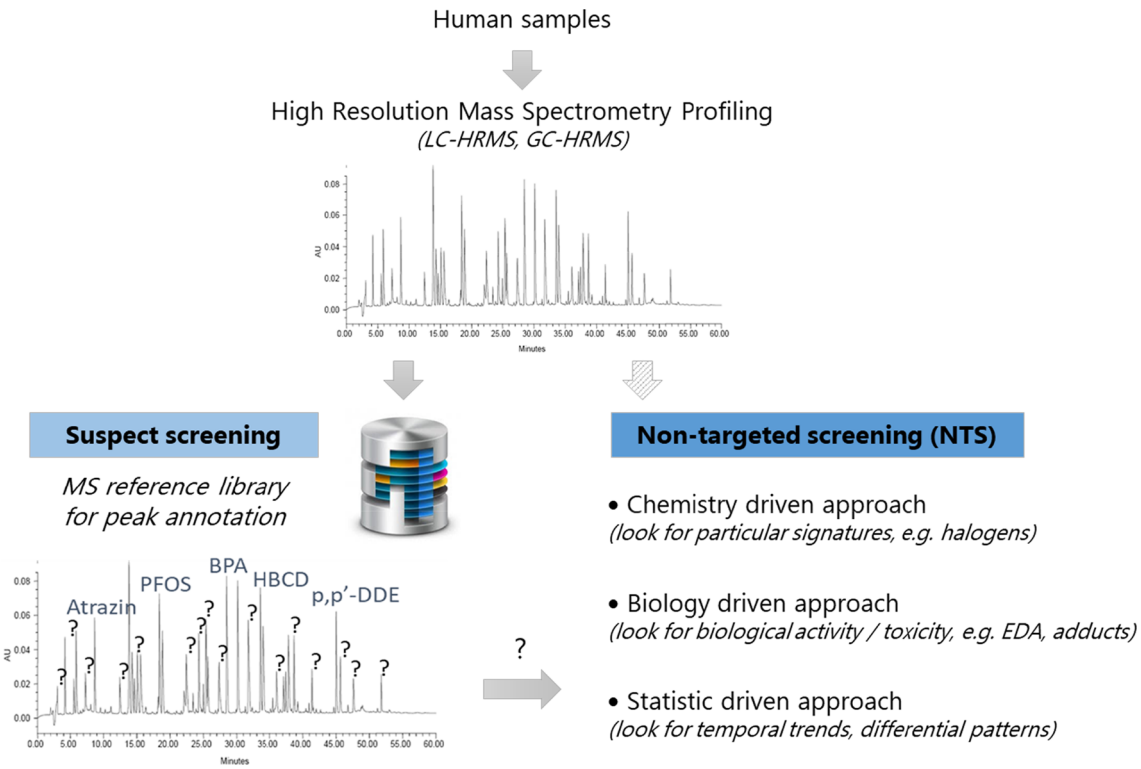


Fig. 2. Global principles of suspect and non-targeted screening approaches applied to characterisation of human internal exposure.

complementarity between methods that can give access to different subsets of chemical markers and would impair the discovery aspect of NTS. Conversely, harmonising quality assurance/quality control (QA/QC) measures and criteria, as well as the result reporting, appears necessary to reinforce the description of method performances (sensitivity, reproducibility, range of accessible markers…) for better comparability.

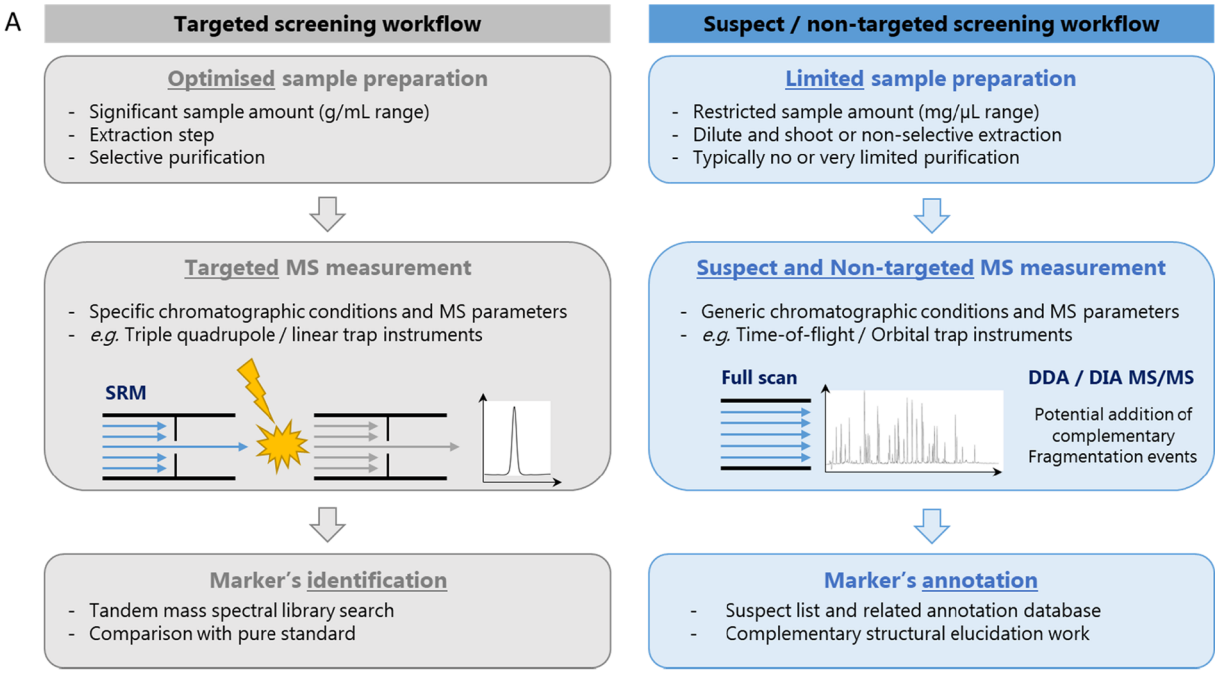
3. Sample preparation

3.1. The selectivity versus sensitivity compromise

Suspect and non-targeted screening analytical workflows involve multiple steps ranging from sample preparation and data acquisition to data mining, expert reviewing and interpretation. Irrespective of the matrix of interest (*i.e.* conventional HBM matrices such as urine or blood, or alternative matrices including breast milk, placenta, meconium or other tissues…), the first sample preparation step is critical and a compromise between selectivity and sensitivity has to be sought. In order to cover a wide range of potential markers of exposure, extraction and purification should be as non-selective as possible. On the other hand, the sensitivity of the method is partly correlated to the concentration of matrix interferences which may impair the detectability and reproducibility of the signals of interest, for instance through ion suppression (Cote et al., 2009; Antignac et al., 2005). This also applies to ultra-HRMS instruments (*e.g.* Fourier transform ion cyclotron resonance devices – FT-ICR) in direct introduction mode where in-source signal disruptions caused by matrix effects cannot be compensated by the high MS resolution of the detection system. Consequently, to achieve a minimal level of compatibility of the prepared sample extracts with the instrumentation used for signal detection generally means extraction step(s) followed by a certain level of purification are desired. One of the main challenges associated with NTS is to achieve a balance regarding purification selectivity to limit matrix interferences, whilst preserving as many compounds of interest as possible with sufficient sensitivity. This new concept of cleaning to remove most abundant interferences is based on similar practical procedures used for targeted method to enrich low concentrated analytes by purification but it is facing new issues, especially when the nature of the markers to be detected are not fully known in advance. In the context of the HBM4EU project, this selectivity issue is suggested to remain a matter of flexibility to be adapted according to each specific application with regards to a particular interest toward certain chemical classes.

3.2. The starting sample volume compromise

The pre-analytical phases consist of sample collection and storage, which may also impact on the results obtained but which are not covered in the present paper focused on the analytical steps. After the pre-analytical phase, a first crucial step that directly impacts the selectivity/sensitivity ratio is the selection of an appropriate starting sample volume for analysis. This volume depends on both the contamination level of the sample, which is partly unknown in NTS, and on the detection capability (sensitivity) of the instrumentation intended to be used. This parameter directly influences the possible enrichment factor of both markers of exposure (both known and unknown) and matrix interferences, as well as the efficiency required for the extraction and purification steps. As a general principle, the higher the sample volume considered for analysis, the more effective the purification step can be to concentrate markers of exposure and negate matrix interferences. This approach is commonly used for targeted analysis; especially for markers present at low concentration levels in complex biological matrices for which relatively high quantity of sample is available (*e.g.* sentinel animals, food, *etc.*). Conversely, human biomonitoring matrices are typically available in lower amounts than environmental or food matrices, limiting possibilities for pre-concentration. Furthermore, the concentrations of environmental pollutants and/or their metabolites are typically orders of magnitude lower than concentrations of endogenous compounds, food constituents and drugs (Rappaport et al., 2014). Accordingly, as a general rule, NTS methods for human matrices should be preferentially based on low sample volumes and limited sample preparation. This paradigm shift from conventional targeted



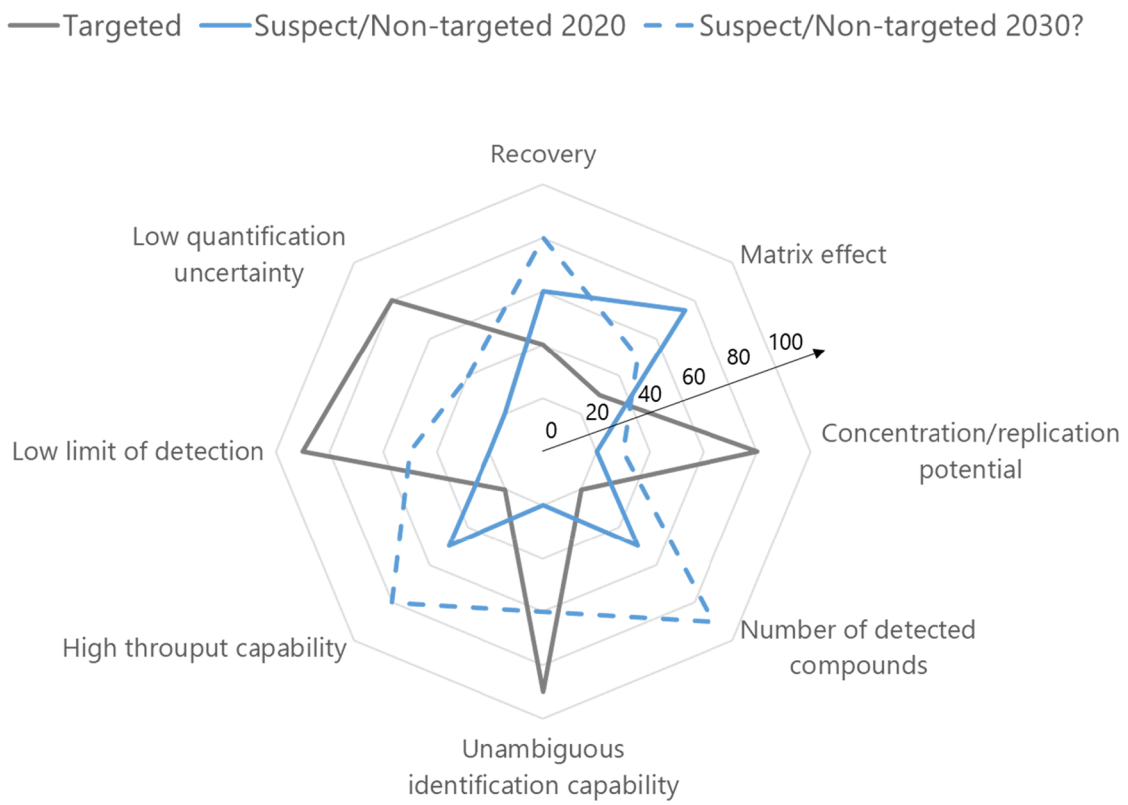


Fig. 3. Summarised conceptual comparison of the analytical workflows typically applied for conventional targeted (left) versus non-targeted (right) methods (A), and of the resulting global performances expected with both approaches (B). Radar chart axis scale is in arbitrary units with 0 correspond to low performance and 100 a high performance.

approaches leads to new analytical challenges, especially with regard to the sensitivity required for the MS detection systems, pushing toward high level HRMS instrumentation for accessibility to lowest concentrated exposure markers (Fig. 3).

3.3. Deconjugation versus no deconjugation

Another important aspect for consideration regarding the sample preparation protocol for suspect and non-targeted screening, especially

when applied to urine, is whether to include a deconjugation step for hydrolysing the phase II metabolites of the considered exposure markers, *i.e.* glucuronide and sulphate conjugates (as mainly concerned species, although not exclusively) (Plassmann et al., 2015). Deconjugation (via enzymatic or chemical hydrolysis) is often favoured for conventional targeted methods, especially with regard to the determination of reference exposure values under regulatory context, since it enables the quantification of the total (free + deconjugated) forms of the considered markers, monitored as a single diagnostic entity. This strategy leads to an aggregated indicator of the global internal dose with optimal sensitivity. However, this analytical step may lead to a significant intra- and inter-laboratory variability in terms of quantitative results, considering the wide range of possible hydrolysis conditions and the difficulty to achieve a reproducible deconjugation rate for the markers anticipated to be measured. This step is also susceptible to introduce an external background that can impact the robustness of results if not appropriately managed.

Conversely, the direct detection of phase II metabolites without deconjugation appears more appropriate in the context of suspect and/or non-targeted screening, considering the high polarity and the usually good signal response observed for conjugated metabolites in LC-HRMS. Additionally, advanced MS acquisition modes, such as neutral loss monitoring and mass defect filtering, allows the semi-selective detection of all glucuronide and sulphate chemical species present in a sample. This can permit the identification of more markers of exposure than the relatively limited targeted (deconjugation) strategy. This option also offers simplification of the sample preparation protocols, which further facilitates harmonisation and limits interlaboratory variability. On the other hand, this non-deconjugation strategy requires the elaboration of an appropriate annotation library, through the experimental determination (*in vitro* assays) or prediction (*in silico* metabolism modelling) of the "MS ready" information required to detect such not yet confirmed metabolites. In other words, this second strategy appears simplified in terms of sample preparation, but more demanding in terms of data processing and structural elucidation.

Finally, the definition and application of a single harmonised technical protocol in the context of suspect and non-targeted screening is probably not realistic, since the inclusion of a deconjugation step may be justified on a case-by-case basis depending on the purpose of each study. Conversely, a more systematic and harmonised reporting regarding deconjugation should be promoted for better documenting the rationale behind the inclusion (or not) of such deconjugation step and for reliable inter-study comparisons.

3.4. Extraction approaches

Minimal sample preparation procedures, such as "dilute and shoot" or ultrafiltration, which are used in the metabolomics field (Khamis et al., 2017; Fernández-Peralbo and Luque de Castro, 2012), may also be applied to suspect and non-targeted screening of conventional HBM biological fluids, such as urine or blood. These quick, simple and non-selective approaches have the advantage of preserving the sample integrity, limiting sample preparation related variability, and facilitating interlaboratory harmonisation. However, they are susceptible to significant matrix effects that can impair both the detectability of low concentrated markers of interest (Lin et al., 2010) and method repeatability. Online treatment is another option for limited sample preparation, for instance through turbulent flow chromatography (Couchman, 2012) or online solid phase extraction (Zhang et al., 2016).

Other human matrices, especially those with high protein and/or lipid content (breast milk, faeces, adipose tissue, placenta, hair, etc.) require a more elaborated treatment prior to analysis. For solid matrices, lyophilisation (freeze-drying) and/or grinding (*e.g.* via tissue lysers, freezer mills) allow sample homogeneity and extraction efficiency. Liquid-liquid and solid-liquid extraction (LLE/SLE) are the most commonly used approaches, where the nature and proportions of the applied solvent mixtures directly determine the range of extracted compounds according to their physicochemical properties. In the context of suspect and non-targeted screening, the selected extraction/partitioning solvents should allow the solubility of a wide range of compounds of differing polarity (Cajka and Fiehn, 2016).

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 partitioning allows collection of two or three complementary fractions from each analysed sample and is an efficient way to both divide (and so dilute) the whole matrix effect and permits characterisation of fractions by complementary technologies, *e.g.* LC-HRMS (predominantly hydrophilic compounds) and gas phase chromatography (GC)-HRMS (more hydrophobic compounds), respectively. The Bligh and Dyer approach originally developed for lipid extraction (Bligh and Dyer, 1959; Ulmer et al., 2018), consisting in applying a ternary solvent system with water, methanol and chloroform, is an example of such partitioning commonly applied and/or adapted for suspect and non-targeted screenings. Other closely related alternatives are the Folch method (Folch et al., 1957) or more recently methanol/methyl *tert*-butyl ether (MTBE) approach (Matyash et al., 2008). As a general rule, such non-selective procedures may be privileged to preserve sample integrity with maximal potential for exposure marker detection without *a priori*. Another approach consists in the Quick, Easy, Cheap, Effective, Rugged, Safe extraction (QuEChERS), which is well established for contaminant multi-residues analysis (Cloutier et al., 2017) and metabolomics (Garwolińska et al., 2019).

Liquid-based extraction may also be accelerated by using high temperature and/or pressure devices such as pressurised liquid extraction (PLE). This approach is adequate for (semi-)solid matrices and has proven its efficiency for targeted measurement of lipophilic contaminants (dioxins, PCBs, BFRs…) from environmental and food matrices (Vazquez-Roig and Picó, 2015). Microwave assisted extraction (MAE) is another alternative compatible with either solid or liquid matrices (Llompart et al., 2019). Introduction of external contamination background not previously visible through the targeted approaches, or the possible degradation of some exposure markers of interest under these extraction conditions, are additional concerns for suspect and non-targeted screening. These techniques may induce some selectivity with regard to certain classes of compounds, but also higher efficiency of the extraction process, which can be relevant in case of more oriented researches or applications. Finally, a rigorous assessment of the applied protocol appears necessary and should be promoted in order to systematically document the application scope of the applied methods, *i.e.* evaluate and communicate on its suitability to detect only a certain range of compounds.

3.5. Additional purification and fractionation

To achieve better sensitivity and detectability of some markers of interest in complex matrices, an additional purification may be required following the extraction step. Introducing selectivity towards particular classes of chemicals can be justified by the own research priorities of the developing laboratories. This may also be a pragmatic choice to maximise the sensitivity compared to generic preparation. However, the introduction of supplementary sample preparation steps may impair the global ambition of a non-selective, large coverage analysis due to the loss of some exposure markers of possible interest. Additional purification strategies may also compromise the fast and high throughput objectives expected for these NTS approaches.

Solid Phase Extraction (SPE) or d-SPE (dispersive-SPE, Bakhytkyzy et al., 2020) is an example of purification techniques widely used for targeted analyses that can be also applied for NTS (Samanipour et al., 2018). The selectivity of the resulting purification may be adapted according to the nature of the stationary and mobile phases, with a very

large number of options today available. This approach is selective of compounds of interest which is not fully compatible with NTS. In this context, purification should be selective of matrix interferences instead of compounds of interest. Historical options are available such as organic solvent-based protein precipitation but also more recent options are developed to selectively remove particular matrix components such as lipids (*e.g.* Captiva ND/EMR SPE) (Zhao et al., 2018). Other approaches used in omics fields, such as chemoselective probes to target chemical groups (*e.g.* halogens) (Mitchell et al., 2014) or deproteination by applying magnetic beads (König et al., 2013) will maybe more developed in the future and could be adapted to NTS. In the same idea of a purification selective of matrix interferences, sample fractionation can also be used for NTS, as a conservative clean-up strategy and/or for the confirmation of chemical structure besides the detected markers. Semi-preparative HPLC or Size Exclusion Chromatography (SEC) may be employed to this end (Saito et al., 2004). Sample fractionation is particularly applied when the chemical NTS is coupled to effect directed analyses (EDA) aiming to characterise a biological activity associated to the corresponding fractions, to facilitate the chemical elucidation of compounds responsible for the biological activity (Simon et al., 2015).

3.6. Extract reconstitution

After extraction, the extract is usually concentrated by solvent evaporation (mostly to dryness, or by introducing a keeper solvent to preserve the loss of some volatile compounds). Subsequently, the last step before injection into the analytical instrument used for detection is reconstitution of the final extract. Technical consideration of reconstitution is not trivial and appropriate solvent(s) selected based upon the capability to re-suspend the extracted compounds and compatibility with the separation and detection system. If the solvent (mixture) used is not appropriate, not all compounds will be dissolved and therefore not detected. Because suspect and non-targeted screening aim to cover compounds with a wide range of physicochemical properties, the use of a mixture of solvents with complementary polarities and solubilisation capabilities is an appropriate strategy to reconstitute the final sample extract. In addition, the reconstitution can be ultrasonically assisted to reduce compound's adsorption on glass vial. The solvent (mixture) also needs to be compatible with the chromatographic system used for MS analysis, as the injection solvent system may greatly influence retention times, as well as peak shapes. If no definitive guideline can be proposed at this stage, the systematic evaluation of the used reconstitution conditions on a set of QA/QC reference compounds covering the range of markers susceptible to be addressed by the method is a good option, as described in Section 6.

4. Instrumental analysis

Other necessary guidelines to improve data comparability are related to the instrumental analysis, where several options exist for chromatography coupled to HRMS. LC-HRMS is remains at the present time the most commonly used technology for suspect and non-targeted screening, either with Orbitrap or Time-of-Flight (TOF) HRMS devices. The present paper focuses on the main LC-HRMS approaches for laboratories interested to start implementing suspect and non-targeted screening analyses. That said, using a combination of LC-HRMS and GC-HRMS to cover a wider range of compounds in terms of molecular size, polarity and volatility (Pico et al. 2020) should be promoted as an integrated and comprehensive workflow (Fig. S3).

4.1. Chromatographic separation

For LC separation, a large diversity of stationary phases, mobile phases and solvent additives are available. Despite this diversity, reversed-phase columns (mainly C_{18}) remain the most commonly used due to their efficiency over a wide hydrophobicity range. Their widespread use also allows methodological comparisons and harmonisation. However, hydrophilic interaction phase (HILIC) and polar embedded reversed-phases are increasingly emerging alternatives for highly hydrophilic compounds, due to their orthogonality to C_{18} phase and their compatibility with common ionisation sources (Jandera and Janás, 2017). Regarding the mobile phase composition, conventional water/methanol or water/acetonitrile binary systems are most commonly used, and both seem suitable (Yusa et al., 2015). A ternary system water/methanol/acetonitrile may also be suggested to take simultaneous benefit of the respective properties of both organic solvents. Because of the typically applied limited sample preparation, the use of a generic elution gradient can be recommended as a general rule to reach a satisfying separation of the analytes and to limit matrix effects. The introduction of a final flush of the column (*e.g.* with acetone/isopropanol) can also be advised to avoid carry-over between injections. Modifiers (acetic acid, formic acid, ammonium acetate, ammonium fluoride, *etc.*) are often added to mobile phase to stabilise the pH, to increase peak shape or to promote ionisation or specific adduct formation (Kruve and Kaupmees, 2017). The nature of the solvent modifier directly impacts the obtained chemical profiles: the distribution of the different ionic species formed for the expected exposure markers (*e.g.* (de)protonated molecules *vs.* formate and ammonium adducts) makes the annotation process of the detected signals more complex and has to be handled by the data processing component of the analytical workflow (see Section 5).

Supercritical fluid chromatography (SFC) also constitutes another interesting tool to separate a broad range of polar molecules ($-2 < \log P > +2$) and may be considered as a "green" analytical technique (Losacco et al., 2019). However, this technique is not yet implemented routinely in laboratories that may limit its current capabilities in term of international harmonisation and primary focus for labs willing to develop these approaches.

Importantly, the observed chromatographic retention time (rT) of the detected MS signals is an important piece of information with regard to the identification of the corresponding markers. Although the marker's identification is primarily based on spectrometric characteristics (exact mass, isotope and fragmentation patterns) as described in Section 4.2, the experimentally observed *vs* reference or predicted/modelled rT is helpful to decrease the number of candidate chemical structures possibly fitting with a given detected accurate mass. With this regard, harmonising the chromatographic systems used in different laboratories does not appear as a realistic, nor relevant approach. Conversely, the introduction of rT index (Celma et al., 2018) or retention projection (Abate-Pella et al., 2015) criteria within the annotation process should be promoted, in a comparable manner to that of GC–MS profiling.

4.2. Ionisation and detection

First of all, electrospray ionisation (ESI) is commonly used as ion source. The present paper will then focus on this technological option, although alternatives such as chemical ionisation and photo-ionisation at atmospheric pressure (APCI/APPI) may have some advantages in term of marker's coverage. Using ESI with both positive and negative ionisation modes would maximise the number of detected markers, either through two separate injections or using a single injection in the polarity switching mode for MS devices with sufficiently high scan rates.

Then, high-resolution mass analyser/detector is required for NTS to reach unique elemental composition in order to facilitate data processing and improve marker's identification. LC-HRMS coupling usually refers either to Orbitrap or to time-of-flight (ToF) HRMS devices. Fourier-transform ion cyclotron resonance (FT-ICR) instruments may be also mentioned to be possibly used, especially with regard to their structural elucidation capabilities based on ultra-high resolution (Kind and Fiehn, 2006). However, FT-ICR may face some limitation with

regard to in-source matrix effects and related signal non reproducibility that impair their application for complex matrices where signals of interest are of very low abundance, as it is the case for HBM. Their elevated cost also limits their large scale implementation, and so this option cannot be really considered in the current state as a priority for laboratories aiming to implement NTS, nor suitable for short term method harmonisation.

In term of mass ranges, priority can be given to the *m/z* 50–1000 range, fitting with the properties of exposure markers typically expected in HBM. However, the *m/z* 1000–2000 range may also be informative and optionally covered to detect additional markers, as well as contribute to confirm marker identity through the detection of supplementary adducts for high molecular weight compounds.

Besides the ionisation polarity and mass range criteria, the full scan mode acquisition represents the starting recommendation for NTS. At this stage, ensuring maximal reliability of the generated data appears crucial especially in terms of resolution and mass accuracy. Appropriate control and adjustment for these two settings are necessary, through appropriate calibration procedures, as well as through the recurrent analysis of appropriate mixtures of reference compounds/ material. For state-of-the-art instrumentation, mass resolution typically exceeds 30,000 and mass accuracy is below 5 ppm. More advanced/latest generation of instrumentation reach rather higher performances and had to be preferred for NTS in order to facilitate data processing.

Data dependent acquisition (DDA) and data independent acquisition (DIA) are more advanced options that have to be considered for generating structural information in the context of NTS (Oberacher and Arnhard, 2015). These acquisition modes require hybrid MS instruments equipped with fragmentation capabilities. Briefly, DDA is more restrictive than DIA, by fragmenting the "n" only ions passing a specific threshold (*e.g.* abundance, neutral loss, *etc.*) of the MS full scan. In contrast, all ions are fragmented in DIA and dedicated data treatment tools are required (*e.g.* SWATH developed by Sciex or HRM by Biognosis (Ludwig et al., 2018)) to properly assign the various fragment ions to their respective precursors. This increases the complexity of deconvolution of DIA and becomes a major limitation in the data processing step. The additional information related to the structure of the compound, in addition to its exact mass (elemental composition, isotopic pattern), is the basis of an increased confidence level for compound identification. Nevertheless, the advanced data acquisition is currently still a matter of research and development, and depends on the considered generation of instrumentation.

Finally, developing methods based on non-selective data acquisition modes is in agreement with the objectives for suspect and non-targeted screening. As for development of the sample preparation method, the analytical approach appears as a paradigm shift with conventional analytical approaches, leading to new challenges (Fig. 3). For suspect and non-targeted screening, no global harmonisation related to the data acquisition can be recommended. Conversely, complementarity and orthogonality of various analytical methods present obvious advantages to identify as many markers as possible. The effort should preferably focus on the development of annotation MS reference libraries with sufficient flexibility to cover the different ionic species potentially expected for the different markers under various analysis conditions.

5. Data processing

5.1. Post-acquisition processing

The post-acquisition data processing step in the context of suspect and non-targeted screening consists of shifting from raw instrumental data to a curated tabulated file (peak list) used for subsequent annotation and statistical analyses. This appears as the main component of non-targeted analyses and represents a substantial effort, since it can be very labour intensive and time-consuming often requiring manual intervention/oversight. This component crucially depends on the availability and performance of bio-informatics tools. A wide range of software is available to perform the extraction of information from the raw data (Stanstrup et al., 2019; Hu et al., 2016). Some of these tools are integrated solutions from MS vendors, *e.g.* Metaboscape from Bruker, Progenesis QI from Waters, Trace finder, Sieve and Compound Discoverer from Thermo, Mass Profiler Professional from Agilent, MetID from Shimadzu, or XCMSplus from Sciex. Other options are open source software (Spicer et al., 2017b), many largely implemented in the metabolomics community, *e.g.* XCMS in the R computational environment/online (Tautenhahn et al., 2012), MZmine 2 (Pluskal et al., 2010), Workflow4Metabolomics (Giacomoni et al., 2014), MS-DIAL (Tsugawa et al., 2015) and MetAlign (Lommen and Kools, 2012). Other in-house developed solutions complete this panel of existing offers, such as HaloSeeker (Leon et al., 2019), an open access tool designed for the specific screening of halogenated markers.

These data processing tools aim to detect any signal present in the generated chemical profiles (peak picking), to align common peaks found in the different samples (peak alignment) 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 and even induce some pitfalls on the generated results. Even after years of use and experience with some of these tools, there are still no consensus guidelines regarding both a preferable selection from this panel or their fine appropriate parameterisation in the context of NTS. As settings also depend on the analytical configuration (LC and MS settings), a harmonised procedure is also hard to implement. This absence of comprehensive and universal data processing solution still appears as a main bottleneck of NTS approaches (Baran, 2017; Tugizimana et al., 2016). For HBM, the limited possibility of sample replication, commonly applied in metabolomics to manage variability, complicates this component of the NTS workflows. Therefore, the establishment of common QA/QC measures to reach a better level of confidence on the produced results and a better comparability between different data processing approaches appears to be necessary (Considine et al., 2018). Defining and reaching correct data processing outputs for a set of QA/QC reference compounds covering the range of markers susceptible to be addressed by the method here appears as a good option, as described in Section 6.

5.2. Compound annotation

Importantly, the confidence level associated to the identification of the detected markers depends on the type and extent of structural information collected and available through the implemented analytical workflow. A harmonisation proposal was elaborated in the water analysis and metabolomics communities to clearly distinguish the levels of confidence, from level 5 where only exact mass is available to describe the considered marker, to level 1 where full mass spectrometric pattern (MS/MS data) are available and successfully compared to a analytical standard (Schymanski et al., 2014). Intermediate confidence levels are reached through the querying of databases where chromatographic and MS descriptors (so-called "MS ready information") are indexed for a list of *a priori* defined chemical compounds (McEachran et al., 2018; Oberacher et al., 2019; Schymanski and Williams, 2017). Spectral properties can be either experimentally determined from analytical standards, or theoretically calculated/modelled through biocomputing tools (*e.g.* MS/MS spectral similarity networks oft termed "molecular networking" or fragment tree correlations, network propagations via *e.g.* substructure/motif searching or *in silico* fragmentation). The same marker' ID confidence scale should be then more largely adopted and harmonised in the exposomic community.

Such databases are well developed in the metabolomics community primarily focussed on endogenous compounds acting as markers of effect (*e.g.* Human Metabolome DataBase (HMDB), and METLIN (Warth et al., 2017)). Although not directly suitable for annotating markers of chemical exposure, these metabolomics-related databases can be useful

in the annotation pipeline to reveal and then discard from further processing, endogenous compounds (metabolites of endogenous substances, markers of effect…). The US-EPA Comptox Dashboard is another existing resource in the field (McEachran et al., 2017) incorporating an environmental compounds component, as well as some particular sub-databases focusing on particular classes of substances, for instance psychoactive substances (Mardal et al., 2019; Lung et al., 2016). Yet, no extended and consolidated MS reference library exists at European scale to annotate markers of chemical exposure (either parent compounds and/or their metabolites) and to accompany the development of the exposomics field. One ambition of the component of the HBM4EU initiative dealing with these new methodological approaches is to build this ambitious and QA/QC consolidated database dedicated to markers of human internal exposure to CECs (Fig. S4). It also aims to develop a data processing methodology to prioritise the way to analyse the generated data (peak-picking, pairing, alignment, background subtraction etc.), in the spirit of NTS based on non *a priori* assumptions.

Finally, the confidence level associated to the markers identified through suspect and non-targeted screening approaches may be highly variable from one study to the other, as well as from one given marker to the other within the same study. Until the availability of more consensual standards, a careful documentation of the real level of identification associated with each reported marker appears mandatory in this emerging field. Harmonised reporting of suspect and non-targeted screenings results also appears as a priority and a way to clarify and makes transparent and comparable, from a given study to another one, this crucial issue of marker's identification, especially in regulatory and support to policy contexts. Development of a common reporting template for European exposure is an on-going activity developed within the HBM4EU initiative to report suspect and non-targeted screening results, as it was already done by EPA with ENTACT initiative (Ulrich et al., 2019).

6. Method performance assessment

The analytical chemistry community was proficient for many years in the assessment of the performance of conventional targeted methods with appropriate QA/QC measures (Fig. 4). One of the main approaches uses one or more reference standard compounds to evaluate (then possibly validate) various analytical criteria including efficiency, selectivity, recovery, accuracy, linearity, limits of detection and quantification, *etc*… Non-targeted method assessment is facing a more complex situation, since some of the signals of interest are still unknown. However, several concepts from target analysis can be transferred to the non-targeted methods. Therefore, non-targeted 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 materials at concentration levels typically observed in humans exposed to those chemicals (Sobus et al., 2019). An appropriate set of such QA/QC samples should include system suitability test samples, fortified and/or naturally contaminated matrix samples, as well as procedural blank samples. This QA/QC aspect is well implemented in the last generation of metabolomics studies (Dudzik et al., 2018), and consequently, it should also be better developed in the exposomics area.

Samples fortified with a set of known substances are useful for various QA/QC purposes. 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 to monitor performances over a batch of samples and for batch-to-batch corrections (*i.e.* stability of retention times, chromatographic performance and peak shapes, mass accuracy and resolution, detection sensitivity, stability of signal intensities). A set of known markers of exposure covering a broad range of physicochemical properties, selected to be representative for the expected diversity of marker compounds can be used as indicators of the method performances at various levels, covering sample preparation (recovery, matrix effect…), data acquisition (chromatographic and mass spectrometric resolution, mass accuracy…) and data processing (peak picking and alignment…) steps. A set of relevant test compounds for QA/QC purposes was elaborated in the HBM4EU project and is presented in Table S1. Global coverage in terms of molecular weight and polarity (illustrated in Fig. S5) shows good representativeness of the list of HBM4EU markers of interest. This harmonised QA/QC compound test set remains a matter of further individual adjustments justified by the focus of each study, but facilitates further methodological comparisons, including the implementation of inter-laboratory studies.

Another QA/QC related issue associated to NTS approaches is the assessment, control, and management of the external contamination encountered in the procedural blank samples. The issue proves to be more problematic for NTS than for conventional targeted approaches because non-selective sample preparation and data acquisition can lead to detection of various compounds originating from sources other than the sample itself (*e.g.* plasticisers, plastic additives, solvent/reagent impurities…). It is important to define which part of the generated information relates to the analysed sample and which to instrument noise or external contamination. In practice, there are a number of difficulties related to the characterisation of the background noise and the ways to manage it through a well-established, reliable, and documented blank subtraction process (Caesar et al., 2018). Several

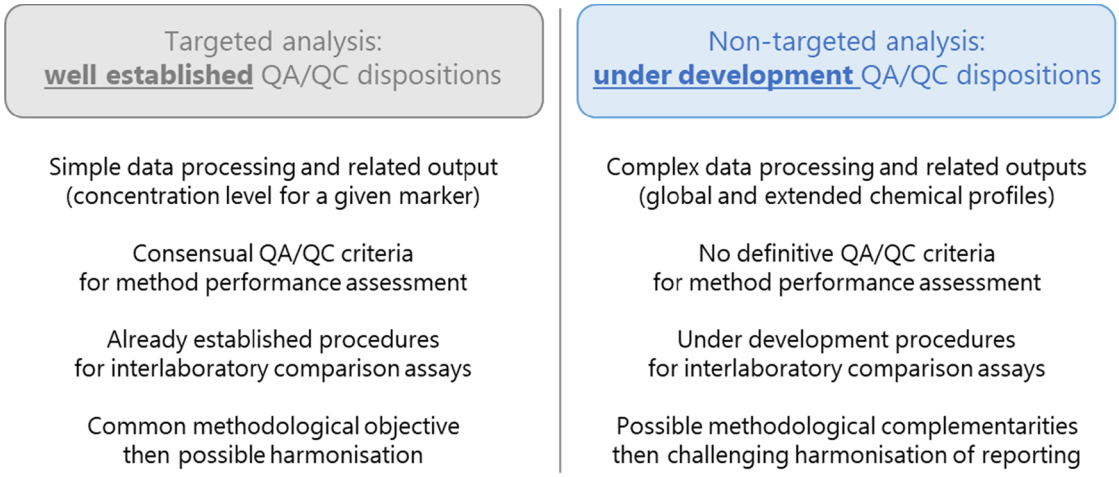


Fig. 4. Summarised conceptual comparison of the QA/QC current state of development typically observed for conventional targeted (left) versus non-targeted (right) methods.

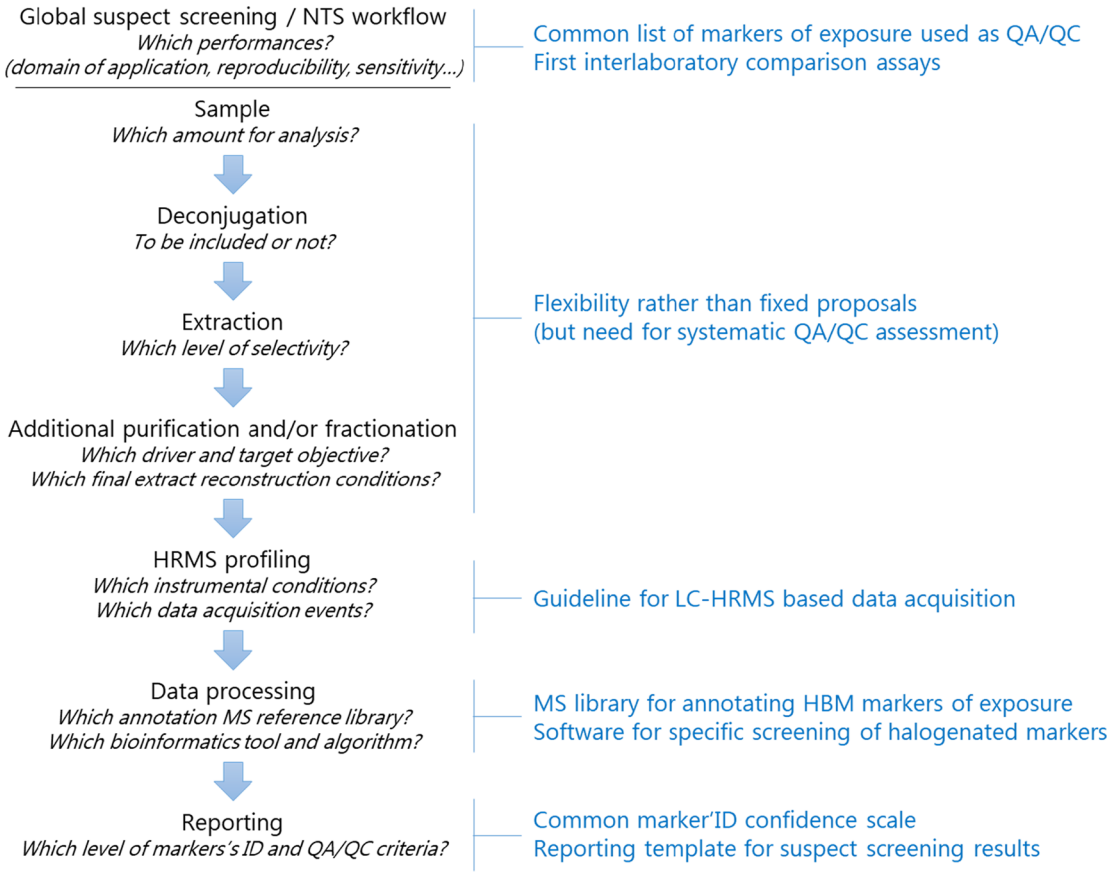


Fig. 5. Main harmonisation outputs developed within the HBM4EU project with regard to suspect and non-targeted analytical workflows applied to human samples.

parameters need to be established, such as the number of procedural blanks introduced in each analytical batch and method to assess 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? All these points require significant efforts in targeted methods and are clearly also relevant for NTS. Even in established workflows, these issues are not always adequately considered (Considine et al., 2018; Dudzik et al., 2018; Boccard et al., 2010). It requires not only strict analytical precautions, but also new conceptual and computational solutions with regard to data handling, normalisation, statistical treatment etc., and will require additional collaborative work in order to achieve better harmonisation. Meanwhile, appropriate documentation of the procedures followed to characterise and manage the external procedural contamination in NTS is needed.

Evaluation of NTS approaches can also be performed through comparison to results obtained by targeted approaches. This is recommended to be implemented during method development and represents a valuable way to consolidate the NTS 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 NTS performances, including importantly a first evaluation of the false negative and false positive rates (McCord and Strynar, 2019; Herrera-Lopez et al., 2014). Coupling of traditional and emerging methodologies will permit an efficient mutual benefit for both biomonitoring and exposomics areas (Dennis et al., 2016).

7. HBM4EU activities and harmonisation

The component of the HBM4EU initiative dealing with suspect and non-targeted screening approaches is paving the way to an integrated and sustainable framework to address the CEC topic at European level, through collaborative and harmonised actions as well as proof-of-concepts with dedicated outputs (Fig. 5 and https://www.hbm4eu.eu). To summarise, technical needs appear in this field for: novel minimal selectivity sample preparations, complementary instrumental platforms, joint libraries, strong data processing capacities, as well as harmonised data management, reporting, and interpretation tools. In particular, harmonising some QA/QC criteria to enable better assessment of method performances (marker's ID related confidence level, sensitivity, reproducibility, range of accessible markers…) and to improve data comparability. Links with already existing initiatives, for instance from the metabolomics or environmental monitoring, have to be used in that respect. However, a global methodological harmonisation in this field is estimated to potentially lead to the loss of useful complementarity between different methods that can give access to different subsets of chemical markers and so to impair the discovery aspect of NTS. A reasonable position should be then to combine (1) the elaboration of technical and analytical guidelines to support less experienced laboratories that may want to start with NTS and (2) a high degree of innovation in more advanced laboratories already involved in NTS for further advancing this field to the benefit of all the regulatory and research community.

8. Conclusions

The emerging NTS area is characterised by both a contextual scientific background of high complexity and an underlying necessary methodological framework of high technicity. There are significant challenges for this field that entail major analytical developments related to each step of the workflow. The analysis of human samples requires specific methodologies and processes compared to other fields of application, such as environment or food. On one hand, rigorous harmonisation measures are required to achieve better consolidation and comparability of data generated from various studies, especially

regarding their further use in a regulatory and support to policy context. On the other hand, this evolving field requires considerable flexibility in order to maintain its capacity in discovery and exploratory research. Considering all challenges described above, we are proposing a specific approach to harmonisation which is based on the following principles:

(a) Besides particular cases, sample preparation should provide minimal selectivity to encompass the desired diversity of exposure markers and an acceptable purification for limiting matrix interferences and their detrimental impact on the overall method performances.
(b) Considering the wide range of exposure markers of interest in a whole NTS context, the combination of several complementary sample preparation methods is beneficial and global harmonisation on that point does not appear a priority.
(c) The implementation of these approaches requires high-level equipment and significant technical expertise, with an optimal combination of both LC-HRMS and GC-HRMS to achieve broad coverage of markers with various physicochemical properties.
(d) Considering the large number of possible technical choices and parameterisation options, no strict guideline with regard to HRMS data acquisition for NTS can be elaborated, yet some harmonisation is desirable and appears to be possible.
(e) Data processing applied to NTS requires advanced computational tools; many still under development, and represents one of the major challenges due to the highly complex data requiring specific expertise to manage data complexity and a critical view on the generated results to ensure consolidated outputs. There is a critical need in the field for multidisciplinary as well as for high level and sustainable competences which are not traditionally present in analytical laboratories.
(f) The development of an extended and qualitatively consolidated MS reference library for annotating markers of exposure is a key strategical element for operational and harmonised implementation of these approaches at the European scale.

CRediT authorship contribution statement

Mariane Pourchet: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Laurent Debrauwer:** Conceptualization, Methodology, Writing - review & editing, Project administration. **Jana Klanova:** Conceptualization, Resources, Writing - review & editing. **Elliott J. Price:** Methodology, Formal analysis, Writing - review & editing. **Adrian Covaci:** Methodology, Resources, Writing - review & editing. **Noelia Caballero-Casero:** Methodology, Formal analysis, Writing - review & editing. **Herbert Oberacher:** Methodology, Formal analysis, Writing - review & editing, Visualization. **Marja Lamoree:** Methodology, Formal analysis, Writing - review & editing. **Annelaure Damont:** Methodology, Formal analysis, Writing - review & editing. **François Fenaille:** Writing - review & editing. **Jelle Vlaanderen:** Writing - review & editing. **Jeroen Meijer:** Methodology, Software, Formal analysis, Data curation. **Martin Krauss:** Methodology, Writing - review & editing, Visualization. **Denis Sarigiannis:** Resources, Writing - review & editing. **Robert Barouki:** Conceptualization, Writing - review & editing, Funding acquisition. **Bruno Le Bizec:** Resources, Writing - review & editing, Funding acquisition. **Jean-Philippe Antignac:** Conceptualization, Methodology, Software, Investigation, Writing - original draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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