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Noelia Caballero-Casero [a, **], Lidia Belova [a], Philippe Vervliet [a], Jean-Philippe Antignac [b], Argelia Castaño [c], Laurent Debrauwer [d, e], Marta Esteban López [c], Carolin Huber [f], Jana Klanova [g], Martin Krauss [f], Arjen Lommen [h], Hans G.J. Mol [h], Herbert Oberacher [i], Olga Pardo [j], Elliott J. Price [g, k], Vera Reinstadler [i], Chiara Maria Vitale [g], Alexander L.N. van Nuijs [a, 1], Adrian Covaci [a, *, 1]

[a] Toxicological Center, University of Antwerp, Universiteitsplein 1, 2610, Wilrijk, Belgium
[b] Oniris, INRAE, LABERCA, Nantes, France
[c] National Centre for Environmental Health, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain
[d] TOXALIM (Research Centre in Food Toxicology), Toulouse University, INRAE UMR 1331, ENVT, INP-Purpan, Paul Sabatier University, 31027, Toulouse, France
[e] Metatoul-AXIOM Platform, National Infrastructure for Metabolomics and Fluxomics: MetaboHUB, Toxalim, INRAE, F-31027, Toulouse, France
[f] UFZ, Helmholtz Centre for Environmental Research, Permosers Straßsse 15, 04318, Leipzig, Germany
[g] RECETOX Centre, Masaryk University, Brno, Czech Republic
[h] Wageningen Food Safety Research, Part of Wageningen University & Research, Wageningen, the Netherlands
[i] Institute of Legal Medicine and Core Facility Metabolomics, Medical University of Innsbruck, Muellerstrasse 44, 6020, Innsbruck, Austria
[j] Foundation for the Promotion of Health and Biomedical Research of the Valencia Region, FISABIO-Public Health, Av. Catalunya, 21, 46020, Valencia, Spain
[k] Faculty of Sports Studies, Masaryk University, Brno, Czech Republic

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ABSTRACT

Although the exposure assessment of chemicals of emerging concern (CECs) has taken a decisive step forward through advances in (bio)informatics, statistics, and the development of highly sophisticated analytical instruments, the lack of standardisation and harmonisation of analytical workflows and method performance assessment for suspect and non-target screening hampers the interpretation of results, their comparability and thus, its transmission to policymakers. To date, unlike in other research fields such as forensics or food analysis, there is a lack of guidelines for non-target analysis in human risk assessment and quality assurance and quality control (QA/QC) protocols. Moreover, the majority of efforts have been focused on the development and implementation of QA/QC actions for data acquisition, data analysis and mining, largely neglecting the sample preparation necessary for determination of CECs by suspect and non-target screening methods.

In this article, we propose a set of QA/QC measures that covers sampling, sample preparation and data acquisition, as an aspect of work conducted within the European Biomonitoring for Europe initiative (HBM4EU). These measures include the use of standardised terminology and the implementation of dedicated QA/QC actions in each stage of the analytical process. Moreover, a framework for the analytical performance assessment has been developed for the first time for the identification of CECs in human samples by suspect and non-target approaches. Adoption of the actions proposed here for the identification of CECs in human matrices can significantly improve the comparability of reported results and contribute to the (challenging) Exposome research field.

* Corresponding author.
** Corresponding author.
E-mail addresses: Noelia.CaballeroCasero@uantwerpen.be (N. Caballero-Casero), Adrian.Covaci@uantwerpen.be (A. Covaci).
[1] Shared last authors.

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1. Introduction

During 2018, more than 300 million tonnes of chemicals were consumed in the 28 member states of the European Union (EU), from which 200 million tonnes are known to be hazardous to health [1]. This extensive use of chemicals results in increased exposure of the European population to potentially harmful chemicals, which have been linked with, among other, cancer, reproductive problems, and adverse effects on the endocrine system [2]. In 2012, the World Health Organization (WHO) estimated 1.4 million annual deaths in Europe linked to environmental pollution [3]. In this context, the EU has adopted appropriate legislative measures as the European Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) [4], the Stockholm convention [5], the Rotterdam Convention on the Prior Informed Consent Procedure for Certain Hazardous Chemicals and Pesticides in International Trade [6] and the Minamata Convention [7] which are a significant step forward in the protection of human health and the environment to commercialised chemicals. However, the current European legislation does not cover all the chemicals neither their possible generation of transformation products in the environment and their release from multiple sources.

The *Exposome* concept introduced by C.P. Wild in 2005 describes the combination of all life-course chemical exposures and body responses [8,9]. As the exposome is a matter of high inter- and intra-individual variability [10], developing reliable measurement tools to map the full spectrum of human exposures is challenging. The recent advances in statistics, bioinformatics and analytics can face the new challenges arising within the Exposome research field. With regard to the chemical composition of the exposome, the use of latest generation high-resolution mass spectrometry (HRMS) techniques [11], which have outstanding properties, such as high mass accuracy, mass-resolving power > 30,000 (R, defined at full width at half maximum, FWHM), high scan speed and broad dynamic range [12], represents the main advance to address this challenge. Coupled to a separation technique, such as liquid and/or gas chromatography (LC and GC), HRMS has been successfully applied for the detection, annotation and identification of new chemicals in different fields, including forensic toxicology [13,14], water quality monitoring [15], food safety [16], environmental toxicology [17] and human exposure to environmental contaminants [18]. However, the study of the Exposome requires the development and establishment of a comprehensive list of chemicals or biomarkers to be investigated in population studies and human biomonitoring campaigns. It is assumed that a large number of chemicals present in the environment remain unknown to the scientific community, or the information related to their identity and physicochemical properties is limited [19]. These chemicals of emerging concern (CECs) are suspected to exhibit adverse health effects in humans [20].

Although the analytical workflow for non-target methods depends on several factors, such as the aim of the study, groups of CECs to be investigated or type of matrix, there is a general scheme to perform sample analysis and identification of compounds. Analytical steps, including sample preparation, separation and detection of CECs should strike a balance between reducing the potential matrix effects and extracting analytes with a wide range of chemical properties. Sample preparation and data acquisition applied in non-target methodologies are basically independent of the subsequent data analysis approaches. However, once the samples have been analysed and based on the available information about the groups of CECs included in the research hypothesis, these data mining approaches can be divided into *suspect screening* for *known-unknowns* and *non-target screening* for *unknown-unknowns* [21].

The suspect screening approach aims to identify *known-unknowns ("suspects") which are compounds expected to be* present in the sample. For these compounds available information, usually only chemical name and formula, is limited and no analytical standards are accessible. In most cases, observed features are compared against lists of potential candidate compounds (only name and formula) or libraries (MS or MS/MS spectra) with the help of data processing software. The aim of non-target screening is the holistic and all-encompassing coverage of chemical space through structural elucidation of compounds without any prior information (*unknown-unknowns)* [22]. This process should be supported by the analyst's strong knowledge of chemistry and by using advanced data acquisition and processing approaches, together with bioinformatics and modelling tools [21–23].

The development of analytical approaches for non-target methods to reflect the global risk of simultaneous exposure to the wide variety of chemicals present in the environment is challenging, in particular for the identification of the diverse groups of CECs in human matrices (e.g. urine, blood, serum, placenta and adipose tissue). The major analytical challenges for human exposure assessment include: i) expected concentrations of CECs and their biotransformation products are low (below ng/mL level) and might vary by orders of magnitude between compounds groups and samples; ii) CECs typically present themselves at lower concentration levels than endogenous compounds CECs that might interfere and suppress detection; iii) high intra and inter-individual variabilities exist, i.e. sample composition affected by the sampling time and sampled individual conditions; vi) spectral databases are incomplete, culminating with a lack of MS/MS spectra and scarce coverage of metabolites to facilitate structural identification [24]; and v) establishment of a cause − effect relationship between chemical exposure and health effects is difficult [10,25,26]. As such, there is an urgent need to develop new wide scope analytical approaches able to detect, annotate and identify the diverse groups of CECs currently present in humans and to provide accurate and reproducible results, for further adequate exposure assessment.

The lack of standardisation for non-target approaches, in terms of analytical methods, method performance assessment, and result reporting, is a sensitive topic within the scientific community [27–29]. Two fields in which progress has been made are metabolomics [30,31] and the identification of CECs in environmental samples [32]. As such, the elaboration of akin harmonisation guidelines is also needed for the identification of CECs in human matrices [18,33]. For this reason, several worldwide initiatives have been launched to address this harmonisation issue of the identification of CECs in human matrices by non-target approaches.

The United States Environmental Protection Agency (USEPA) has led a collaborative trial on non-target analysis (ENTACT) [34] that involved international both private and public laboratories and was supported by EPA's ToxCast [35] and ExpoCast programs [36]. The results of the trial, which was based on the analysis of mixtures of ToxCast chemicals, pointed out the lack of accuracy, precision, and reproducibility of the reported identified compounds across analytical platforms and laboratories [37]. In a European context, the Network of Reference Laboratories for Monitoring and Biomonitoring of Emerging Pollutants (NORMAN) aims to promote communication and information exchange on CECs between research teams from different countries through the validation and harmonisation of non-target methods and data-analysis tools [38,39]. The network has organised several collaborative trials to harmonise CECs detection in environmental and biological samples [32,40–42].

The Human Biomonitoring for Europe initiative (HBM4EU), started in 2017, aims at the coordination, advancement and harmonisation of EU human biomonitoring programs to provide a realistic assessment of the current exposure of European citizens to chemicals and the resulting risks for human health [43]. Significant advances have been achieved in the HBM4EU project with regards to the harmonisation of analytical workflows for CECs in human matrices by non-target approaches [21] and acquisition of MS/MS spectral libraries [22].

To ensure harmonisation of non-target results for reliable identification of CECs in human samples, a set of QA/QC measures covering entire analytical workflows is needed (sample collection, sample preparation, acquisition and data processing) [33,44]. However, to date, sample preparation has been neglected, despite it is key stage to achieve real harmonisation of non-target methods for CECs in human matrices.

Within the HBM4EU project, the main principles and challenges of the harmonisation in non-target approaches specifically applied to CEC detection in human matrices have been thoroughly discussed [21]. Besides, QA/QC measures have been developed for spectral library compilation using liquid chromatography coupled to HRMS [22]. This paper aims to propose a set of QA/QC measures for the harmonisation of non-target methodologies for the detection of CECs, for the metrological traceability of results [45–47]; and to complement the previous work presented by the HBM4EU project (Fig. 1). This paper presents a set of QA/QC actions for covering the analytical workflow stages of sample collection (pre-analytical actions), sample preparation and (partially) acquisition method, as well as a framework for establishing an analytical performance of the method that allows results comparison between laboratories in the same research field.

2. Survey of QA/QC implementation status for non-target approaches

The development of a specific set of QA/QC measures starts with identifying both the current situation in the identification of CECs in biological matrices and the limitations of the main common analytical workflows. For this purpose, a dedicated questionnaire on QA/QC measures was circulated between five HBM4EU partner laboratories selected due to their expertise and involvement in non-target screening in the exposome research field. Respondents detailed the QA/QC actions that they usually apply for the analytical stages other than the data analysis. The major findings drawn from the questionnaires pointing to a current lack of QA/QC measures and of harmonisation were largely in agreement with the information reported in other research fields, such as metabolomics [48,49] (Table 1):

i) Lack of consistency in the used analytical terminology, unlike target approaches where well-established analytical terms are used [50,51], which impedes the comparability and interpretation of results. Therefore, technical terms and concepts are defined in this paper for more unified understanding of non-target methods. All definitions are drawn from European legislation [51], international organisations, such as UNODC and IUPAC [50,52–55]; scientific networks, like NORMAN, Eurachem and CITAC [56,57], and specialised literature [22,58–62]. To facilitate the reading of this manuscript, the complete glossary is presented in the Supplementary Information (SI-1).
ii) Lack of QA/QC actions covering the whole analytical workflow and harmonised method performance assessment.
iii) Undefined criteria for the selection of type (isotopic labelled or native compound or both) and number of internal standards (ISs), even though their use is common.

3. Quality assurance & quality control measurements

Although great progress has been made on the implementation of QA/QC actions for analytical workflows in metabolomics and environmental fields, there are substantial differences in the non-target analysis of CECs in human matrices that introduce new challenges. Thus, an appropriate set of QA/QC measures based on metrological traceability of results is crucial to understand the causes of undesirable variations and to be able to reduce them to the minimum. For appropriate use of the set of QA/QC actions, the

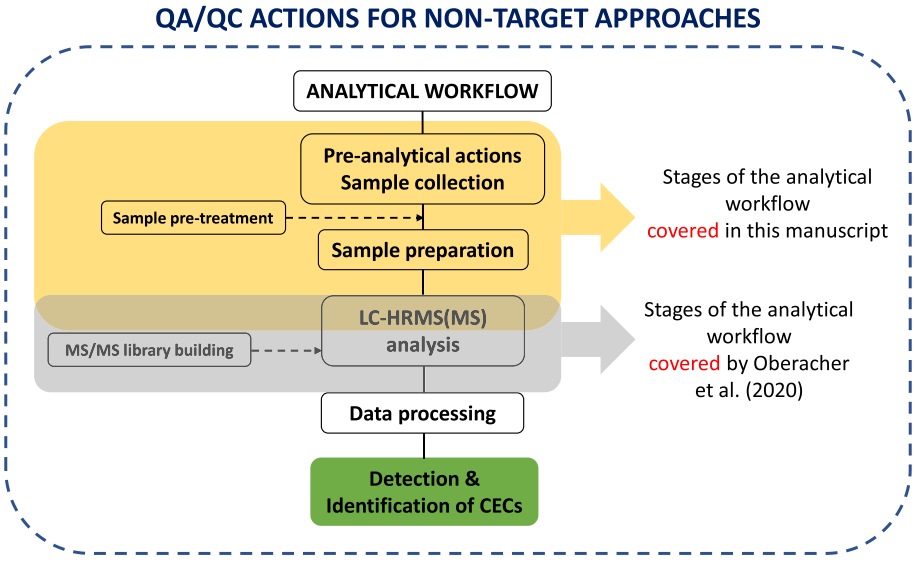

Fig. 1. Analytical stages addressed for the proposal of dedicated QA/QC actions in the context of suspect and non-target screening workflows developed for the detection of CECs in human studies. The dashed arrows show those stages or actions from the analytical workflow, which may be optional.

Table 1
Overview of the QA/QC actions and analytical performance conducted in serum and urine analysis by non-target approaches based on the questionnaire filled by the five partner laboratories.

Analytical stage	QA/QC action	Laboratory				
		1	2	3	4	5
Sample clean-up and extraction	ISs (Total number)	✓ (40)	-	✓ (>40)	✓ (2)	✓ (>10)
	ISs before extraction (name given)	✓ (pre-extraction standard)	-	✓	✓	✓ (pre-extraction standard)
	ISs after extraction (name used by the laboratory)	✓ (post-extraction standard)	-	-	-	✓ (post-extraction standard)
	ISs fortified samples before extraction (%)	100	-	100	100	100
	ISs fortified samples after extraction (%)	100	-	-	-	100
	Procedural blanks (%) (name used by the laboratory)	20 (solvent blank)	-	2 (preparation blanks)	5	10
	Analysis of sample by duplicate	✓	-	✓	-	✓
	Pooled QCs samples (name used by the laboratory)	✓	✓	✓	✓ (sample blank)	✓
	CRM QCs samples	✓	-	-	-	-
LC-HRMS(MS) analysis	Solvent blanks	-	✓	✓	✓	✓
	ISs solution (RT shift & mass accuracy)	✓	-	-	✓	✓
	Signal normalisation (ISs)	✓	-	✓	-	✓
	Samples reinjection	✓	✓	✓	✓	✓
	Randomised batch run	-	✓	✓	-	✓
	Daily system check calibration	✓	✓	✓	✓	✓
Analytical performance	Calculation of recovery (ISs)	✓	-	✓	✓	✓
	Calculation of selectivity (ISs)	-	-	✓[a]	-	✓
	Calculation of reproducibility (ISs)	✓	✓[a]	✓	-	✓
	Calculation of repeatability (ISs)	-	✓[a]	✓[a]	-	-
	Calculation of stability (ISs)	-	-	-	-	-

[a] Calculation based on the signal of non-isotopically labelled compounds. ISs: internal standards; QCs: quality controls; CRM: certified reference material; RT: retention time.

Table 2
Summary of the proposed QA/QC measures for each analytical stage of suspect and non-target methodologies used for the CECs analysis in human studies covered in this article.

	QA/QC measures	Optimisation	Analytical parameter evaluated
Sample collection	Selection of the sampling material Define a minimum set of data Field blanks: assessment of external and pre-analytical contamination QC-pooled sample production Fortified samples with ISs: assessment of CECs stability/degradation Fortified samples with parent/metabolite compounds: assessment of post-sampling (bio) transformation of CECs	Testing the sampling material Use SOPs and training	Stability of the analyte Semi-quantification of ISs-parent and ISs-metabolite
Sample pre-treatment	Procedural blank: assessment of external procedural contamination	Fortified pooled sample with ISs Fortified synthetic matrix samples with ISs (optional)	Subsample representativeness Detection frequency of ISs Total number of detected features Semi-quantification of ISs-parent and ISs-metabolite
Sample clean-up & extraction	Procedural blank: assessment of external procedural contamination QC samples (CRMs, pooled samples)	Fortified pooled sample with ISs before clean-up and extraction Fortified pooled sample with ISs before instrumental analysis QC samples (CRMs, laboratory reference samples) Fortified pooled sample or synthetic matrix with ISs	Extraction efficiency SSE Extraction efficiency and variability Extraction efficiency, reproducibility and SSE(%) between batches
LC-HRMS method	Periodical system calibration Solvent blank: assessment of procedural contamination and carry-over Sample reinjection: signal measurement variability System suitability Randomised batch run	ISs solution: mobile phase composition, elution program, analytical column, number and speed of scans and dynamic range Fortified pooled sample with ISs: operational concentration range of the method Sample reinjection	Retention time shift Chromatographic resolution and peak shape Total number of detected features Mass accuracy, mass resolution and signal to noise ratio
Other (common) measures	System suitability Control chart: faster control over the process Use of high purity chemicals Exhaustive cleaning of labware Written SOPs and training		

ISs: internal standards; QCs: quality controls; SSE: signal suppression or enhancement; CRM: certified reference material; SOPs: standard operational protocols.

analytical laboratory should define specific criteria for the implementation and assessment of each individual QA/QC action, describing the parameter under evaluation, as well as defining how these parameters can be influenced. The parameter evaluated for each QA/QC action and its optimal and decision value should be previously established by the analyst taking into account the specific requirement of the study (i.e. goals, number of samples, matrix and expected compounds) [63]. While the implementation of all QA/QC actions proposed is not a requirement and depends on the project's goals, the adoption of dedicated actions for each analytical stage is necessary to ensure reliable results. Table 2 summarises the proposed measures.

3.1. Internal standards

Either analytical standards of native compounds not expected to be present in the matrix or isotopically labelled compounds can be used as internal standards (ISs). Due to the difficulty of finding compounds that are always absent in human matrices, the use of isotopically labelled ISs for assessing method capability is more advantageous. Since they normally do not occur in nature, they can be distinguished from the matrix components. For more effective utilisation of ISs as part of the QA/QC strategy, it is crucial to select the appropriate compounds according to both the research goals and data processing strategy. As a general rule, and where possible, the selection of compounds as ISs should be governed by the following criteria:

i) *Chemical structure similarity.* Ideally, ISs are structurally identical to the compounds to be determined, in order to ensure the same analytical behaviour and response. When suspect screening is performed, the ISs should be representative of the compounds classes of CECs expected (i.e. screened) in the samples. For non-target screening analysis, the selection of ISs presents an even greater challenge. Due to the intrinsic lack of chemical information of non-target methodologies, this is difficult or even impossible to achieve. Thus, the purpose leading the ISs selection should be to strike a balance between available chemical information, compound representativeness, matrix components information and project's aims. A smart strategy is selecting a set of ISs based on the data processing approach (e.g. chemicals with halogens, hydrophobicity, bioavailability) and the aims of the project (e.g. pharmaceuticals, legal pesticides).
ii) *Chemical properties.* The selected ISs should cover a wide range of physicochemical properties, such as the octanol/water partition coefficient (logKow), molecular weight and acid dissociation constant (pKa). In addition, compounds whose hydrolysis rate constants are well-known or have been empirically calculated should be included as well, in order to be able to assess potential degradation processes.
iii) *Type of species.* CECs and/or their transformation products, as well as typical human metabolite-types (e.g. glucuronidated-type for urine matrix) should be represented in the ISs selection.
iv) *Commercial availability.* Because of requirements of project aims (e.g. occupational exposure studies), the use of specific compound as ISs might be preferred. However, since most of the CECs and/or their metabolites are unknown compounds, it can be difficult or even impossible to obtain analytical standards, in particular isotopic labelled ones, from commercial suppliers. Custom synthesis might be an option, but other factors, such as cost, time, and number of needed ISs can make it unfeasible.

The number of used ISs depends on the aims and individual attributes of each study. A too small number of ISs does not justify their usefulness, while a large number of ISs may hamper the CECs detection and the evaluation of both analytical method and decision-making process (i.e. acceptable/non-acceptable analytical performance results). A smart strategy is having at least one ISs per expected compound group (i.e. food additives, pharmaceuticals, personal care products/cosmetics, plasticisers, pesticides, etc.), including ISs structurally characteristic (e.g. halogen atoms, aromatic groups), and ensuring that their respective elution times span the whole chromatogram or retention time window of interest.

3.2. Sample collection

The implementation of a QA/QC set for sampling may be complicated, because many matrices are collected by healthcare professionals (e.g. blood, plasma, serum, adipose tissue and placenta). The potential systematic error can be reduced by developing detailed standard operational protocols (SOPs) for sampling and storage, and training anyone who performs the sampling. Depending on the type of human matrix, the sample collection varies from single-step to multiple-stages. Subsequently, a risk exists that some of the identified and reported chemicals in samples actually originate from the sample collection procedure (background contamination). Furthermore, the ratio between the parent compounds and their metabolites may change over time because the sample stability could become compromised. Thus, the following dedicated QA/QC actions for sampling are proposed:

i) *Sample information.* Criteria for acceptance/rejection of samples when samples arrive at the lab should be defined. Only samples with guaranteed traceability of the whole sampling process and storage may be included in the considered studies. When samples from biobanks are analysed the use of project-dedicated QA/QC measures at this stage is unfeasible, at least information about the performed sampling process and storage conditions is required (i.e. when and where samples were collected, how and by whom that was carried out). In case of new samplings, all samples should be accompanied by a sampling questionnaire gathering information about the sample, such as the date of sampling, type (e.g. spot urine sample, first-morning urine, 24 h urine), volume, additives, and any details that could influence the results of the analysis.
ii) *Field blanks* should be used to enable the identification then further exclusion of background chemicals coming from the sampling material and handling process. Background contamination originates from both leaching from materials and preservative addition. Since the leaching of chemicals depends on the time-of-contact and storage conditions, the use of field blanks from the beginning of the sampling campaign is crucial and they must be submitted to equal processing as samples. A conservative number of field blanks is about 10% of the total sample number. To obtain field blanks, collection devices containing a synthetic matrix (e.g. simulant solution of saliva or urine [64,65]) or solvent are prepared together with the samples and submitted to the same procedure (e.g. tubes for collection of blood contain chemicals for allowing or blocking coagulation process). Furthermore, field blanks must be stored under the same conditions as the samples. Since the physicochemical properties of the matrix may affect both the leaching and contamination processes, the use of synthetic matrix rather than solvents is preferable.

iii) *Fortify samples with ISs.* The main advantage of fortifying samples with ISs during their collection is to detect potential losses or degradation issues. However, since human matrices are frequently collected by healthcare professionals, the implementation of fortified samples at this stage might be unfeasible. In this case, the samples could be fortified immediately after sampling, as soon as it can be performed. In this way, the analyte stability is still (partially) evaluable. Ideally, all samples should be fortified with ISs, especially those with well-known hydrolysis rate constants, but the high costs for wide-population studies make this difficult in practice. Therefore, the number of fortified samples can be adjusted based on the goals and cost or even the preparation. The preparation of a pooled sample made up of an aliquot from all the samples (QC-pooled sample) at this stage is strongly advisable, which can be also fortified with ISs. In any case, fortified samples must be representative of the whole batch of samples. When the individual fortified samples approach is performed, one third of the samples is a conservative number, but the minimum number of fortified samples required to obtained representative data about all the samples can be calculated by applying the following equation (Eq. (1)) adapted from the proposed one by Daniel [66].

$$Number\ of\ samples = \frac{2x\left(Z_{(1-\alpha/2)} + Z_{\beta}\right)^2 xSD^2}{\Delta^2} \quad (1)$$

where Δ is the margin of error (%), α the significance level, β is the probability of detecting a significant result, SD is the maximum standard deviation allowed; and *Z-score* is the number of standard deviations a given proportion is away from the mean. For the Normal distribution, Z-values are 1.96 ($\alpha_{0.05}$) and 1.28 ($\beta = 90\%$). The maximum values of Δ and SD, and the minimum value of β are previously defined by the analysts based on their experience, the project aims and the matrix-type.

iv) *Fortify synthetic matrix with analytical standards of parent compounds and metabolites (QC samples).* Since the obtaining of both blank samples (that means samples without any of the compound of interest (CECs)) and enough volume/amount may be unfeasible, the use of a synthetic matrix is advisable. The synthetic matrix can be either commercially available (e.g. horse serum) or *in*-house prepared (e.g. a salt mixture solution to simulate human saliva [65]). Collection conditions, such as type or material of devices, storage conditions, and freeze/thaw cycles can modify the parent/metabolite ratio originally present in the samples. This effect has been widely studied for plasma and serum in the metabolomics field [67], and is strongly dependent on the chemical properties of the matrix (pH, salts, etc.). This action can be combined with the use of field blanks. Albeit the number of fortified synthetic matrix QC samples depends on the intrinsic characteristic of the study, for keeping the consistency with the use of field blanks, a minimum of 10% of the total sample number is advisable.

3.3. Sample preparation

3.3.1. Sample pre-treatment

A pre-treatment stage, such as homogenisation and deconjugation, is frequently performed for the analysis of human samples. Sample pre-treatment may be crucial and strongly affects the outcomes of exposome studies. Thus, in addition to the use of procedural blanks, the pre-treatment process should be optimised, and other QA/QC actions should be implemented. Since human samples are usually limited in volume, a pooled sample prepared from volunteers or preferably a QC-pooled sample is advisable for optimisation purposes.

Solid matrices, like adipose tissue, meconium, and placenta are submitted to particle size reduction and/or homogenisation [18]. Furthermore, in many studies, only a sample portion is analysed, so subsample representativeness should be studied. The whole matrix should be ideally fortified with ISs before the homogenisation and partitioning process. After the analysis of the subsamples, the detection frequency of the ISs and the total number of mass features can be used for assessing (optimisation) the process and the study of representativeness along sample batches.

The need for a deconjugation step in urine is a controversial topic in the human biomonitoring field of CECs. The advantages and disadvantages of performing deconjugation in non-target approaches during CECs determination have been discussed by Pourchet et al., 2020 [21] and it is not the subject of this paper. However, when deconjugation takes place, the efficiency and suitability of the process for a specific metabolite-type can be studied. Thus, pooled or synthetic matrix samples are fortified with ISs of conjugated metabolites and submitted to the deconjugation process. Both the deconjugation and biotransformation process can be evaluated based on the detection frequency of the ISs-conjugated/ISs-free, a semi-quantitative approach and the total number of mass features (to assess the potential contamination background of this stage). Whether the enzymatic deconjugation mode is selected, in addition to the reaction conditions, the type and the number of enzyme units per mass unit and the lot number should be reported to facilitate batch comparison.

As for the rest of the analytical stages covered in this manuscript, the decision-making is based on the criteria previously established and described by the laboratory in charge of the analyses. So, when the pre-treatment step under optimisation or the batch sample does not accomplish with optimal or decision values, it is considered as failed and the analysis should be performed again.

3.3.2. Clean-up and extraction

Human tissues and biofluids are complex matrices owing to their composition rich in proteins, lipids and/or salts. An ideal sample preparation suitable for the CECs detection should be able to extract a wide range of chemicals and to eliminate potential matrix effects. Yet in practice, the balance between extraction and purification is complicated and presents numerous challenges: i) the wide (unknown) diversity of chemical properties of CECs may lead to losses of interesting compounds by inappropriate sample preparation; ii) CECs are present at different concentration levels in the same sample leading to difficulties to determine them in a single analysis; iii) some endogenous compounds are typically present in higher concentrations than CECs and may cause matrix effects; and iv) some endogenous compounds, such as lipids and proteins may interfere with the CECs detection in both LC and MS [21,68–72].

Although relatively simple and consensual sample preparation has been proposed in other fields such as metabolomics mainly focused on endogenous markers of effect, the determination of exogenous markers of chemical exposure or their biotransformation products (metabolites), typically at trace concentration level, usually requires more complex methods such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE), as well as a thorough sample preparation optimisation. The evaluation of extraction efficiency and matrix effects are two key factors in this

development. For this purpose, the following QA/QC actions should be implemented:

i) *Sample clean-up method* optimisation. Endogenous molecules, such as proteins and lipids, should be removed to overcome their negative effects on the CECs determination. The use of organic solvents and acids for protein precipitation is frequently used for bioanalysis. Chemical filtration (e.g. Captiva® or Isolute®-PDL cartridges) can be used to specifically eliminate lipids. Clean-up stages should be optimised by using fortified samples with ISs to ensure the non-exclusion of potential CECs or their chemical stability. For this purpose, pooled samples are advisable, but naturally contaminated samples should also be used preferably when possible.

ii) *Extraction method optimisation based on extraction efficiency and reproducibility.* Synthetic matrix samples, pooled samples, naturally contaminate samples, or even procedural blanks fortified with ISs can be used for method development reducing the inter-variability factor. Based on the project goals, the optimisation can be performed using a semi-quantitative or qualitative approach.

iii) *Extraction method optimisation based on matrix effects.* The signal suppression or enhancement (SSE) caused by matrix components can be evaluated by comparing the instrumental response obtained for the ISs solution and the same amount of ISs spiked in a sample after extraction and prior to LC-HRMS analysis. Equation (2) [73] calculates the percentage of matrix effects:

$$SSE(\%) = \frac{Signal\ of\ IS\ in\ spiked\ sample after\ extraction}{Signal\ of\ ISs\ in\ standard\ solution} x100 \qquad (2)$$

A calculated SSE value above 100% represents an enhancement of the ISs signals, while values below 100% indicate suppression of the ISs signals. For the identification of CECs, signal suppression effects could have direct consequences precluding their detection. Signal enhancement is also an undesirable effect, since it makes semi-quantitative analysis through normalisation of signals difficult to perform.

iv) *Procedural blanks.* During sample preparation, many compounds may be unintentionally introduced into the sample extract from solvents, labware and/or consumables. Any instrumental signal in the procedural blanks can be identified as a contaminant and then submitted to evaluation of removal from data processing.

v) *QC samples.* Certified reference materials (CRMs) allow quality assessment across different laboratories. Certified reference materials of CECs in human matrices are not available. However, laboratory reference samples, which are another type of QC, can be prepared by fortifying pooled samples with ISs [74,75]. Fortified synthetic matrix samples and samples previously analysed in other batches may be also used as QC samples. QC samples should be included in each sample batch to assess method suitability. After batch analysis, extraction efficiency, reproducibility and SSE (%) are calculated for QC samples. If the calculated values for QC samples fail to comply with the previously defined acceptance criteria (i.e. the proposed guideline in this article or the defined and stablished by the laboratory performing the analysis), the sample batch needs to be re-submitted to the analytical process.

3.4. LC-HRMS analysis

The identification of CECs in human matrices usually requires the use of high-resolution tandem mass analysers that provide both full-scan MS and MS/MS [21,22], such as quadrupole-quadrupole time of flight (QqTOF), ion trap-Orbitrap (LTQ-Orbitrap) and Q-Exactive Orbitrap (QqOrbitrap). These detectors, combined with LC, mainly use electrospray ionisation (ESI) owing to its high versatility, albeit atmospheric pressure chemical ionisation (APCI) and matrix-assisted laser desorption ionisation (MALDI) have also been applied [18]. Both LC and MS methods should be optimised to make a maximal number of potential CECs accessible.

i) *LC method optimisation.* The development and optimisation of chromatographic conditions has to consider several factors. Solvents, pH and buffer concentration may directly affect elution reproducibility and resolving power (e.g. tailing peaks). The formation of different adducts in the ionisation process (e.g. typically ammonium, sodium or potassium adducts in the positive mode) depends strongly on the mobile phase modifiers selected and may have a significant impact on the CECs identification capability. On the other hand, the stationary phase, length, internal diameter, and particle size of the analytical column determine the efficiency and selectivity of the separation. For human matrices (e.g. urine), the presence of metabolites, which are usually more polar, can be more important than of parent compounds. So, other stationary phases than reversed-phase, such as modified reverse-phase or HILIC may be considered. An appropriate IS mixture should be used for testing (system suitability) and optimising of LC conditions, including the elution program and injection volume, which should be kept as general as possible. Once the LC method has been optimised, the total ion chromatogram (TIC) of a fortified sample should be visually inspected to check the lack of compound signals at the solvent front or the end of the chromatogram, as well as to assess potential retention time shifts between injections.

ii) *MS(/MS) method optimisation.* An appropriate ISs solution should be used for HRMS method development based on the optimisation of mass accuracy, mass resolution, and signal to noise ratio. For this purpose, parameters such as the number and speed of scans (MS and MS/MS level) and dynamic range have to be optimised. On the other hand, after method optimisation, pooled samples fortified with ISs should be used to assess the operational concentration range of the method, from the estimated detection limit to the maximum signal to noise ratio without detector saturation.

A dedicated set of QA/QC is proposed for LC-HRMS acquisition methods to ensure system suitability, as is the case in other stages of the analytical procedure (Fig. 1).

i) *System calibration.* Periodical tune and calibration of the HRMS instrument should be performed before sample batch analysis or at least, calibration should be daily checked [22].

ii) *Background contamination.* System components and mobile phase solvents can introduce potential contaminants that belong to chemical groups included in the CECs category. Thus, it is crucial to detect and minimize them as much as possible by running solvent blanks within each sample batch. To set a specific number of required blank solvents is complex, because factors such as length of the batch (in terms of numbers of samples but also time duration) or mobile phase composition can have a major influence. As a general rule, the optimal number of solvent blanks is which gives a significant statistical difference on the total of

detected features lower than 20% (or the pre-established in-house threshold based on the project's aims) [76]. In the same way, a cleaning procedure should be developed and applied before and after performing the analyses to avoid any cross-contamination.

iii) *Carry-over.* This well-known and undesirable effect should be investigated and eliminated during acquisition method development [22,77]. It has been proven that hydro-organic mobile phase composition is more effective for carry-over reduction that pure organic compositions [78]. However, the complexity of human matrices and highly variable chemical concentrations require the inclusion of solvent blanks after the analysis of samples in each sample batch for routine system monitoring of carry-over [48]. To set the number of solvents blank within a sample batch, similar criteria as for background contamination assessment can be followed [76]. On the other hand, the autosampler may be a source of carry-over in LC-MS, especially in bioanalysis. Thus, the use of a full loop injection instead of a partial loop might be of help for the total removal of this undesired effect, especially in the analysis of complex samples or when a high concentration of compounds is expected [79]. Besides, the use of cleaning samples (i.e. water:isopropanol mixture) along the sample batch reduces or even eliminates the adverse effects of carry-over [77,78].

iv) *Signal variability and retention time shift assessment.* Variations in signal intensity and retention time shifts are usual phenomena, especially in large-scale experiments caused for example by the precipitation of matrix components in the ion source or the column. Sample reinjection allows assessing the instrumental signal variability and retention time shift within a sample batch. This instrumental evaluation should be performed both in the absence of matrix (ISs solution) and in the presence of the studied matrix (QCs samples). When sample reinjection is not feasible (i.e. low sample volume, low analytes stability), these phenomena can be evaluated through the monitoring of the ISs and well-known endogenous compounds signal and retention time in the samples analysed along the batch. Although this strategy is suitable for the evaluation of these phenomena, it can be misleading because more factors are affecting it (e.g. extraction efficiency, ISs stability).

v) *The order solvent blanks injection within a sample batch.* Even in a randomised batch, the order of solvent blanks should be decided by the analyst based on the research aims, complexity of matrix, and batch length. As a general recommendation, solvent blanks (for background contamination and carry over assessment) are analysed at the beginning and end of the batch, and before and after QC samples.

3.5. Other QA measures

In addition to the above mentioned set of QA/QC measures for the identification of CECs in human samples, other good practices can be implemented:

i) *System suitability.* Regular (preventive) maintenance actions prevent measurement errors and major instrumental issues [22]. This general provision indeed appears of even high importance in the context of sample preparation of low selectivity typically applied for suspect and non-target screening methods for which the medium-term stability of the measurement system may be impaired by a higher dirtiness of the injected extracts. The daily analysis of the ISs solution is advisable for checking the system suitability.

ii) Control chart. The use of a graph is an easy way to control process stability. The lower and upper limit is set for each variable, so any measure outside from these limits is considered an outlier and requires an intervention or action. It is commonly used for QC samples, but it can be useful for LC-HRMS-MS system suitability as well (tune, calibration, and pressure profiles) [77].

iii) Use of high purity chemicals. The use of chemicals with high levels of purity (>95%) and LC-MS grade solvents reduces potential contamination. As well as, the material-made up labware should be carefully selected in order to avoid potential contamination (e.g. polysiloxane, silicone, etc.).

iv) Exhaustive cleaning of labware. All the material and labware used for sample preparation and standards solutions preparation should be submitted to a strict protocol of cleaning for removal potential background contamination.

v) Standard operational protocol and personnel training. The development of in-house SOPs significantly reduces bias errors due to slight variations at multiple steps and handling. The SOP document should provide a complete description of the QA/QC actions, as well as, the criteria for both the QA/QC actions and the analytical workflow assessment. Moreover, a record of lot numbers for the chemicals used with each SOP facilitates the traceability of the analyses. On the other hand, training of the staff involved in any of the analytical stage (if

Table 3
Proposed minimum criteria of the performance characteristics for suspect and non-target screening of CECs in human biological matrices.

Performance characteristics	Parameter	CECs (expected) mass fraction (ng mL^{-1} or ng g^{-1})				
		<1	1–10	10–100	100–1000	>1000
Sensitivity	Limit of identification (ng mL^{-1} or ng g^{-1})	As low as possible	≤1	≤5	≤ one order of magnitude lower	≤ one order of magnitude lower
Selectivity	Matrix effect (SSE %)	70–150	60–150	50–150	50–150	40–150
Accuracy	Trueness/Recovery (%)	≥60	≥40	≥30	≥30	≥20
	Reproducibility (RSD %)	As low as possible	≤40	≤30	≤15	≤15
	Repeatability (RSD %)	As low as possible	≤30	≤25	≤20	<20
Stability	RSD %	≤40	≤20	≤15	≤10	≤10
Representativeness	RSD %	As low as possible	≤40	≤30	≤15	≤15

SSE: signal suppression or enhancement; RSD: relative standard deviation.

needed), as well as the potential weakness detected during the training, should be described in detail in the SOP as well. Once the SOPs are developed, in order to ensure their correct interpretation and application, personnel should be trained by an experienced member of the laboratory.

4. Framework for analytical performance reporting

According to the strict IUPAC definition [54,55], the concept of validation does not apply to non-target approaches, for which compounds are not preselected or even totally unknown. Therefore, the different sources of error affecting the analytical performance cannot be individually evaluated for them. Nevertheless, as it has been shown by Simonet [63] the most relevant quality control actions in quantitative analysis are applicable to non-target methodologies with the appropriate modifications and adaptations. On these premises, we propose a framework for establishing and document the analytical method performance. This is a tool for harmonisation of results based on a wider validation concept to prove that a method complies with previously established criteria.

The development of this framework, as well as the suggested limits for each analytical parameter, are based on the existing validation guidelines for quantitative, qualitative and metabolomics analytical methods [31,38,48,50,51,53–55,60,63,80]. The combined use of ISs and the evaluation of the method potential for CECs identification with quantitative markers allow the comparison of results from different non-target methods, as well as increase the level of confidence for the reported results. While it is true that analytical performance is estimated only for a group of representative compounds (ISs), it may explain why some expected compounds in a matrix cannot be detected. Since IS compounds have been selected according to the intrinsic characteristic of each project (see Section 3.1.), when samples do not accomplish with minimum performance characteristics, method optimisation should be revised and samples re-analysed. The acceptable number of samples that failed with the criteria should be established and reported by the analyst on each project. Table 3 shows a proposed minimum criteria of the performance characteristics based on ISs response, according to the expected CECs concentrations present in the sample. Assessing and reporting the application range of a given method proposed for suspect/non-target screening therefore appears as an important element of comparability.

4.1. Sensitivity. Limit of detection/limit of identification

The CECs can be present at different concentrations depending on the extent of the exposure and the studied matrix. It is therefore crucial to define the detection ability of the proposed method. For this purpose, decreasing ISs concentrations in both solution and fortified pooled QC samples are analysed. The initial concentration for the ISs depends on the CECs expected concentration in the samples (e.g. therapeutic range) either the aims of the research. Lower amounts of ISs should be analysed until the peak area of at least one of the IS reaches a signal to noise ratio of 10. The signal to noise ratio is a key factor for the identification of compounds during data processing where signal to noise thresholds can be set as filter. Thus, the method detection ability must be sufficient to later allow the identification of CECs. It is desirable that the ISs limits of detection are as low as possible, but in practice the ISs limits of detection only need to be lower than CECs expected concentration (limits of identification), in order to enhance the detection and potential CECs identification (Table 3).

4.2. Selectivity

Despite being one of the most important analytical parameters, selectivity is not commonly evaluated in non-target approaches [81]. Due to the complexity of human matrices, both matrix effects and interferences are common undesirable issues in bioanalysis that affect the selectivity [54]. It is not possible to evaluate the specificity for all compounds due to practical limitations, but the method selectivity for the ISs can be assessed. According to the selectivity definition, it can be calculated through two different approaches, matrix effects and interference effects.

The measurement of the matrix effect for ISs through the calculation of SSE (%) (see Section “3.3.2 Sample clean-up and extraction”) gives an estimate of the influence that the matrix components may have on the CECs present in the sample, under the analysis conditions. To overcome this negative effect on the CECs identification, the lowest acceptable SSE (%) value for the ISs at representative concentration is 40% (Table 3). Although this value is lower than the accepted range for target bioanalytical methods, it may still be considered a restricting limit for a non-quantitative method. This value has been proposed as a result of considering the combined effect of (potentially) low recoveries (even so low as 20%) and measurement variability, that could negatively affect the identification procedure. While an upper limit does not seem necessary for identification purposes, it is important to consider that the signal enhancement may hamper any semi-quantitation based on signal normalisation. Therefore, a general upper limit of 150% is proposed, which is based on the combination of measurement variability (up to 30–40%), and the recovery error allowed for target methods in bioanalysis (+10–20%) [50,51]. The matrix effect evaluation for all samples is strongly advisable, but for harmonisation purposes, reporting matrix effects for QC samples is adequate, too.

The evaluation of potential interferences in the samples can be accomplished by analysing blank samples or samples fortified with ISs [80]. Once the samples have been analysed, data processing is performed and checked for false and true positive identifications, respectively. The use of fortified samples is preferable over blanks because it gives a more realistic approximation of the existence of interference [80]. Since an interference can be systematically present in the matrix or be specimen-dependent, it is advisable to evaluate this effect for all analysed samples. However, in large batches, it can be unfeasible for practical reasons. In such a case, the analysis of fortified QC pooled samples is a smart strategy. Once the samples have been analysed, data processing is performed and the number of true and false positive identifications is calculated for the spiked compounds. Ideally, no false positive identifications occur and all ISs are truly identified. However, a small margin of error can be tolerable, albeit the acceptance criteria of true and false positive ratio should be pre-established by the laboratory in charge of the analysis and subsequently reported with the results.

4.3. Accuracy

As previously defined (see “SI-1-Harmonised definitions1”), accuracy may be described by both trueness (recovery when a suitable certified reference material is not available) and precision. To evaluate the extraction efficiency of the method (recovery), at least six pooled QC samples fortified with ISs at a relevant concentration according to the expected CECs concentration range should be analysed within the same batch. Since recovery and precision can strongly affect the CECs identification, both parameters should be evaluated as a whole. Acceptable lower limits for recovery according to the concentration range are given in Table 3. Although an extraction efficiency of 20% is commonly accepted in

non-target approaches a minimum recovery of 30% is strongly advisable and in any case, it must be higher to precision values.

Precision can be evaluated in terms of reproducibility (intra-day precision) and repeatability (inter-day precision) and is usually expressed as the relative standard deviation (RSD) or the coefficient of variance (CV). In non-target methods, the linearity between the analyte area and concentration is often not addressed. This may introduce an error in the precision evaluation. Thus, the substitution of SD of the ISs area by median absolute deviation (MAD) is strongly advisable [48]. The reproducibility can be addressed by analysing at least six QC samples spiked with ISs at a representative concentration within the same batch. The QC sample must be simultaneously treated with the samples and under the same conditions by the same operator. To evaluate the repeatability, the procedure must be repeated on three different days. Both reproducibility and repeatability are calculated according to the following equation (Eq. (3)) [48].

$$RSD(\%) = \frac{1.4826\, xMAD}{median_{IS\ area}} x100 \quad (3)$$

where 1.4826 is the scaling factor described by Hoaglin et al. [82]. The RSD tolerance range under reproducibility and repeatability varies with the concentration, as shown in Table 3.

4.4. Stability

Due to the general chromatographic conditions, non-target acquisition methods often tend to have a long run time. Furthermore, many QA/QC samples (i.e. ISs solution, solvent blanks, reinjected samples, QC samples, etc.) are included in the batch, which increases the analysis time. Furthermore, the batch should be analysed twice, for positive and negative polarity, thus the batches run often entails several days, depending on its length. Therefore, the stability of the sample extracts in the autosampler should be ensured, at least until the end of the batch analyses. For this purpose, both the ISs solution and a QC pooled sample fortified with ISs should be reinjected by duplicate at least three times along the same batch of samples (at the start, middle and end of the batch).

Stability can be evaluated through two different approaches: i) qualitative, by comparing the entire number of detected features in three replicates; and ii) semi-quantitative by calculating RSD for ISs areas according to Eq (3). Since the number of detected features is affected by the variability of the instrumental measurement, this factor needs to be considered during the comparison. The calculated RSD of ISs for stability should always be lower than the reported reproducibility for the same batch, or method repeatability when it was not possible for the same ISs, and in agreement with the criteria presented in Table 3.

4.5. Representativeness

To evaluate the representativeness of the subsample size used in the analysis of a solid matrix, the mean RSD obtained for the measurement of the ISs area in the QC samples individually fortified (at least 6 samples) is compared against the mean RSD obtained from the measurement of QC subsamples taken from a larger pooled QC sample fortified at the same concentration. The calculated RSD (Eq. (3)) has to agree with the reproducibility of the method (Table 3). When the characteristics of the matrix (i.e. nails or meconium) hinder its homogenisation, the sample representativeness can be addressed through the comparison of the variances by applying the Fisher test. Though it is stricter than the RSD method, the Fisher test gives specific information about the significance of the association between both sets of subsamples [83].

5. Interlaboratory comparability investigation

Up to now, there are no standardised analytical methods for CECs determination and identification in human matrices. Due to the importance of accuracy and comparability of the reported results, especially in large-scale projects where samples are analysed by different laboratories, an interlaboratory comparability investigation (ICI) is strongly advisable. Although there are several guidelines on the ICI criteria (i.e. ISO-17043-2010 [84], EUROCHEM-2012 [85]), these are dedicated to quantitative analytical methods (target approach). For non-target methodologies (qualitative) different criteria and good practices covering several aspects of the methods by organising interlaboratory trials have been proposed [28,34,37,63]. When the comparability of different analytical workflows and subsequently produced data appears as a major objective, then this interlaboratory process has to be promoted. Defining a common set of QA/QC reference compounds and assessing the individual method's capacity to effectively detect and identify those QA/QC markers at a given concentration level can be proposed as a general procedure.

6. Conclusions

A set of QA/QC measures for sample collection (pre-analytical actions), sample preparation and the acquisition method (partially) has been proposed. These actions can be applied entirely or partially. However, all analytical stages should be covered to ensure the required quality of the results for Exposome research. A detailed SOP of the analytical method, including all QA/QC measures and the evaluation criteria, the metadata (i.e. materials and reagents, list of ISs, a description of what is to be measured, how this is to be carried out, etc.), together with the obtained results should be reported, since all these parameters can significantly affect the CECs determination, identification and the results' comparison.

Also, for the first time a framework for the assessment of analytical method performance in the identification of CECs in human samples has been developed and presented for non-target approaches. This framework is an objective and reliable tool for method harmonisation towards the comparison of results obtained by different laboratories. Whilst more effort from the scientific community is needed to reach the same level of standardisation for the determination of CECs by non-target methods as in target bioanalysis, the current set of QA/QC measures in the frame of the HBM4EU initiative is a valuable starting point for dealing with this challenging topic.

The Exposome research field is very promising, but challenging. The amount and variety of chemicals in the environment to which humans are exposed, is experiencing continuous growth. Thus, Exposome needs analytical strategies to obtain a holistic view of external (non-genetic) factors affecting human health, through high quality and comparable results. The development of dedicated QA/QC actions for the determination of CECs in human matrices contributes greatly to the achievement of these objectives. From an analytical point of view, some challenges need to be addressed, such as increasing the capability for exogenous chemicals screening in humans, which also requires the achievement of the same harmonisation degree in non-target methodologies as for target analyses. Consequently, future efforts must be directed to ensure the quality of the results and protection against false negative/positive compounds detection. Thus, deeper research in terms of QA/QC and harmonisation through the organization of collaborative trials at the European and/or international level may allow the establishment of stricter and precise QA/QC actions and specific minimum criteria of the performance characteristics for suspect and non-target screening of CECs in human biological matrices. Moreover, development necessity of complementary QA/QC guidelines that fully cover the acquisition method, data-processing and results' reporting has to be more specifically

addressed in connexion with similar initiatives in other fields such as environment or food safety. Networks including experts in theory, medicine and toxicology must be established in order to elucidate CECs biological consequences on humans.

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 data

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