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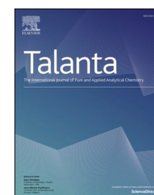

Evaluating human serum for trace levels of lipophilic organic chemicals: A novel GC-(Q)Orbitrap methodology

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ABSTRACT

The exposome encompasses all environmental exposures across the lifespan, with chemical pollutants representing a major component. A robust sample preparation and multiresidue analytical method based on GC-(Q) Orbitrap technology was developed and validated for comprehensive characterization of the human chemical exposome in serum. The method demonstrated high accuracy and satisfactory performance at validation levels of 2 and 10 ng mL^{-1}, enabling the simultaneous quantification and confirmation of 198 GC-amenable compounds from diverse physicochemical properties (e.g., OPEs, PCBs, PAHs, and alkyl-PAHs). The validated methodology was applied to 24 serum samples from pregnant women participating in the INSULIN cohort (Hospital Joan XXIII, Tarragona, Spain), detecting ten chemicals (three PAHs, six alkyl-PAHs, and one synthetic antioxidant) at concentrations between 1 and 4 ng mL^{-1}. Furthermore, nontarget screening tentatively identified 64 additional compounds from various chemical families, highlighting the potential of GC-(Q)Orbitrap for broad-spectrum non-target analysis and holistic exposome characterization. This approach overcomes LC-based analytical limitations by enabling detection of low-to-medium polarity and semi-volatile compounds, demonstrating its value for expanding chemical coverage in human biomonitoring. Overall, this validated methodology provides a powerful tool for exposome-wide association studies (ExWAS) and public health assessments by enabling more comprehensive evaluation of human chemical exposures.

1. Introduction

The exposome encompasses all environmental exposures that humans encounter from birth onward, including the internal exposome (e.g., biological processes, inflammation, gut microflora), the general external exposome (e.g., psychosocial factors, climate, social networks) and the specific external exposome (e.g., chemical pollution, infectious agents, lifestyle factors) [1]. Among these, chemical pollution is a critical component, as increasing evidence links even low-level exposure to both persistent and non-persistent chemicals with adverse health effects outcomes in the general population [2,3].

In recent years, the investigation of the environmental factors affecting human health has gained significant attention, driven by advances in analytical instrumentation and research frameworks [4]. Consequently, numerous studies have underscored the need to develop wide-scope screening workflows based on high-resolution mass spectrometry (HRMS) [5–7] to expand the understanding of the chemical exposome, including its composition and potential environmental and health impacts [5,8–11].

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Abbreviations:

DI-SPME Direct immersion solid phase microextraction
EDC Endocrine disrupting chemicals
ExWAS Exposome-wide association studies
GC Gas Chromatography
HMW-PAHs High molecular weight polycyclic aromatic hydrocarbon
HPLC High performance liquid chromatography
HRF High Resolution Factor
HRMS High-resolution mass spectrometry
ILIS Isotopically labelled internal standards
LC Liquid Chromatography
LCL: Lowest calibration level
LLE Liquid-liquid extraction
LMW-PAHs Low molecular weight polycyclic aromatic hydrocarbon
LOD Limit of detection
LOQ Limit of quantification
MS Mass spectrometry
OPEs Organophosphate esters
PAHs Polycyclic aromatic hydrocarbon
PB Procedural blanks
PBDEs Polybrominated diphenyl ethers
PCBs Polychlorinated biphenyls
PCPs Personal care products
PSA Primary secondary amine;
Q Quadrupole
QC Quality control
QTOF Quadrupole Time-of-Flight
QuEChERS Quick, Easy, Cheap, Effective, Rugged and Safe
RSD Relative standard deviation
RI Retention index
Rt Retention time;
SI Search Index
SPE Solid phase extraction
TCA Trichloroacetic acid
UV Ultraviolet;
ΔRI Deviation of the retention index

Comprehensive characterization of the chemical exposome requires unbiased, non-selective sample preparation methods to minimize information loss. However, biological matrices, such as human biofluids, are inherently complex, containing a wide diversity of compounds (e.g., metabolites, proteins and lipids) spanning broad concentration ranges and physicochemical properties [12]. Depending on the intended degree of purification for serum and plasma, previous studies have employed various extraction methods, including liquid-liquid extraction (LLE) [9,10] and solid-phase extraction (SPE) [11].

Despite these advancements, accurately capturing the full exposure history remains challenging due to the chemical exposome's substantial variability and dynamic nature. While substantial efforts have focused on the polar and semipolar fractions using liquid chromatography-HRMS (LC-HRMS), this approach cannot encompass the entire exposome because volatile and nonpolar chemicals fall outside its analytical window. To address this gap, gas chromatography coupled with mass spectrometry (GC-MS) remains the technique of choice for the analysis of small volatile organic molecules in biological and environmental samples. Target analytes include organochlorine pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), brominated flame retardants (PBDEs), and perfluoroalkylated substances, among others [13–15].

This approach is particularly relevant considering that air pollution ranks among the leading causes of death globally, and the complexity of airborne chemical mixtures is steadily increasing [16,17]. Nonetheless, the volatile and nonpolar fraction of the chemical exposome in humans remains understudied, with limited research on compounds such as alkyl-PAHs despite their relevance in petrochemical exposure contexts. This highlights an urgent need for robust methodologies capable of capturing these understudied contaminants [6,18–20].

The integration of both hybrid quadrupole time-of-flight (QTOF) and Orbitrap analyzers in GC-HRMS has demonstrated significant potential across diverse scientific and analytical domains, enabling comprehensive analysis of environmental and biological samples [21–23]. While atmospheric pressure chemical ionization (APCI) sources combined with GC-QTOF instruments have introduced novel applications by exploiting abundant molecular or protonated ions [22,24], electronic ionization (EI) remains the preferred technique in GC-HRMS due to its robustness, reproducibility, and the availability of extensive commercial spectral libraries. While both QTOF and Orbitrap analyzers are now compatible with EI sources, it is notable that GC-(Q)Orbitrap systems only became commercially available in 2015, almost a decade after QTOF platforms [25]. Orbitrap instruments offer higher resolving power (up to 120,000 at *m/z* 200) and sub-ppm mass accuracy compared to QTOF (60,000 at *m/z* 200). Yet, their potential for non-target contaminant detection has only recently been explored, positioning them as a promising and intriguing avenue within GC-HRMS that warrants further investigation [26]. HRMS-integrated approaches that combine target, suspect, and non-target analyses are essential for enabling simultaneous quantification or identification of specific chemicals while also detecting unknown compounds in human samples. This addresses the need for comprehensive chemical exposome and biomarker analysis [20,27,28].

This study aimed to develop and validate a comprehensive GC-(Q) Orbitrap-based methodology for the trace-level analysis of lipophilic organic chemicals in human serum. The extraction protocol was optimized directly in real human serum, ensuring consistency between validation and application for real-life studies. Validation was performed in a wide-scope target approach covering GC-amenable chemicals such as organophosphorus compounds (OPEs), PCBs, and organochlorine and organophosphorus pesticides, and notably, PAHs and alkyl-PAHs, which are of growing public health concern and remain poorly studied in human matrices. The method was subsequently applied in both target and non-target screening approaches within the INSULIN cohort, a population-based study of pregnant women residing near the largest petrochemical complexes in Southern Europe conducted at Hospital Joan XXIII (Tarragona, Spain). This validation lays the foundation for future exposomics applications, enabling the expansion of the chemical coverage universe and the detection of previously overlooked compounds to achieve a more comprehensive understanding of the human chemical exposome and highlights the relevance of this approach for future exposome-wide association studies (ExWAS).

2. Methods

2.1. Chemicals and reagents

The array of organic pollutants investigated in this work encompasses several chemical groups (Table S1). Reference standards for pesticides (organochlorine (OC) and organophosphorus (OP)), PCBs, PAHs, and alkyl-PAHs were acquired from Dr. Ehrenstorfer (Augsburg, Germany) and Chiron (Trondheim, Norway). OPEs standard mixtures were purchased from Chiron (Trondheim, Norway). Individual stock standard solutions were prepared (500 μg mL^{-1}) in acetone and stored at −20 °C. Working solutions were obtained by volumetric dilution in

acetone for sample fortification and n-hexane for instrumental analysis. Alkane standard solution C_8–C_{40} (Sigma-Aldrich, Germany) was used to determine Linear Retention Index.

Four isotopically labelled internal standards (ILIS) for PAHs (acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}) and ten ILIS for pesticides (atrazine-desethyl-d_6, hexachlorobenzene-$^{13}C_6$ (HCB–$^{13}C_6$), atrazine-d_5, carbaryl-d_7, prometryn-d_6, terbutryn-d_5, penconazole-d_7, p,p'.DDE-d_8, propiconazole-d_3, and tebuconazole-d_6) were employed. Two mixed working solutions (1 μg mL^{-1}) were prepared: one for PAHs-ILIS and another for pesticide-ILIS, through volumetric dilution of individual stocks with hexane (calibration) or acetone (sample fortification). All solutions were stored at −20 °C until use.

High-purity organic solvents such as n-hexane (99 %, HPLC grade) and dichloromethane (GC ultra-trace analysis grade, stabilized with ethanol), along with sodium chloride (content ≥99.5 %), were sourced from Scharlab (Barcelona, Spain).

2.2. Sample treatment

2.2.1. Sample treatment optimization

Three extraction methods were evaluated (Fig. S1). Methods A and B involved liquid-liquid extraction (LLE). Method A combined LLE with a final clean-up via freezing [13,29]. Method B used ethyl acetate and $MgSO_4$ to extract non-polar compounds and reduce water content, followed by a QuEChERS clean-up with primary secondary amine (PSA) for the removal of organic acids, fatty acids, and sugars, and an additional freezing step [30]. Method C consisted of direct immersion solid-phase microextraction (DI-SPME) based on literature [31] using a DVB/Carbon WR/PDMS fiber, suitable for mixed polarity compounds. In this approach, serum was diluted and acidified with methanol and trichloroacetic acid (TCA) before extraction. Recovery was assessed in triplicate at 2.5 ng mL^{-1} for LLE methods (A and B) and at 0.25 ng mL^{-1} for DI-SPME (C), correcting with ILIS.

2.2.2. Sample treatment selected

Method A was ultimately selected (Fig. 1). Briefly, serum samples were thawed at room temperature. Aliquots of 250 μL were transferred into 2 mL centrifuge tubes and fortified with 10 μL each of PAHs-ILIS (250 ng mL^{-1}) and pesticides-ILIS (200 ng mL^{-1}, 100 ng mL^{-1} thiabendazole-d_4, and 40 ng mL^{-1} p,p'-DDE-d_8). Samples were equilibrated for 2 h at 4 °C, followed by the addition of 750 μL hexane:dichloromethane (80:20, v/v) and 0.1 g sodium chloride. After vortexing (1 min), ultrasonication (5 min), vortexing again (1 min), and centrifugation (8 min at 6010×*g*), samples were frozen overnight at −80 °C. The organic layer was transferred, evaporated under nitrogen to dryness, and reconstituted in 100 μL n-hexane. Extracts were stored at −20 °C and protected from light until analysis. Procedural blanks (PB) were prepared using MilliQ-water instead of serum and processed identically to assess potential contamination.

2.3. Human serum samples

Twenty-four serum samples were randomly selected from the INSULIN cohort, an ongoing population-based cohort study of pregnant women residing near the largest petrochemical complex in southern Europe (Tarragona, Spain). Participants were recruited between March 2021 and January 2022 at Hospital Joan XXIII (Tarragona, Spain). Blood was collected post-delivery using Vacutainer® Rapid Serum tubes and stored at −80 °C in the INSULIN biorepository, located at the Pere Virgili Health Research Institute biobank. The study was approved by the Ethics Research Committee of Pere Virgili Health Research Institute (Ref. 225/2020), and all participants provided informed consent and fulfilled some questionnaires on lifestyle and socioeconomic characteristics.

2.4. Instrumental analysis

The analysis was performed using a Thermo Scientific™ Q Exactive™ GC hybrid quadrupole-Orbitrap mass spectrometer (commercial name), which is referred to throughout the text as GC-(Q)Orbitrap, equipped with a TriPlus RSH autosampler (Thermo Scientific, Bremen, Germany). Separation was achieved on an HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Thermo Fisher Scientific™, Palo Alto, CA, USA) with helium (99.9999 % purity) as the carrier gas at a constant flow rate of 1 mL min^{-1}. The injector temperature was set at 300 °C, with a split flow rate of 50 mL min^{-1} and a purge time of 1.0 min. The oven temperature program initiated at 90 °C(1 min), ramped to 330 °C at 5 °C min^{-1}, and held for 4 min, resulting in a total runtime of 53 min.

The mass spectrometer operated in positive electron ionization mode at 70 eV with full scan acquisition at a resolution of 60,000 FWHM (at m/z 272). The scan range was set from m/z 40 to 750 with an AGC target of 1×10^6. Source and transfer line temperatures were maintained at 270 °C and 330 °C, respectively. Daily calibration was conducted using an internal calibration gas (Thermo Fisher Scientific™, Palo Alto, CA, USA). The C_8–C_{40} alkane series was used to determine the external non-isothermal retention index (RI). Nitrogen gas (Praxair, Spain) was used

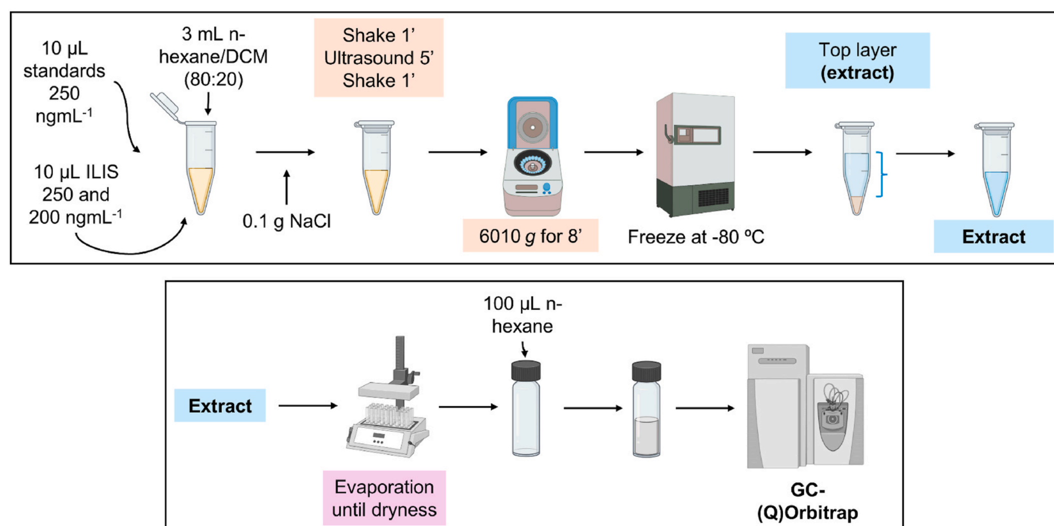


Fig. 1. Sample preparation method A applied to serum samples, detailing the extraction, cleanup, and processing steps designed to optimize analyte recovery. Evaporation was performed using a gentle nitrogen stream. DCM: Dichloromethane, *g*: relative centrifugal force, ILIS: Isotopically labelled internal standards.

for the C-Trap supply.

2.5. Validation of the quantitative method

The quantitative method was validated based on the criteria from the European Commission guidelines (SANTE/11312/2021/v2) [32] for a proprietary database of selected chemicals (Table S1). Validation parameters included linearity, accuracy, precision, limit of quantification (LOQ), and confirmatory capabilities.

Accuracy was assessed via recovery experiments in human serum (n = 6) fortified at two concentration levels (2 and 10 ng mL^{-1}, corresponding to 5 and 25 ng mL^{-1} in extract, respectively). Bracketing calibration ensured stability before and after sample analyses. Linearity was evaluated from triplicate calibration curves prepared in solvent across six concentration levels (2.5, 5, 10, 25, 50, and 100 ng mL^{-1}). Linearity was considered satisfactory when the regression coefficient (R^2) was greater than 0.99 and the residuals were less than 30 %. Precision, as repeatability, was calculated from relative standard deviation (RSD%) of recovery replicates at each fortification level, with acceptance criteria of mean recoveries between 70 and 120 % and RSD ≤20 %. Exceptions were allowed for mean recoveries outside 70–120 % if consistent (RSD ≤20 %) and within 30–140 %. LOQs were established as the lowest spiked concentration meeting the accuracy and precision criteria. Procedural blanks and solvent blanks were analyzed within each batch to monitor potential contamination.

2.6. Target and nontarget screening methodology

Instrument control and data acquisition were conducted using Xcalibur 4.0 software (Thermo Scientific, Waltham, MA, USA), and data processing was performed with TraceFinder™ 5.1 (Thermo Scientific, Waltham, MA, USA).

Quantitative data for target compounds were processed in TraceFinder™ 5.1, using areas relative to internal labelled standards (ILIS) to correct potential variability arising from sample handling and matrix effects (n = 14). For identification of target compounds, a retention time (Rt) tolerance of ±0.1 min from the standard was applied. The criteria included the presence of at least two ions measured at their accurate mass (mass error ≤5 ppm or <1 mDa for m/z < 200), preferably including the molecular ion. In the case of compounds that met these criteria but had concentration values below LOQ, they were labelled as detected ("d"). For quality assurance, quality control (QC) samples were incorporated into the batch at two concentration levels (2 ng mL^{-1} and 10 ng mL^{-1}) to verify the sensitivity and performance of the instrument. In addition, procedural blanks (PB) were analyzed in triplicate to assess potential sources of contamination.

For nontarget screening, the Deconvolution Plugin 1.5 for Trace Finder™ 5.1. was used for comprehensive peak detection and tentative compound identification. Processing parameters were set to: signal-to-noise ratio (S/N) greater than 3, mass error of ±5 ppm, total ion chromatogram (TIC) intensity threshold of 1.000.000, ion overlap window of 98 %, and retention time (Rt) aligning window ± 10 s. Peaks were then matched against the National Institute of Standards and Technology (NIST) mass spectral library and search software (NIST/EPA/NIH EI-MS library 2020, comprising over EI spectra of 306.643 compounds and retention index (RI) values of 139,382 compounds), and other GC-HRMS libraries available. For this process of identification, peaks were filtered by a search index (SI) greater than 700, a high-resolution filtering value above 80, and linear retention index deviation percentage criteria (ΔRI) with the threshold set at 5 %, aligned with previous studies using GC-(Q) Orbitrap under a non-target approach [21,33]. Post-processing, compounds were filtered using criteria of SI > 700, HRF >80 %, and RI deviation (ΔRI) <5 %, as in previous GC-(Q)Orbitrap non-target studies. In the non-target workflow, procedural blanks (PB) were included to identify compounds arising from external sources rather than being intrinsic to the sample. Peaks were then aligned and grouped across samples using the Rt alignment tool, which also excluded peaks found in PB. This generated a cross-sample peak list for further refinement.

Subsequently, potential identifications underwent manual curation to minimize false positives by reviewing fragment ions, Rt, and exact m/z values, along with visual comparison of fragmentation patterns to library spectra. Although EU SANTE 2021 guidelines [32] recommend including the molecular ion among at least two confirming ions for HRMS identification, its absence is common in EI; thus, at least three fragment ions were reviewed to strengthen identification confidence. Additionally, isotopic patterns were evaluated for certain fragments to further support compound assignments. Fig. 2 illustrates the non-target screening workflow implemented in this study, highlighting the key steps involved in achieving potential identifications. Furthermore, each potential annotation was verified using the PubChem and REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals) databases to assess compound properties and previous reports. Compounds previously described as natural products from plants or bacteria were excluded from further consideration. Potential annotations were classified according to the identification confidence levels proposed by Schymanski et al. [34], adapted here for GC-HRMS analyses: Level 2a corresponds to a unique probable structure based on a library match (e.g., NIST), while Level 3 indicates a tentative candidate with multiple possible matches and insufficient evidence to confirm a single structure.

3. Results and discussion

3.1. Sample treatment optimization

For the optimization of sample preparation, real human serum samples were used to test the method directly in the same matrix that will be applied in real-life analyses. Therefore, an optimal extraction approach was crucial to minimizing the co-extraction of endogenous interferences (such as proteins, lipids, salts, and phospholipids) while maximizing recovery of exogenous chemicals with high precision, thereby enabling accurate non-target measurements [31]. To address this dual requirement, three distinct extraction methods (A, B, and C) based on bibliography were selected to treat serum samples.

The compounds included in Fig. S2 were selected because the sample preparation was primarily optimized for PAHs and alkyl-PAHs, which are commonly released from petroleum refining processes and were the initial focus of this exploratory study. This study aimed to develop and validate the extraction method before its application to a cohort of pregnant women who live near to an oil refinery industry. Therefore, the optimization was guided by these compounds, while additional xenobiotics with similar non-polar properties, such as pesticides, OPEs, and PCBs, were subsequently included and their performance confirmed during method validation.

As a result, relative recoveries were studied (Fig. S2). When comparing methods A and B (both based on liquid-liquid extraction with ILIS correction), method B consistently yielded recoveries exceeding 120 % for most compounds (54 %), while two compounds had poor recoveries below 70 %. In contrast, method A achieved recoveries within the 70–120 % range for all compounds except 2-methylnaphthalene (44 %) and indeno[1,2,3-*cd*]pyrene (65 %), suggesting that method A provided more consistent results across a wide range of chemical properties, facilitating effective sample treatment. The use of PSA as a clean-up step in method B may explain the poor recoveries observed, as this sorbent is designed to remove polar co-extracted substances (e.g., organic acids, sugars, fatty acids, ionic lipids, and polar pigments). Given the wide polarity range of analytes in this study, some moderately polar compounds may have been inadvertently removed during PSA clean-up [35]. The freezing step in method A facilitated sample handling without affecting compound recoveries, and n-hexane effectively extracted analytes within its polarity range [13]. Consequently, method A was selected for further work.

Method C (DI-SPME), which was initially based on literature [31]

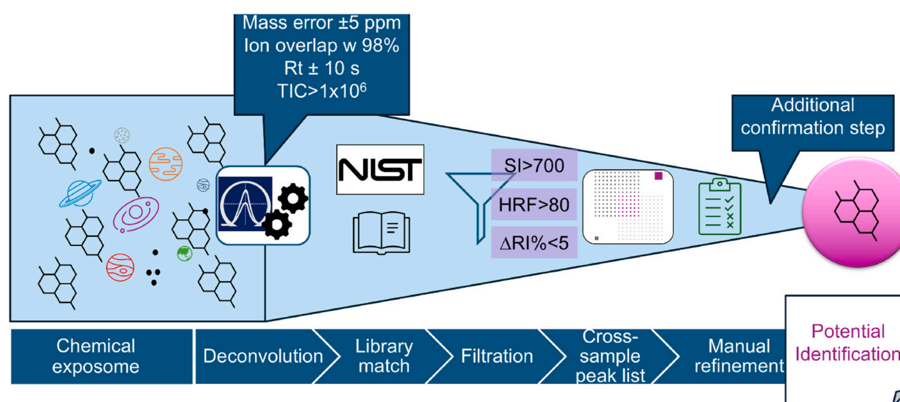


Fig. 2. Workflow for Compound Identification in Serum Samples Using Non-Target Screening Approach. The process begins by analysing the sample, which potentially contains a wide chemical universe, followed by compound deconvolution using TraceFinder™. Parameters for deconvolution included S/N > 3, mass error ±5 ppm, ion overlap window of 98 %, retention time (RT) window ± 10 s, and a TIC intensity threshold of 10^6. Subsequently, a library match step is conducted using databases such as NIST. Filtering criteria include SI > 700, HRF >80, and RI deviation <5 %. A cross-sample peak list is generated, followed by manual refinement to exclude compounds that fail to meet criteria for peak shape, spectrum, among others. An additional confirmation step was performed as well. This comprehensive approach culminates in potential compound identification.

and previous laboratory experience, yielded unsatisfactory detection limits, producing no signals due to inadequate sensitivity. Its optimization was not further pursued, as the other sample preparation methods already provided satisfactory results, and additional effort on DI-SPME was deemed unnecessary; consequently, results are not shown. Nevertheless, both the fiber materials and post-extraction rinsing step are key steps to improve the sensitivity and robustness of the extraction method, as these aspects need to be considered for upcoming research on SPME as an extraction method, as demonstrated with multilayer fibers composed of polydimethylsiloxane and divinylbenzene (PDMS/DVB/PDMS), for multiclass xenobiotics in plasma [36].

3.2. *Validation of the target method*

The analytical potential of the GC-(Q)Orbitrap is illustrated in Fig. 3, showing well-defined quantification and confirmation peaks at low QC concentration (2 ng mL^{-1} in sample) for 1,7-dimethylfluorene, dichlofenthion, PCB 28, and bifenthrin, representing different chemical groups.

Following the optimization of sample treatment, recovery experiments were conducted in sextuplicate to verify the quantitative approach at two concentration levels (2 and 10 ng mL^{-1}). Additionally, fourteen internal standards were added as surrogates to reduce analytical variability, excluding dichlorvos, chinomethionate, and aldrin, for

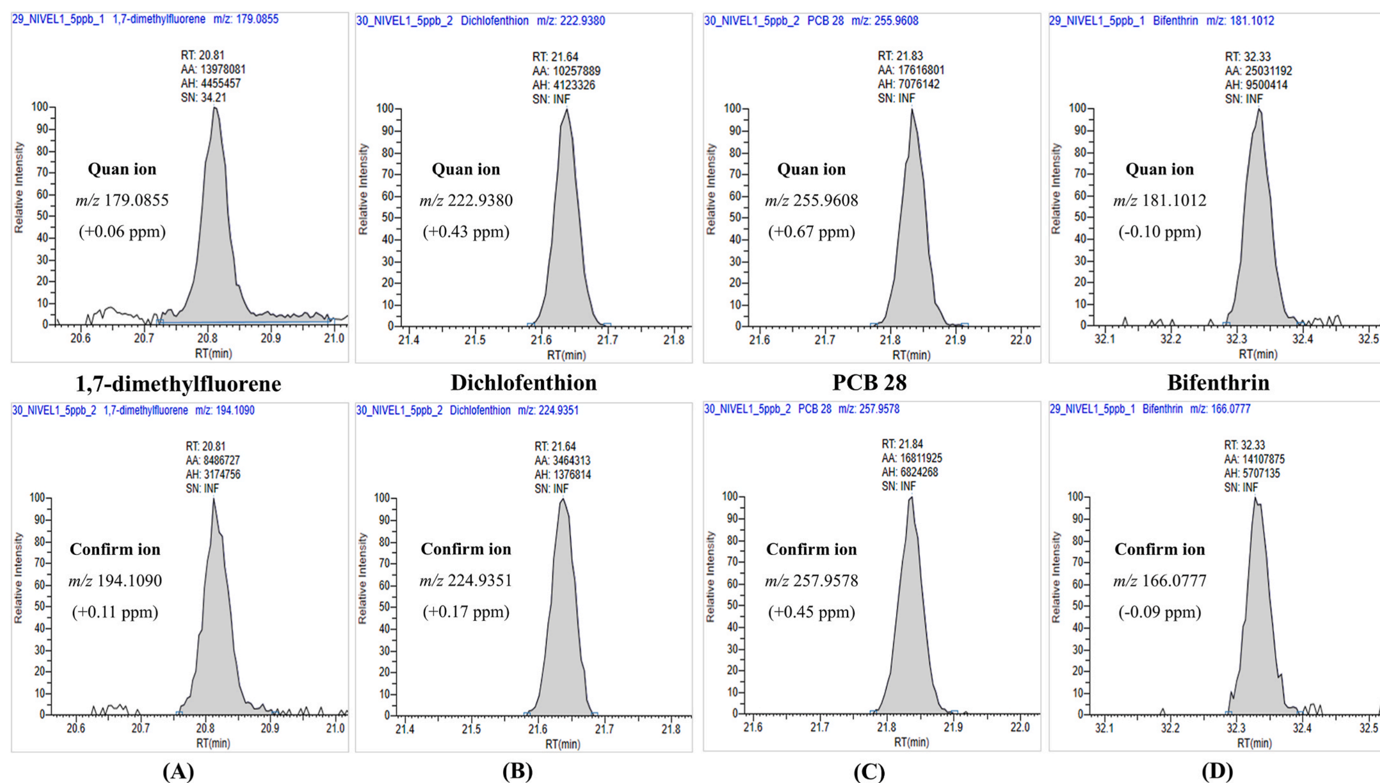


Fig. 3. GC-(Q)Orbitrap chromatograms obtained for the quantification (quantification and confirming ion at low QC (2 ng mL^{-1} in sample) of (A) 1,7-dimethylfluorene, (B) dichlofenthion, (C) PCB 28, and (D) bifenthrin.

which better recoveries were obtained without ILIS correction (Table S1). ILIS were assigned to each compound based on their chemical affinity. For instance, acenaphthene-d_{10} was assigned to certain compounds belonging to the PAHs and alkyl-PAHs families, as it shares the same chemical family and demonstrated better recovery correction within this group.

Linearity of the relative response (corrected with the corresponding ILIS) of the analytes was determined by analyzing triplicate calibration curves in an enriched solvent, covering six concentration points within the range of 2.5–100 ng mL^{-1} (excluding those specified in Table S1). All correlation coefficients (R^2) exceeded 0.99, with residuals below 30 %.

A total of 198 compounds were validated, comprising 9 OPES, 15 PAHs, 24 alkyl-PAHs, 143 pesticides, and 7 PCBs. A total of 229 fortified compounds were evaluated, of which 198 were successfully validated (Table S1). Precision and recoveries were satisfactory for most compounds, with recoveries falling within the range of 70 %–120 % and RSD below 20 %. However, some exceptions were noted with recoveries of some compounds (propoxur, phosphamidon, thiabendazole, methidathion, famphur, DDT, and TPhP H) above 120 % or lower than 70 % at the lowest fortification level. Nevertheless, in certain exceptional cases, mean recovery rates beyond the 70 %–120 % range may be deemed acceptable if they exhibit consistency (RSD ≤20 %) and remain within the bounds of 30 %–140 %. At the fortification level of 2 ng mL^{-1}, the mean recovery rate corresponds to 102 ± 26 % for all the target compounds.

Fig. 4 graphically represents the mean recovery rates ± standard deviation (SD) for validated compounds across different chemical groups, including PAHs, pesticides, alkyl-PAHs, OPEs, and PCBs, evaluated at two concentration levels (2 and 10 ng mL^{-1}) in serum samples. The figure also illustrates the distribution of recovery rates in relation to the LOQ classification for each compound, providing insights into the method's performance and recovery efficiency across diverse chemical groups. LOQ was defined as the lowest concentration of the analyte that could be validated with acceptable accuracy by applying a complete analytical method and identification criteria, resulting in LOQ values of 2 ng mL^{-1} for 139 compounds and 10 ng mL^{-1} for 59 compounds. A total of 6 OPES and 53 pesticides had the highest LOQ, denoting that most PAHs and alkyl-PAHs were available to detect and quantify at low concentration levels, relevant given their prevalence in petrochemical contexts. At the LOQ of 10 ng mL^{-1}, some compounds obtained a recovery rate exceeding 120 %, namely pesticides Cyfluthrin and Cypermethrin, along with their analyzed isomers, among others.

3.3. Human serum sample analysis

3.3.1. Quantitative target analysis

Human serum samples spiked at the two validated concentration levels (2 and 10 ng mL^{-1}) were included as quality control (QC), along with three procedural blanks and solvent blanks to avoid potential false positives. All QC samples reached acceptable recovery tolerances, confirming the validity of the results for the entire batch of samples. Regarding the limit of detection (LOD), in high-resolution Orbitrap instruments, the LOD cannot be robustly estimated using the conventional signal-to-noise ratio, as baseline noise is often negligible and may result in artificially infinite S/N values [37]. Therefore, consistent with the SANTE guideline, which defines the LOQ and limit of determination but not an LOD, only the LOQ was established in this study.

The results obtained for the 24 samples analyzed are shown in Table 1. A compound was considered detected "d" when its quantification and confirming ions were clearly present in the sample (Fig. 3), retention time is aligned within the defined tolerance window, but the concentration was below the validated LOQ. Additionally, for those compounds which showed concentration between the lowest calibration level (LCL) and LOQ were reported in the table with an asterisk (*). Although these concentrations are below the lowest validated level, this information highlights that lower sensitivity may be achieved for those species in future research. As illustrated in Fig. S3, these peaks below the LOQ displayed retention time and peak shape consistent with those of the LCL. Conversely, a compound was classified as "nd" (not detected) when it did not meet these detection criteria.

Ten chemicals were detected in total: three PAHs, six alkyl-PAHs, and one synthetic antioxidant. Diphenylamine, a synthetic antioxidant widely used in the fruit industry to prevent superficial scalding in apple and pear crops, is also employed across multiple industries due to its antioxidant properties. However, this compound has been banned in the EU due to its ecotoxicity and human toxicity, as exposure can occur via inhalation, skin absorption, ingestion, or eye contact [38]. In the present study, diphenylamine was detected in one sample at a concentration below its LOQ.

Regarding PAHs, naphthalene and phenanthrene were detected in 100 % of the samples in concentrations below 2 ng mL^{-1}, whereas pyrene was detected in two samples. These are classified as lower molecular weight PAHs (LMW-PAHs), which tend to remain dissolved in air and are readily absorbed through the respiratory system, posing risks in cases of acute exposure, while chronic low-level exposure has been

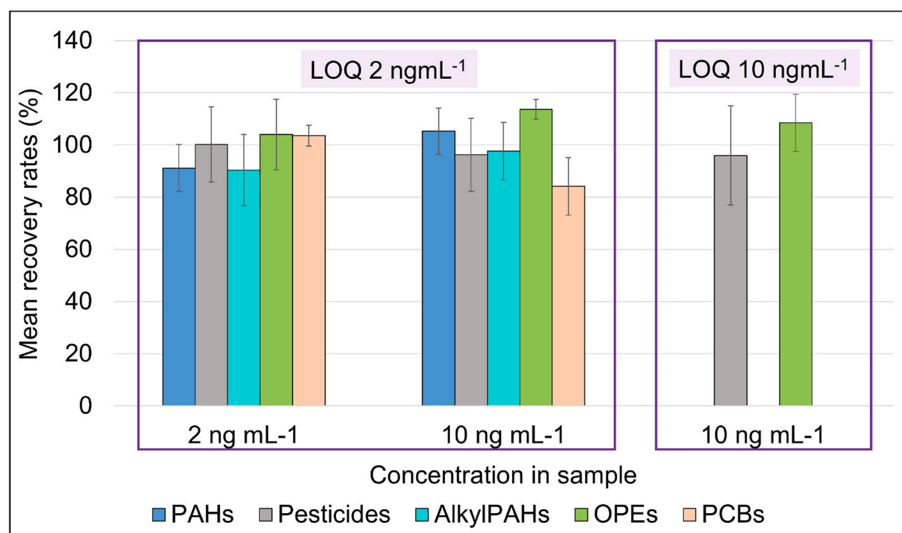


Fig. 4. Mean Recovery Rates of Validated Compounds by groups. The figure illustrates the mean recovery rates (±SD) for validated compounds from different groups (PAHs, pesticides, alkyl-PAHs, OPEs, and PCBs) at two concentration levels (2 and 10 ng mL^{-1} in serum samples). Additionally, it displays the distribution of recovery rates according to the LOQ classification for each compound.

Table 1
Concentrations of the chemicals found in the samples using the target methodology (ng mL^{-1}). Compounds labelled as "nd" were not detected, while "d" indicates those that were detected but the concentration was below LOQ. Values between the LCL and LOQ are reported with an asterisk (*).

Compound name	Sample concentration (ng mL^{-1})																							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
Naphthalene	d	d	d	d	1.3*	d	1.3*	1.1*	d	d	1.1*	d	d	d	d	1.3*	d	d	1.2*	d	1.0*	1.3*	1.0*	1.0*
Naphthalene, 2-methyl-	1.2*	1.4*	1.5*	1.5*	1.5*	1.4*	**2.1**	1.5*	1.4*	1.3*	1.4*	1.1*	1.1*	1.6*	1.0*	1.4*	1.2*	1.4*	1.4*	1.5*	1.4*	1.5*	1.5*	1.4*
Naphthalene, 2,6-dimethyl-	d	1.1*	d	1.5*	1.0*	**2.3**	1.4*	1.2*	1.1*	d	1.2*	1.1*	1.4*	d	d	1.5*	1.3*	d	**2.0**	1.0*	1.2*	1.1*	1.0*	1.0*
Diphenylamine	nd	nd	nd	nd	nd	nd	d	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fluorene, 1-methyl	nd	nd	nd	nd	nd	nd	nd	nd	d	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Phenanthrene	d	1.2*	1.2*	1.5*	1.3*	1.1*	1.7*	1.1*	d	1.2*	1.3*	d	1.2*	1.4*	d	1.2*	1.0*	1.3*	1.1*	1.8*	1.1*	1.2*	1.4*	1.0*
Phenanthrene, 1-methyl-	**2.2**	**2.5**	**2.8**	**2.3**	**2.7**	**2.8**	**3.8**	**2.4**	d	**2.6**	**2.2**	1.6*	**2.2**	**2.6**	**2.4**	**2.6**	**2.6**	**3.3**	**2.9**	**2.7**	**2.4**	**3.2**	**2.6**	**3.2**
Phenanthrene, 1,3-dimethyl-	nd	nd	nd	nd	nd	nd	**2.3**	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Phenanthrene, 1,2-dimethyl-	d	1.0*	1.1*	1.0*	1.0*	1.0*	1.3*	1.0*	d	1.0*	d	d	d	1.1*	d	1.1*	1.0*	1.2*	1.0*	1.1*	1.0*	1.2*	1.1*	1.1*
Pyrene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	d	nd	d	nd	nd	nd	d

linked to adverse health outcomes [39–41]. Comparatively, Morsina Çipa et al. [40] reported naphthalene concentrations averaging 23 ng mL^{-1} in serum samples from individuals working in or residing near oil refineries, levels notably higher than those observed in the current study. Conversely, Singh et al. [42] reported mean naphthalene concentrations of 1.99 ng mL^{-1} in blood samples from children in India, aligning more closely with our findings.

Six detected chemicals belonged to the alkyl-PAHs family, compounds of petrogenic origin that are widespread in the environment [43]. Four out of the six detected chemicals were identified in all samples. For instance, 2-methylnaphthalene was detected in every sample, but concentration was higher that LOQ value in one sample (2.1 ng mL^{-1}), 2,6-dimethylnaphthalene (2.3 ng mL^{-1}), 1,2-dimethylphenanthre (<LOQ) and 1-methylphenanthrene (2.2–3.8 ng mL^{-1}), while 1,3-dimethylphenanthrene was only quantifiable in one sample at 2.3 ng mL^{-1} and 1-methylfluorene was detected at a concentration lower than LOQ. Concerns surrounding alkylated PAHs have heightened recently due to their potentially greater endurance, water solubility, bioaccumulation potential, toxicity, mutagenicity, or carcinogenicity compared to the parent PAHs [44,45]. Despite this, environmental and biomonitoring data for alkyl-PAHs remain limited, in part due to the lack of robust analytical methods capable of efficiently distinguishing and quantifying their numerous homologous isomers [44–46]. The identification of these compounds in the present study using GC-(Q) Orbitrap is therefore notable, underscoring the need for future studies with larger sample sizes to validate these results and explore potential associations with health outcomes.

While discerning distribution trends of PAHs and alkyl-PAHs in serum based on this limited batch of 24 samples is not feasible, more extensive studies could help elucidate exposure sources, including inhalation and dietary intake. Interestingly, the higher prevalence of LMW-PAHs and alkyl-PAHs compared to high molecular weight PAHs (HMW-PAHs) suggests a tendency of LMW-PAHs and alkyl-PAHs to dissolve in serum at detectable concentrations, due to their increased solubility. This pattern implies that inhalation may be the predominant exposure route, although other sources cannot be excluded in the present study. Exposure to PAHs during pregnancy may contribute to an increased risk of maternal complications, including gestational diabetes mellitus (GDM) and gestational hypertension [47], highlighting the importance of further research to identify additional potential associations. Alternative biospecimens, such as adipose tissue, placenta, nails, or hair, may offer improved insights into HMW-PAH presence in humans.

3.3.2. Non-target screening analysis

The results obtained with the optimized target methodology demonstrate the high robustness and sensitivity of the analytical setup for determining and quantifying a broad range of lipophilic contaminants with diverse physicochemical properties in serum matrices. These analytical capabilities enable the implementation of the current workflow for the discovery of unknown contaminants through non-target screening approaches.

Following deconvolution of the GC-HRMS data, each sample yielded between 1000 and 1400 features, corresponding to 7563 tentatively assigned compounds based on software-matched fragmentation patterns with the NIST library or HRMS libraries provided by the supplier. Application of multiple filters (HRF >80, SI > 700, and ΔRI <5 %) reduced this to 150–190 peaks per sample, representing around 500–540 chemical compounds potentially present in at least one sample (6–7 % of total features with confident assignments). Cross-sample compound alignment based on retention time (Rt) was then performed to compare annotations across the batch, while the Rt alignment tool also facilitated the exclusion of peaks found in procedural blanks. The resulting compound list contained 246 items per sample (approximately 3 % of the full feature list). Subsequent manual inspection removed peaks with poor shape. Chemicals were then examined using open

databases such as PubChem and ECHA CHEM with information from all REACH registrations received by the European Chemicals Agency (ECHA) to assess their chemical properties, environmental pathways, and toxicological profiles. The selected compounds represent substances to which participants could be exposed via multiple pathways, including environmental, industrial, or pharmaceutical sources. While exposomics encompasses both internal and external exposures, this study focused exclusively on chemicals associated with the general external exposome.

The non-target workflow developed here enabled the tentative identification of a broad range of compounds, as illustrated in Fig. 5. As a result of manual refinement, 64 potential chemicals were identified (see Table S2). These included potential annotations from diverse groups such as PCPs, UV filters, pesticides, PAHs and petroleum derivates, pharmaceuticals, plasticizers, and industrial additives, as well as some stimulants, among others. Among these, approximately 70 % were classified as Level 3, while the remaining 30 % were classified as Level 2a [34]. Furthermore, compounds identified through the targeted approach were also incorporated into this figure as Level 1 identifications, to provide a more comprehensive and integrated overview of the chemical space covered in this study. It is worth noting that the classification levels proposed in the literature are more likely to lead to the classification of data coming from soft ionization sources [48], since the presence of the unfragmented parent ion is usually required. This underscores the importance of developing and proposing confidence levels specifically tailored to the characteristics and requirements of hard ionization, thereby enabling more reliable communication regarding compound identifications.

These compounds demonstrate the method's robustness and adaptability for broad-spectrum chemical screening, including compound name, Rt, SI, HRF (%), Level of identification, library, and library ID number (Table S2). The area across the sample set as a result of the additional confirmation step is available in the supplementary material (Table S3). It was observed that some compounds were initially reported in only a few samples; however, upon performing an additional confirmation step based on molecular and/or fragment ion presence, their detection frequency increased. This discrepancy may stem from the strict filters applied after deconvolution, particularly SI and HRF thresholds, which reduce false positives but may also exclude compounds that are present yet do not fully meet these criteria. Consequently, this additional confirmation and manual curation step proved valuable, allowing for verification of compound presence across the batch even in the absence of analytical standards and despite not meeting initial identification thresholds. It is important to emphasize that the deconvolution process differs from targeted compound searches based on exact mass and retention time, which can enhance detection across samples [33].

The number of detected compounds and their diverse physicochemical characteristics highlight the versatility of the sample preparation method, which enables not only targeted identification but also supports non-target detection, thereby broadening the scope for identifying and detecting chemical compounds within the exposome. The method demonstrated extended detection capability, successfully identifying compounds from various chemical families, including halogenated and non-halogenated species with different polarities and volatilities. Notably, among the potential annotations, PAHs, and petroleum-derived compounds, as well as plasticizers and industrial additives, were the most frequently detected groups, underscoring their relevance and raising interest in their potential links to human exposure. Details of deconvolutions and database comparisons for selected chemicals are provided in the supplementary information (Fig. S4-S7), including molecular ions ($M^{+\bullet}$) when available, two or three fragment ions (F1, F2), their formulas, theoretical masses, and mass errors (ppm), with most ions consistently exhibiting mass errors below 1 ppm.

Regarding the detected chemicals, two alkyl-PAHs not included in

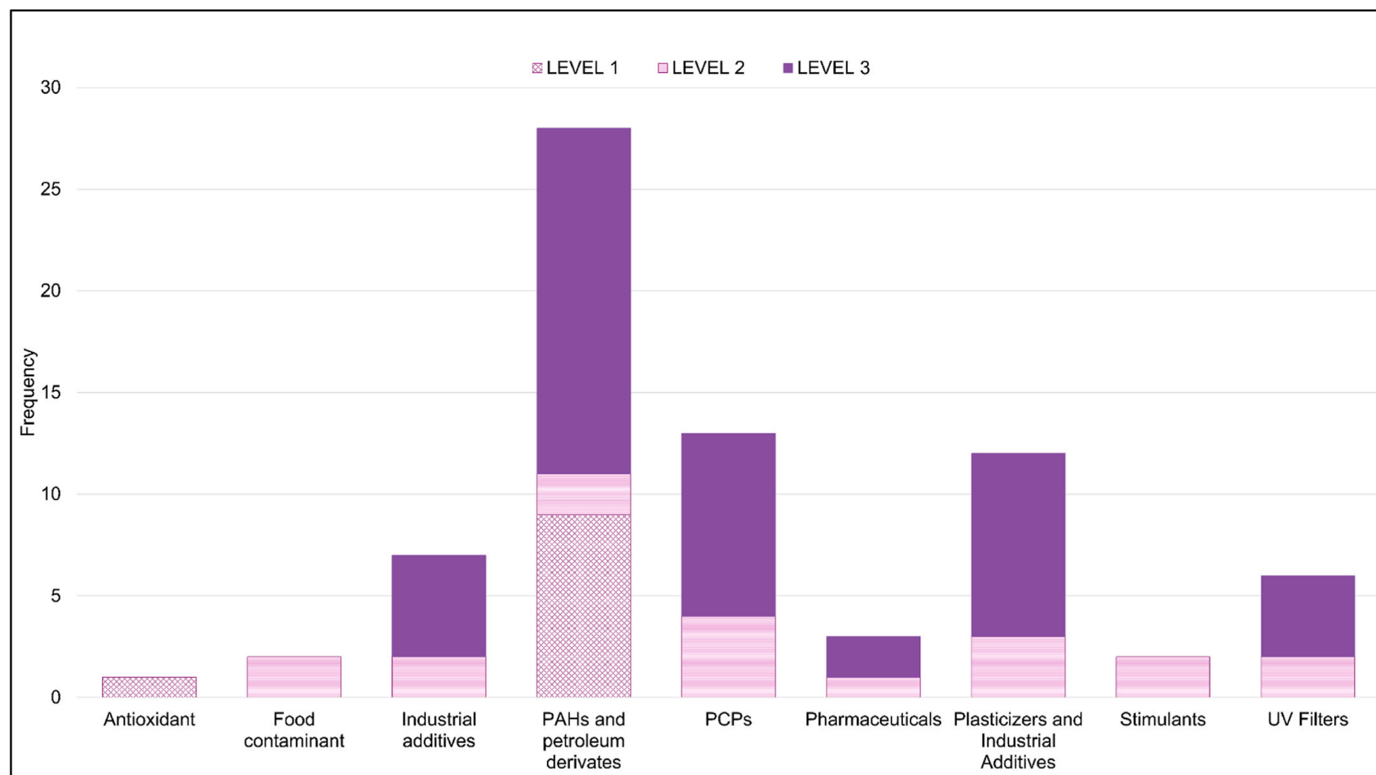


Fig. 5. Distribution of Potentially Identified Compounds by Groups and Confidence Levels. The figure presents the distribution of potentially identified compounds across different groups, including PCPs, PAHs and petroleum derivates, pesticides, pharmaceuticals, stimulants, and others. Legend distinguishes the assigned identification confidence levels: Level 2a (probable identification) and Level 3 (tentative identification). Compounds identified by the target approach were also included in this figure as Level 1.

the in-house database (1,3-dimethylnaphthalene and 2,6-diisopropylnaphthalene) were identified in 25 % of the samples using a non-target approach with the NIST. These compounds have previously been reported in the food sector, primarily due to migration from cellulosic food packaging materials, suggesting dietary intake or possibly gaseous transport as exposure routes [49]. Similarly, several compounds classified as PAHs or petroleum derivatives were tentatively or putatively identified. The presence of alkylated PAHs and other petroleum-based chemicals not initially targeted highlights the value of non-target studies for comprehensive characterization of the human exposome, as highly concentrated chemicals may be overlooked in a purely targeted approach.

Another noteworthy finding was caffeine, detected in all samples with a reliable match. Its presence reflects consumption of caffeine-containing products such as coffee and tea, making exposure ubiquitous and difficult to avoid. Similar results have been reported in serum samples analyzed by LC-HRMS [50], supporting the validity of our findings. Furthermore, lidocaine (a local anesthetic) and bupivacaine (an epidural anesthetic used during delivery) were detected, corroborating previous literature reports in pregnant women (Fig. 6) [51] and previously detected in serum [50]. Specifically, lidocaine was detected in five women, while bupivacaine was found in three of these five.

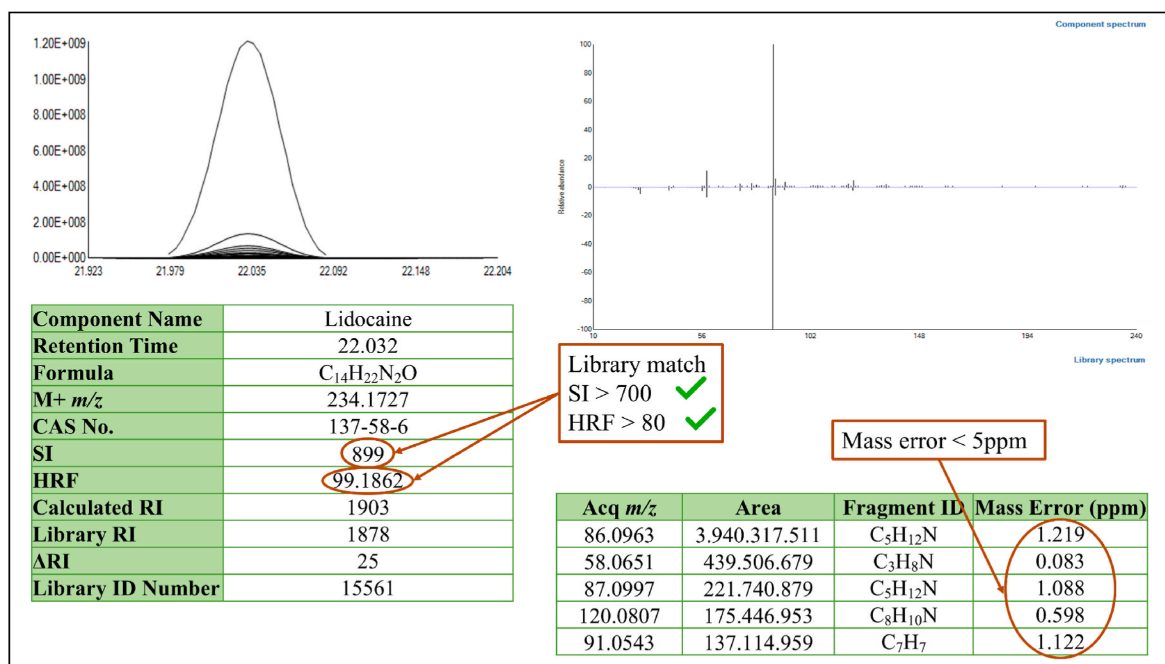
Other compounds identified included bumetrizole (UV-326), and 2-ethylhexyl *trans*-4-methoxycinnamate known as octinoxate, personal care products (PCPs) used as organic UV-filter and fragrances. Although their environmental occurrence and bioaccumulation in animals have been documented [52,53], their presence in human serum has been poorly reported. Given their detection in 50 % and 70 % of samples, respectively, these UV filters warrant further investigation, monitoring, and assessment to better understand their potential toxicological impacts. Additional PCPs compounds commonly used as UV filters were also identified, underscoring the need for their surveillance in human biomonitoring studies. Research on UV filters such as octinoxate has highlighted their potential health relevance as endocrine-disrupting chemicals (EDCs), with numerous studies conducted in animal models. Evidence in humans, however, remains limited and not fully understood. Notably, some studies have reported the absorption of EDCs, including octinoxate, into the bodies of reproductive-age Black women through the use of hair care products containing these chemicals. The fact that these compounds act as EDCs in other species serves as a cautionary signal, particularly for pregnant women, who represent a vulnerable population. Potential effects on the fetus via placental transfer, as well as postnatally through exposure via breast milk, have yet to be fully elucidated [54,55].

Interestingly, no overlapping compounds were identified between the non-target and target analyses. This discrepancy may arise from limitations in non-target workflows, differences between human and automated data inspection criteria, and challenges in deconvoluting distinct spectra for spiked contaminants, as noted by Izquierdo-Sandoval et al. [33]. The high rate of false negatives could be attributed to two factors: I) the inability of the non-target pipeline to deconvolute a characteristic and consistent spectrum representative of the contaminant's fragment ion, or II) deconvoluted spectra with insufficient similarity to the candidate spectrum in the NIST library. Additionally, stringent filters such as HRF and RI criteria can reduce compound matches if criteria are unmet or if RI data are unavailable in the NIST database. Although tentative or probable identifications were achieved, it is important to recognize the limitations of the NIST library [33], which, despite its extensive coverage, cannot fully encompass the vast and continually evolving chemical universe, including emerging contaminants. Moreover, missed identifications due to spectrum deconvolution issues and strict library matching highlight the need for methodological refinement to enhance identification accuracy. Nevertheless, non-target analysis remains a valuable tool for generating preliminary insights into potential chemical exposures in biological matrices such as serum.

4. Conclusions

This study highlights the utility of GC-(Q)Orbitrap methodologies in overcoming the inherent detection limitations of LC-based techniques, particularly for compounds exhibiting low to medium polarity and medium to high volatility. The integration of non-target screening within the analytical workflow significantly broadened the chemical detection scope beyond predefined targets, thereby enhancing the comprehensive characterization of the human chemical exposome.

Using the validated target approach, the method successfully identified ten compounds in serum samples from pregnant women within the



Component Name	Lidocaine
Retention Time	22.032
Formula	$C_{14}H_{22}N_2O$
M+ *m/z*	234.1727
CAS No.	137-58-6
SI	899
HRF	99.1862
Calculated RI	1903
Library RI	1878
ΔRI	25
Library ID Number	15561

Acq *m/z*	Area	Fragment ID	Mass Error (ppm)
86.0963	3.940.317.511	$C_5H_{12}N$	1.219
58.0651	439.506.679	C_3H_8N	0.083
87.0997	221.740.879	$C_5H_{12}N$	1.088
120.0807	175.446.953	$C_8H_{10}N$	0.598
91.0543	137.114.959	C_7H_7	1.122

Fig. 6. Lidocaine potential identification parameters.

cohort, including PAHs and alkyl-PAHs of environmental relevance. Moreover, the non-target screening tentatively identified an additional 64 chemical annotations, underscoring the capability of combining both target and non-target analyses to expand the exposome coverage and uncover previously overlooked compounds.

Overall, this research primarily focused on the validation and performance assessment of a wide-scope analytical method, demonstrating its potential applicability in population-based studies. Future investigations are recommended to implement this methodology in larger sample sizes and within vulnerable populations, such as pregnant women, to refine the characterization of chemical exposure patterns. Such applications will not only strengthen the utility of this approach within exposome-wide association studies (ExWAS) but also contribute valuable insights towards advancing public health interventions.

CRediT authorship contribution statement

Allison Jiménez-Nieto: Writing – original draft, Visualization, Software, Methodology, Data curation, Conceptualization. **David Izquierdo-Sandoval:** Writing – review & editing, Validation, Software. **Juan V. Sancho:** Resources. **Marcos Granell:** Methodology, Investigation. **Ana González-Ruiz:** Investigation. **Mónica Ballesteros:** Investigation. **Nerea Vilanova:** Investigation. **Ana Megia:** Investigation. **Pablo Gago-Ferrero:** Writing – review & editing, Conceptualization. **José L. Domingo:** Investigation. **Montse Marquès:** Writing – review & editing, Funding acquisition, Conceptualization. **Tania Portolés:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Rubén Gil-Solsona:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT-4o in order to revise grammar and improve the readability of the text. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.128897.

Data availability

Data will be made available on request.

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