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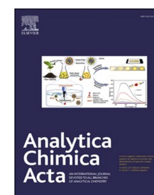

SPME arrow-based extraction for enhanced targeted and untargeted urinary volatilomics

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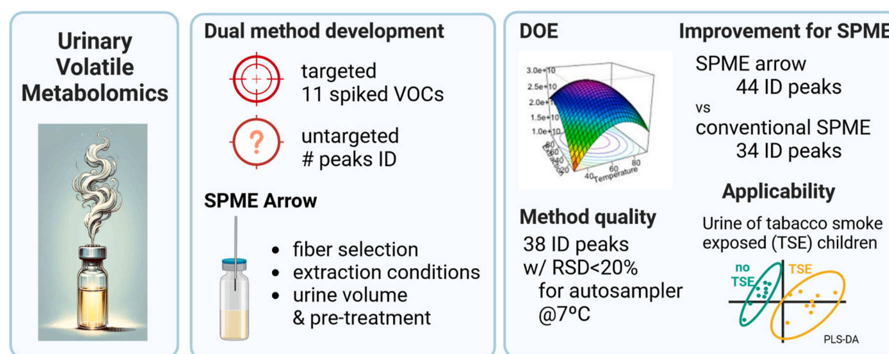
HIGHLIGHTS

- A SPME Arrow-based method for enrichment of human urinary VOCs.
- An untargeted method for urinary VOCs was developed using a targeted approach.
- SPME Arrow outperformed conventional fibers, detecting more urinary VOCs.
- The validated method demonstrated good repeatability and reproducibility.
- Urinary VOCs linked to children's tobacco exposure were successfully identified.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Volatile organic compounds (VOCs) present in human urine are promising biomarkers for various health conditions and environmental exposures. However, their reliable detection is challenging due to the complexity of urinary matrices and the low concentrations of VOCs. Moreover, untargeted approaches present considerable challenges in terms of data interpretation, increasing the complexity of method development. Here we address these challenges by developing a new method that combines solid-phase microextraction (SPME) Arrow with gas chromatography-high resolution mass spectrometry (GC-HRMS), using a design of experiments (DOE) approach for targeted and untargeted compounds. This methodology, specifically tailored for SPME Arrow, represents a significant advancement in untargeted urinary analysis.
Results: The method was developed based on targeted and untargeted outcomes, were ranking results focus on the highest response area of 11 spiked target VOCs representative of urinary volatilomics, and on identifying the maximum untargeted number of VOCs. The method was developed focusing on the highest response area of 11 spiked target VOCs representative of urinary volatilomics and identifying the maximum number of VOCs. A univariate method determined the optimal coating type, urine volume, and salt addition. Subsequently, a central composite design (CCD) DOE was used to determine ideal temperature, extraction, and incubation times. The

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best method obtained has an extraction time of 60 min at a temperature of 53 °C, with an SPME Arrow CAR/PDMS using 2 mL of urine, with 0.25 % w/v of NaCl and a pH of 2. Compared to conventional SPME fibers, the SPME Arrow showed improved extraction efficiency, detecting more VOCs. Finally, the enhanced method was successfully applied to urine samples from children exposed and non-exposed to tobacco smoke, identifying specific VOCs, like *p*-cymene and *p*-isopropenyl toluene related to tobacco exposure.

Significance: By integrating both targeted and untargeted approaches, the developed method comprehensively captures the complexity of urinary metabolomics. This dual strategy ensures the precise identification of known compounds and the discovery of novel biomarkers, thereby providing a more complete metabolic profile. Such an approach is crucial for advancing in non-invasive diagnostics and environmental health studies, as it offers deeper insights into the intricate relationships between metabolic processes and various health conditions.

Abbreviations

(ANOVA) Analysis of variance
(CAR/PDMS) Carbon Wide Range/Polydimethylsiloxane
(CCD) central composite design
(DoE) design of experiments
(DVB/CAR/PDMS) Divinylbenzene/Carboxen/Polydimethylsiloxane
(DVB/PDMS) Divinylbenzene/Polydimethylsiloxane
(GC-HRMS) gas chromatography-high-resolution mass spectrometry
(GC-IMS) gas-chromatography-ion mobility spectrometry
(IS) internal standard
(No-TSE) non-exposed to tobacco smoke
(PA) Polyacrylate
(PCA) principal component analysis
(PLS-DA) partial least square differential analysis
(RI) retention index
(SPME–HS–GC-HRMS) solid-phase microextraction head space with gas chromatography-mass spectrometry
(SPME arrow) solid-phase microextraction Arrow
(SPME Arrow–HS–GC-HRMS) solid-phase microextraction Arrow head space with gas chromatography-high-resolution mass spectrometry
(TSE) tobacco smoke exposed

1. Introduction

In humans, almost 2800 volatile organic compounds (VOCs) are known to be emitted from breath, saliva, blood, milk, semen, skin, feces, and urine [1]. These compounds are of great importance in clinical research, as they can be used as biomarkers to diagnose and monitor various diseases [2]. In addition, VOCs can provide insights into the metabolic processes occurring within the body [3] and can even indicate exposure to environmental toxins or pollutants [4,5].

The analysis of VOCs in human urine samples has become an increasingly popular area in clinical research [6] as it can provide valuable information on the metabolic processes occurring within the body, as well as insight into the diagnosis and monitoring of various diseases [7–10]. This is due in part to the fact that urine is a non-invasive biological fluid, easy to collect and store. In addition, urine is a highly informative matrix for the detection of VOCs, as it contains a wide range of volatile and semi-volatile compounds that can be analyzed.

Sample preparation is a crucial step in analytical procedures, especially when dealing with complex matrices like urine. Effective sample preparation enriches the analytes of interest and minimizes matrix interferences, thereby enhancing the accuracy and reliability of the analytical results. However, capturing VOCs appropriately is challenging due to their volatile nature and low concentration levels. Fortunately, a variety of analytical techniques have emerged in recent years [6], including sorbent-based extraction techniques (SBE) such as solid-phase microextraction (SPME), needle and trap or thin-film [11–14]. Needle-trap or thin-film microextraction, which offer different geometries and larger volumes, require additional thermal units for desorption [15–17].

On the other hand, SPME is a particularly advantageous method for VOC analysis due to its simplicity, minimal solvent requirements, and efficiency extracting a wide range of compounds. This technique is environmentally friendly and cost-effective, representing a significant improvement over traditional methods. SPME stands out for its specific design, facilitating the analysis. SPME followed by gas chromatography-mass spectrometry (GC-MS) [18] is one of the most commonly used configurations [19]. SPME is a simple extraction technique, with minimal requirements, and is efficient in extracting a wide range of compounds which improves the sensitivity. Moreover, the technique is environmentally friendly as it represents a reduction in solvent usage [20].

The recently introduced SPME Arrow [21] improves the conventional SPME by using a larger sorption phase volume with improved mechanical reliability while maintaining all the rest of the functionalities [21,22]. SPME Arrow has shown higher extraction efficiency, greater sensitivity, and improved reproducibility in fragrances [23] and food matrixces [24]. We theorise that the use of a larger sorbent volume will improve extraction efficiency, enhancing sensitivity and detection in complex matrices like urine. However, this process has not been optimized yet.

Biomarker discovery constitutes a critical aspect of biomedical metabolomics, particularly within the domain of untargeted metabolomics, which has demonstrated growing success over the years [25]. Untargeted analysis, also known as fingerprinting, aims at identifying the maximum possible number of compounds in a biological sample. Consequently, the chosen method must be capable of accommodating a wide spectrum of chemical characteristics. Unlike targeted methods, untargeted approaches present considerable challenges in terms of data interpretation, thereby increasing the complexity of method developments [26]. So far there is only one study evaluating the suitability of SPME Arrow-GC-MS in untargeted analysis using urine samples [27]. They obtained no significant differences between SPME conventional fibers and SPME Arrow fibers for the extraction of standard compounds and the number of VOCs detected. After reviewing the report, we found several limitations in the methodology, as different vendors fibers were compared, and the used conditions of extraction where not enhanced for the SPME Arrow.

Not only that, but in our study, we use the optimized method to present the first practical study of the SPME Arrow for untargeted analysis of human urine with a methodology adapted for SPME Arrow. The method was developed based on the response to 11 selected compounds representative of urinary volatilomics, selected because of their biological relevance after a bibliographic analysis [6]. Method development includes fiber coating selection, salting-out effect evaluation, urine volume comparison, extraction parameters evaluation using a central composite design, and performance comparison with conventional SPME Arrow fibers. Finally, the performance of the developed SPME Arrow method was assessed detecting volatilomics signatures of

tobacco smoke exposure digging into children's urine metabolites.

2. Experimental section

2.1. Materials and reagents

The chemical standards of the 11 volatile organic compounds used for method development were obtained from Merck KGaA (Darmstadt, Germany), including 2-pentanone, 2-hexanone, 2-heptanone, 2-nonanone, toluene, dimethyl trisulfide, nonanal, decanal, indole, 1-dodecanol, tetradecanoic acid; 4-fluorobenzaldehyde was used as internal standard (IS) based on bibliography [6,11]. Stock solutions were prepared in methanol at a concentration of 5000 mg L^{-1} for all compounds, except for dimethyl-trisulfide (10,000 mg L^{-1}). Working solutions of 50 mg L^{-1} in methanol were freshly prepared before measurements. A GC grade with a purity >99.9 % Methanol from Scharlab (Barcelona, Spain) was used.

A mixture of C_8–C_{20} hydrocarbons in hexane (40 mg/L, Merck) was used to calculate the linear retention time indexes (RI). Sodium Chloride (NaCl) of >99 % purity supplied by Merck was also used. Chloridric acid 1 M was purchased from Scharlab. Water LC-MS grade was obtained from Thermo Fisher Scientific (Waltham, USA). Helium gas of 99.999 % purity was purchased from Carburos Metálicos (Tarragona, Spain). SPME arrows and conventional SPME fibers were purchased from Restek (Bellfonte, USA). All the tested fibers were conditioned according to the manufacturer's recommendations before their use.

2.2. Urine sample collection

For this study, human urine samples were collected in two batches. The first batch consisted of urine samples used for method development. These were obtained from 4 non-smoker healthy individuals from our research group (aged 22–27 years, 2 females, and 2 males). First morning urine samples were collected in hermetic sterile flasks kept at 4 °C during transportation in February 2022. Upon arrival, the samples were pooled, centrifuged and the supernatant aliquoted on the same day of collection, and subsequently stored at −80 °C until analysis.

The second batch consisted on human urine samples used to assess the applicability of the method developed previously. These samples were from 20 children enrolled in the project THSExposure, which was approved by the Ethics Committee of the University Hospital Sant Joan of Reus, in Catalonia, Spain (Ref. approbation: 15-05-28/5proj4). Participants samples were divided into two groups according to tobacco smoke exposure: exposed (TSE) and non-exposed (No-TSE) participants. The groups were gender-balanced, and the mean age of the participants was 6.3 (2–11) years old for the TSE group and 5.6 (3–9) years for the No-TSE one.

Urine collection was performed between January to October 2017 in the Tarragona area (Spain). The morning void was collected in a hermetic sterile flask by the participant's caregivers, delivered within 1 h to the project researchers and kept at 4 °C during transportation. Upon reception, urine samples were centrifuged, and the supernatant was aliquoted and stored at −80 °C until analysis. Each sample underwent a general urinalysis to identify and exclude those samples presenting any anomaly (presence of blood, urobilinogen, bilirubin, proteinuria, nitrites, ketones, glucose, or leukocytes).

Quality control samples were prepared by pooling equal volumes of urine from all participants and then divided into equal aliquots. All samples were frozen at −80 °C until analysis.

2.3. Sample treatment and extraction

Urine samples were thawed on ice on the day of analysis. Under the developed conditions, we added 0.35g NaCl and 150 μL of HCl 1 M to 2 mL of each sample placed in a 10 mL headspace glass vial and spiked with the internal standard at a final concentration of 0.3 μg mL^{-1}. Extraction of the volatile compounds was carried out with a carboxen/polydimethylsiloxane (CAR/PDMS) SPME arrow fiber (d = 1.1 mm). Before every batch of analysis, CAR/PDMS SPME Arrow fibers were conditioned for 30 min at 280 °C. For the extraction procedure, the SPME arrow fibers were held in the head-space of the vials with the urine samples stirred at 250 rpm for 60 min at 53 °C. Fiber desorption was conducted in the GC injector port at 260 °C for 5 min in the split less mode. To prevent carry-over the SPME Arrow was baked for 10 min at 280 °C. Blank samples were used to ensure system suitability and to test the complete desorption of analytes from SPME fibers in previous measurements.

Extraction fiber and conditions and daily performance were determined by using aliquots of the pooled urine sample spiked with the 11 target compounds at a final concentration of 0.3 μg mL^{-1}. These standards were selected after a curated bibliographic evaluation, to represent the most common volatile chemical classes found in urine and distributed across the chromatogram according to their RI [18,28–33].

2.4. Gas chromatography – high-resolution mass spectrometry (GC-HRMS)

Separation and detection were performed on a GC TRACE 1310 – MS Exactive (Thermo Fisher Scientific, Waltham, MA) equipped with a PAL Autosampler and a temperature-controlled drawer (7 °C). The chromatographic separation was carried out using a ZB-5plus analytical column (5 % phenyl – 95 % dimethylpolysiloxane, 30 m × 0.25 mm i.d.; 0.25 μm film thickness) provided by Phenomenex (Torrance, USA). The oven temperature was as follows: 40 °C hold for 2 min, raised to 300 °C at 10 °C·min^{-1}, and held for 2 min, adapted from Sherman et al. [34]. High purity Helium (≥99.999 %) was the column carrier gas at a constant flow rate of 1 mL min^{-1}. The transfer line and ion source temperatures were both 280 °C. MS spectra were obtained by electron ionization (EI) at 70 eV. The mass range monitored was 35–400 *m/z*.

2.5. Data processing

MS data acquisition was performed with the Xcalibur Software (Thermo Fisher Scientific, Waltham, MA). We processed the targeted analysis data with the Quant browser software (Thermo Fisher Scientific, Waltham, MA) for deconvolution, peak-picking and integration. For the untargeted analysis data, the R package 'metaMS' (v1.28.0) was used for deconvolution, peak-picking and alignment using the default parameters [35].

VOC identification was performed by comparing the obtained mass spectra, and retention time indices (RI) with those in the NIST20 library or pure standards, when available (similarity >0.7 and RI error ±10).

We analyzed blank samples (2 mL ultrapure water) for any of the tested conditions and the final extraction method to remove background peaks. Peaks identified as impurities, such as siloxanes from the GC column were also removed. A median normalization was performed. Statistical univariate analyses (Student t-test and Analysis of variance, Anova), multivariate analysis (principal component analysis or PCA, and partial least square differential analysis or PLS-DA), and plots were performed with R statistics. The response surface of the design of experiments (DoE) was processed using the R package 'rsm' (v2.10.3).

3. Results and discussion

3.1. SPME arrow method development

To determine the optimal extraction conditions, various experiments were performed using the SPME Arrow technique in headspace mode. The experiments were carried out using pooled urine spiked with the 11 standards used in the method development and the IS at a concentration of 0.3 μg mL^{-1}. Supplementary Table S1 details the RIs and the chemical class of the selected standards [6]. The selection of various ketones is

intentional, as they are widely used in gas-chromatography coupled to ion mobility spectrometry (GC-IMS) for analytical method development [36,37] and for batch corrections [38], and therefore, the SPME arrow method developed here will not only be valuable for the GC-MS field but also for GC-IMS users. Initial extraction conditions were selected based on the consensus of the reported conditions for SPME untargeted urinary analysis in the bibliography [6]: 1.5 mL of the urine pool aliquoted in a glass vial with 0.25 % w/v of salt and an acidified pH of 2, incubated for 20 min at 60 °C and then exposed to the fiber for 40 min at 60 °C. Acording to the bibliography, the acidification of samples is favorable when using CAR/PDMS fiber [6].

Two approaches were used to find the optimal extraction conditions: univariate experimental design to select the type of coating, urine volume, and salt addition; and a design of experiment (DoE) following a central composite design (CCD) for temperature, extraction, and incubation time. Selection of the final extraction conditions was based on two parameters combining the best performance for untargeted and targeted analyses: the number of identified VOCs meeting our criteria (Spectral similarity >700, RI error ±10, s/n > 2) found in at least two out of three replicates, and the highest response area of the spiked target VOCs. All experiments were conducted in triplicate.

3.2. SPME arrow fiber coating selection

We compared the performance of five commercial SPME Arrow fibers: Carbon Wide Range/Polydimethylsiloxane 120 μm (CAR/PDMS), Divinylbenzene/Polydimethylsiloxane 120 μm (DVB/PDMS), Polydimethylsiloxane 100 μm (PDMS), Divinylbenzene/Carboxen/Polydimethylsiloxane 120 μm (DVB/CAR/PDMS), and Polyacrylate 100 μm (PA).

Untargeted analysis showed that the DVB/CAR/PDMS arrow was able to identify the highest number of urinary VOCs (41) (Fig. 1A). On the other hand, for the target analysis of the selected VOCs (Fig. S1) the absorption ability of each of the tested arrow fibers was different depending on the target VOC. In general, CAR/PDMS and DVB/CAR/PDMS coatings exhibited the highest extraction efficiency for most of VOCs, whilst the PA coating showed the lowest one.

These findings were consistent with previous studies that used conventional SPME fibers[39–41]. Specifically, it was observed that the CAR/PDMS coating demonstrated higher extraction efficiency for acidified urine samples. On the other hand, for alkaline conditions or non-modified urine samples, the DVB/CAR/PDMS coating exhibited the highest extraction performance.

Considering the stability of the compounds, the acidification of urine samples, and the favorable performance of CAR/PDMS coating in both untargeted and targeted analysis, we chose the CAR/PDMS thereafter as a compromise to ensure the efficient extraction of the different VOC families in urine.

3.3. Urine volume

The analysis of volatile compounds in urine samples is often limited due to their relatively low abundance. To enhance the extraction efficiency, the ratio between the sample volume and headspace is crucial. In this study, we investigated the impact of different sample volumes (1 mL, 1.5 mL, 2 mL, and 3 mL) on the extraction efficiency using the CAR/

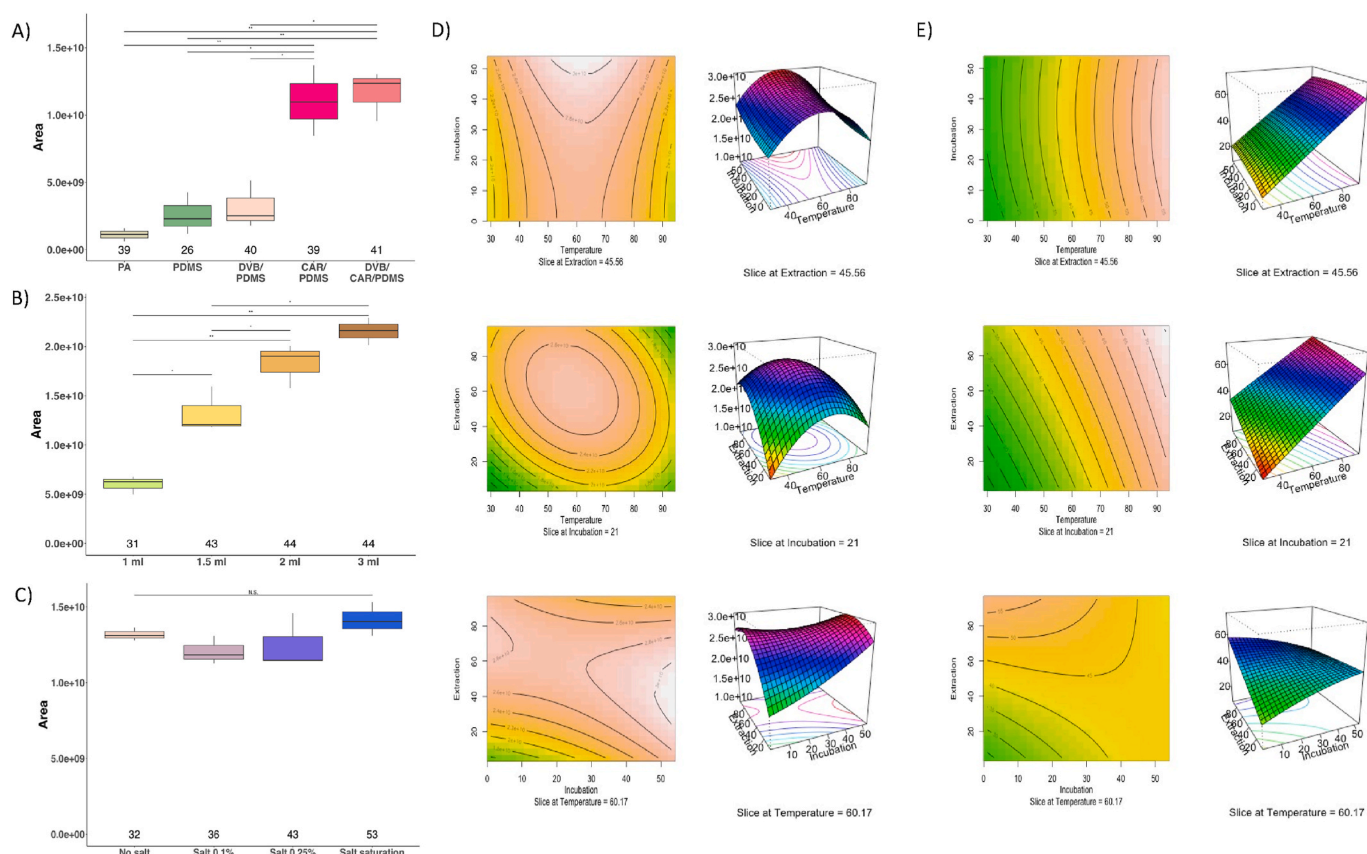


Fig. 1. Results of the SPME Arrow development using a pooled urine matrix spiked with 11 selected VOCs (0.3 μg mL^{-1}). A-C) Show the mean areas of the 11 compounds spiked and the mean total number of untargeted compounds identified, as measurements were performed in triplicates. A) Boxplot for the SPME Arrow fiber comparison and selection. B) Boxplot for the urine volume. C) Boxplots of the salting-out effect evaluation. D-E) Show the response curves for the two designs of experiments (DoE). D) Response curves for the targeted DoE using the 11 spiked compounds. E) Response curves for the untargeted DoE using the information of the total number of peaks identified. Both DoE's are based on a central composite design for temperature, incubation, and extraction time. * indicates anova p-value <0.05 ** indicates anova p-value <0.01.

PDMS SPME Arrow at the initial extraction conditions. Higher volumes of samples were discarded as the aim of the study is to use a large cohort of biobanked samples where the volume is a limitation.

Untargeted analysis revealed that larger sample volumes led to higher extraction efficiency, but not to an increase of the identified VOCs that reached a plateau of 44 identification when using 2 mL of urine (See Fig. 1B). Additionally, there were no significant differences (anova p-value >0.05) between the extraction efficiency of the target VOCs, when comparing 3 mL and 2 mL sample volumes (Fig. S2). Therefore, a sample volume of 2 mL was selected for further analysis.

3.4. Salt addition

The addition of salt is a common technique employed to enhance the migration of compounds to the headspace, thereby facilitating their extraction[42–44]. Given our aim to develop an untargeted analysis and considering that salts like $CaCl_2$ and $MgCl_2$ have less salting out efficiency [45,46], we opted for the widely used sodium chloride for untargeted volatilomics [6]. In this study, we evaluated four different salt conditions: no salt addition, salt added at 0.1 % w/v, 0.25 % w/v, and salt saturation (0.62 % w/v).

The number of peaks detected ranged from 32 when no salt was added to 53 with salt saturation (Fig. 1C). The increase in peak number can be attributed to the enhanced migration of neutral organic compounds to the gas phase due to the variation in equilibrium.

Target analysis also showed the salt addition increases the extraction efficiency of most of the target VOCs (Fig. S3), except for alcohols and indoles. As a compromise, we selected a salt addition of 0.25 % (w/v) for further experiments. This concentration provided a balance between maximizing compound extraction and minimizing potential competition effects, making it a suitable choice for subsequent analyses.

3.5. DOE

The selection of extraction temperature, incubation and extraction time were conducted simultaneously using a central composite design. The CCD ($\alpha = 1.68$) generated 18 experimental conditions, comprising 15 unique combinations and 3 replicates of the central point (Table S2). These conditions were analyzed in a randomized order. Values of temperature and time were selected in accordance with the conditions described in a recent review [6]. Temperature ranged between 50 and 70 °C, extraction time varied from 40 to 70 min and an incubation time ranged between 5 and 30 min. In this section, we assessed the outcomes of the 18 experiments based on the CCD design.

We evaluated the area of the target compounds and also the number of identified peaks and the sum of their peak areas for the untargeted analysis. A mathematical model for prediction was applied by fitting a polynomial equation to the obtained data. The surface response of the target analysis (Fig. 1D) reached a maximum in proximity to the central design conditions, where the temperature ranged between 55 and 65 °C. Specifically, a stationary point of the second order model was identified at 53 °C, 60 min extraction and 20 min of incubation. In contrast, the untargeted DoE did not exhibit a clear maximum point; instead, the response continuously increased specifically with temperature increase (Fig. 1E). Following manufacturer's recommendations about the optimal working condition, the conditions selected for further analysis were based on the targeted approach. Using the targeted stationary point, it was predicted that approximately 40 identified peaks could be obtained per sample. Furthermore, the untargeted DoE model indicated that the incubation step did impact the number of peaks obtained (Supplementary Fig. S4), whereas the number of peaks did not change when incubation time was applied. Therefore, the magnitude of effect was not significant. Consequently, the final conditions selected for the study are an extraction time of 60 min at a temperature of 53 °C with an SPME Arrow CAR/PDMS using 2 mL of urine with 0.25 % w/v of NaCl and a pH of 2.

3.6. Comparison with conventional SPME fibers

To evaluate if the developed SPME Arrow method improves the determination of untargeted urinary VOCs, we compared the performance of a conventional SPME CAR/PDMS (d = 0.6 mm). Using the same conditions as SPME Arrow, conventional SPME fiber was tested for 2 mL of acidified urine with 0.25 % w/v NaCl addition, extracted for 60 min at 53 °C.

The increased phase volume of SPME Arrow enabled the detection of 44 linear peaks, 10 of them not detected by conventional SPME (See Fig. 2 and Table S4). Conversely, only two compounds were detected with the conventional SPME and not in SPME arrow: Bicyclo[3.2.0] hepta-2,6-diene and 3,7-dimethyl-3-Octanol. Mean areas and standard deviation results are detailed in Supplementary Table S3, where the names of the compounds are disclosed. These results clearly indicate that SPME Arrow outperforms conventional SPME in terms of extraction efficiency. Even though, SPME Arrow fibers with a larger diameter (d = 1.5 mm) are available, the larger diameter will increase the extraction volume but also extends the extraction time. The increased sorbent phase of SPME Arrow exhibits enhanced sensitivity and superior capability in extracting a greater number of VOCs [47],a desired characteristic for untargeted analysis. While the larger sorbent volume of the SPME Arrow contributes to improved extraction efficiency, with an increased number of compounds extracted, the greater exposed surface area of conventional SPME fiber results in a higher absolute area of individual compounds (Fig. S5). However, since the primary aim of this study is to maximize the number of compounds detected, the SPME Arrow was selected due to its superior ability to extract a broader range of compounds.

3.7. Method quality parameters

We evaluated the SPME Arrow method repeatability (intra-day) and reproducibility (inter-day) of the identified linear peaks. Repeatability

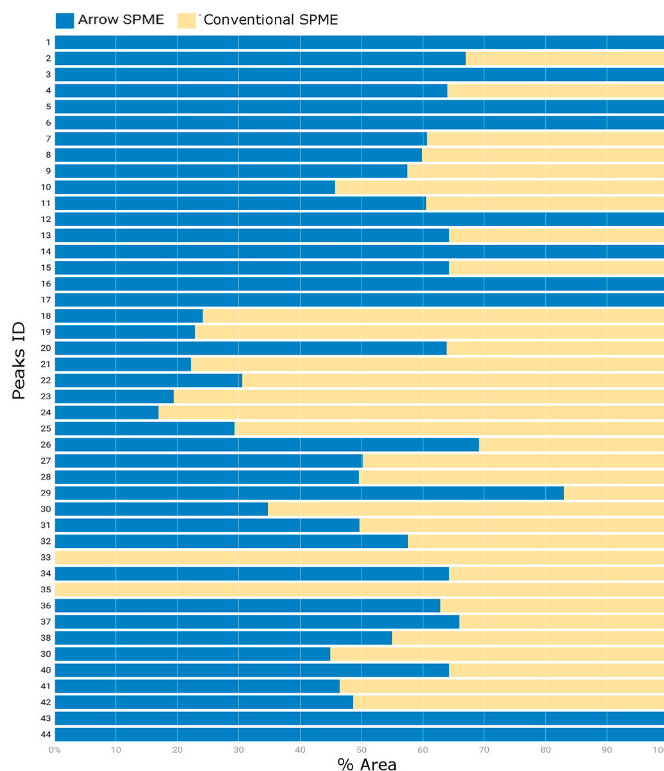


Fig. 2. Stacked bar plot showing peak areas percentage of the 44 compounds identified comparing the performance of SPME Arrow vs. conventional SPME.

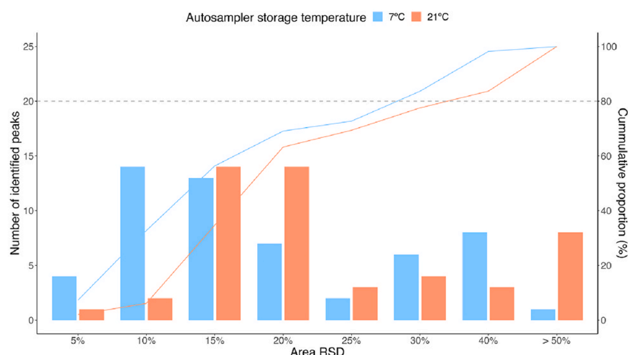
revealed 38 identified peaks with a calculated area RSD <20 %. Similarly, reproducibility yielded 37 identified peaks with an area RSD <20 % (Fig. 3). These results validated the predicted number of peaks obtained by the untargeted DOE model. These results were conducted by keeping the urine samples at 7 °C in a refrigerated vial tray (the minimum possible for our instrument configuration) prior to their extraction. To facilitate comparison with previous studies that did not refrigerate the urine samples prior to the extraction, we repeated the analysis maintaining the urine samples at room temperature (approx. 21 °C), observing a decreased number of peaks with an area RSD <20 % (31 peaks for repeatability, and 16 for reproducibility). Also, the repeatability of the internal standard doubled when the samples were stored at room temperature (8 % RSD at 7 °C compared to 20 % RSD at 21 °C). A similar trend was observed for reproducibility of the IS, where the area RSD increased to 41 % at room temperature but decreased to 22 % at 7 °C.

These findings indicate that storing urine samples in an autosampler at 7 °C leads to a substantial enhancement in reproducibility and repeatability compared to storing them at room temperature. In untargeted analysis, the simultaneous extraction of diverse compounds presents challenges in maintaining data consistency across measurements. By adopting the practice of samples storage in a refrigerating tray prior to extraction, we have observed a reduction in the variability associated with the methodology, thereby contributing to the attainment of more reproducible results. It is worth noting that this practice has not been commonly reported in the literature for volatile analysis.

3.8. Untargeted analysis of children's urines

The developed SPME Arrow method was applied to analyze a subsample of children's urine differentially exposed to tobacco smoke. Following the developed methodology, we identified up to 36 peaks

A) Repeatability



B) Reproducibility

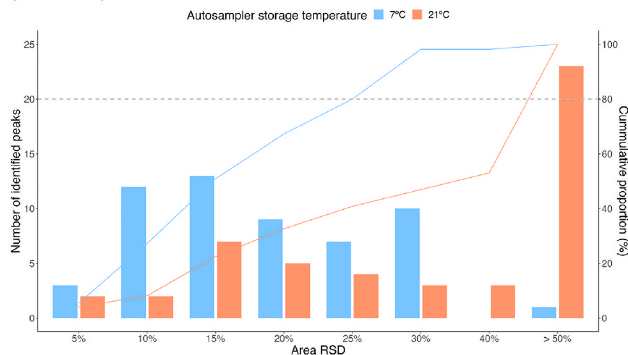


Fig. 3. Bar plots showing the number of peaks identified for each relative standard deviation (RSD), the lines show the cumulative proportion of the identified peaks. A) Repeatability for 5 samples analyzed on the same day; B) Reproducibility for 5 samples analyzed on 5 different days.

(Table S4). We selected those compounds that were present in at least 50 % of samples across one group for evaluating their statistical biological relevance, resulting in a final set of 31 VOCs. The exploratory principal component analysis of the volatilomics data (see Supplementary Fig. S6), showed that the QC samples were aggregated into a tight cluster near the origin of the principal component analysis (PCA) plot, indicating minimal residual instrumental errors.

The univariant statistical analysis with a Student t-test returned two compounds that exhibited significant differences between the urinary volatilome of the children exposed and non-exposed to TSE ($p < 0.05$ and a fold-change FC > 2). The significant compounds were *p*-cymene and *p*-isopropenyl toluene, which increased in the exposed urines. The multivariate statistical analysis with a Partial Least Squares Discriminant Analysis (PLS-DA) (Fig. 4A) returned four compounds with a VIP score >2: *p*-cymene, *p*-isopropenyl toluene, 2-pentanone and 2-methoxy-5-methylphenol, acetate. There are two compounds consistent between the statistical analysis, *p*-isopropenyl toluene and *p*-cymene (Fig. 4B). These are components of tobacco smoke, which were reported from SPME–HS–GC-MS analysis [48]. Other compounds, such as p-cresol and beta-damascenone, showed a large change (FC > 30) but a higher p-value <0.1. This can be attributed to the small sample size used in this exploratory study, but they should be considered as possible targets in future research.

Interestingly, all significant compounds have been previously reported in urine volatilomics samples [6]. However, biomarkers of tobacco exposure, such as nicotine or its metabolite cotinine, were not detected in these samples, which may be attributed to differences in experimental conditions, probably due to the use of a different SPME coating [48,49].

These findings highlight the presence of specific volatile compounds associated with tobacco exposure in urine samples, suggesting their potential as biomarkers for assessing tobacco-related exposure. The use of SPME Arrow–HS–GC-HRMS analysis provides valuable insights into the volatile profile of urine samples, enabling the identification and characterization of compounds related to tobacco usage.

Although other tobacco smoke constituents have been detected in our samples, like 4-heptanone [50] and alpha-terpineol [49], they didn't show a meaningful statistical significance.

3.9. Limitations

The methodology described in this article was tailored to a particular usage, the analysis of large cohort samples. In that sense, we only compared the methodology that is suitable for this purpose. Moreover, some microextraction techniques, such as thin film extraction require dedicated equipment, like thermal desorption units which are beyond our reach [16].

Urine is a high complex matrix, making procedure optimization challenging. One issue is the matrix effect observed with this technique. Except for indole, all the compounds spiked in the sample showed a matrix effect smaller than 20 % (See Supplementary Table S5), indicating a substantial reduction in compound areas. This effect is amplified by the competitive nature of SPME, where a higher number of compounds in sample leads to decreased extraction efficiency. We can also observe similar values of matrix effect for other samples, like tissue using the SPME technique [51].

In this study, we focused on comparing SPME Arrow to conventional SPME fibers due to the resources and technology available in our laboratory. While SPME Arrow demonstrated significant advantages in terms of extraction efficiency, sensitivity, and mechanical reliability, we understand that a comprehensive comparison with other SPME formats and extraction techniques would provide a more holistic view of its performance.

Specifically, SPME formats such as thin film, pins, and blades offer different geometries and sorption capacities that may influence extraction efficiency and sensitivity. Additionally, techniques like needle trap

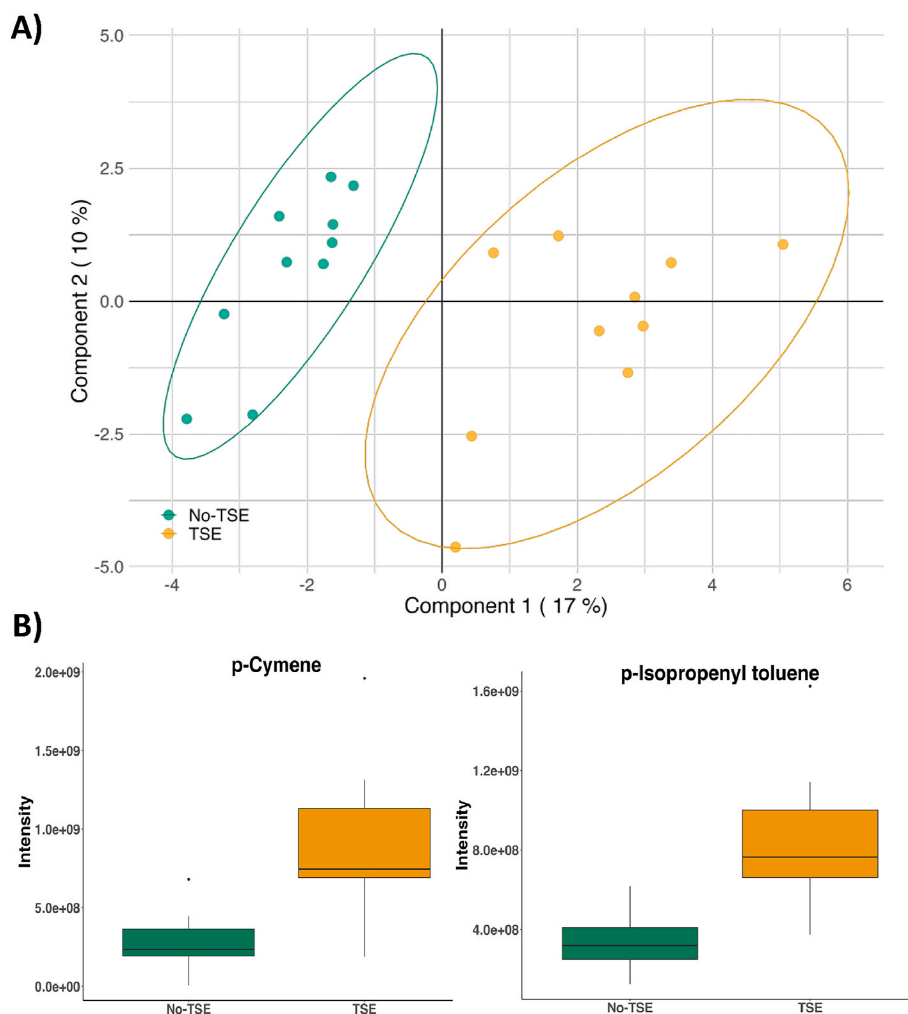


Fig. 4. Enhanced extraction methodology for untargeted volatilome, applied to real samples. A) Partial Least Squares Discriminant Analysis (PLS-DA) for all samples after median normalization. B) Boxplots of the compounds showing significance between children exposed to tobacco (TSE) and non-exposed (No-TSE) children.

and sorbent tubes used for thermal desorption have their own advantages, such as higher sample volumes and potentially greater analyte recovery. However, these techniques often require more complex instrumentation and additional steps, such as thermal desorption units, which were not available in our laboratory.

In the literature comparison of SPME Arrow and conventional SPME fibers, it was observed that the conventional SPME required less sample volume to achieve fiber saturation and shorter desorption times. Despite this, higher peak areas and a greater number of peaks were obtained using SPME Arrow for milk samples, indicating superior sensitivity for volatile organic compounds (VOCs) analysis with SPME Arrow [42]. For spiked drinking water samples, SPME Arrow showed a fourfold increase in response compared to conventional fibers under similar analytical conditions, highlighting its higher efficiency [22]. Both methods detected the same number of VOCs when following the same protocol; however, conventional fibers demonstrated higher sensitivity for low molecular weight compounds, as previously noted by Herrington et al. [22]. In contrast, SPME Arrow exhibited enhanced sensitivity for higher molecular weight compounds [27]. We did not observe these trends in our study, which may be due to differences in experimental conditions and matrices used.

Future studies should include a broader range of techniques to fully elucidate the comparative benefits and limitations of SPME Arrow. Such comparisons would enhance our understanding of the optimal conditions and applications for each technique, providing valuable insights for researchers and practitioners in the field.

4. Conclusions

Volatilomics in urine has been studied for years due to its potential in diagnosing and monitoring various diseases and environmental exposures. However, this study is the first to develop a novel SPME Arrow-based method combined with GC-HRMS to enhance the analysis of VOCs in human urine. Addressing both targeted and untargeted urinary volatilomics challenges through a DOE approach, the SPME Arrow demonstrated superior performance compared to conventional SPME fibers, with improved extraction efficiency and detection of more VOCs. Optimal conditions were determined to be 60 min at 53 °C using 2 mL of urine with 0.25 % w/v NaCl and a pH of 2. By integrating targeted and untargeted approaches, our method ensured precise identification of known compounds and the discovery of novel biomarkers, providing a more complete metabolic profile for non-invasive diagnostics and environmental health studies. The method successfully identified VOCs associated with tobacco exposure in children's urine, such as *p*-cymene and *p*-isopropenyl toluene, highlighting its potential for assessing environmental exposures and related health effects. Exhibiting high repeatability and reproducibility when urine samples were stored at 7 °C prior to extraction, the method significantly enhanced result consistency. Overall, the SPME Arrow-based method represents a significant advancement in urinary volatilomics, offering a robust approach for detecting and analyzing VOCs in human urine, with promising applications in biomarker discovery and environmental health research.

CRediT authorship contribution statement

Maria Llambrich: Writing – original draft, Methodology, Formal analysis. **Noelia Ramírez:** Writing – review & editing, Conceptualization. **Raquel Cumeras:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jesús Brezmes:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

Declaration of competing interest no competing interest to be disclosed.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2024.343261.

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