Title

Analytical methodologies for broad metabolite coverage of exhaled breath condensate

Authors

Aksenov, Alexander A

Zamuruyev, Konstantin O

Pasamontes, Alberto

et al.

Publication Date

2017-09-01

DOI

10.1016/j.jchromb.2017.06.038

Peer reviewed

HHS Public Access

Author manuscript

J Chromatogr B Analyt Technol Biomed Life Sci. Author manuscript; available in PMC 2018 September 01.

Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2017 September 01; 1061-1062: 17–25. doi:10.1016/j.jchromb.2017.06.038.

Analytical methodologies for broad metabolite coverage of exhaled breath condensate

Alexander A. Aksenov[a], **Konstantin O. Zamuruyev**[a], **Alberto Pasamontes**[a], **Joshua F. Brown**[a], **Michael Schivo**[b,c], **Soraya Foutouhi**[d], **Bart C. Weimer**[d], **Nicholas J. Kenyon**[b,c], and **Cristina E. Davis**[a,*]

[a]Mechanical and Aerospace Engineering, University of California, Davis, One Shields Avenue, Davis, California 95616, U.S.A

[b]Department of Internal Medicine, 4150 V Street, Suite 3400, University of California, Davis, Sacramento, CA 95817, U.S.A

[c]Center for Comparative Respiratory Biology and Medicine, University of California, Davis, Davis, CA 95616, U.S.A

[d]School of Veterinary Medicine, 1089 Veterinary Medicine Drive, University of California, Davis, Davis, California 95616, U.S.A

Abstract

Breath analysis has been gaining popularity as a non-invasive technique that is amenable to a broad range of medical uses. One of the persistent problems hampering the wide application of the breath analysis method is measurement variability of metabolite abundances stemming from differences in both sampling and analysis methodologies used in various studies. Mass spectrometry has been a method of choice for comprehensive metabolomic analysis. For the first time in the present study, we juxtapose the most commonly employed mass spectrometry-based analysis methodologies and directly compare the resultant coverages of detected compounds in exhaled breath condensate in order to guide methodology choices for exhaled breath condensate analysis studies.

Four methods were explored to broaden the range of measured compounds across both the volatile and non-volatile domain. Liquid phase sampling with polyacrylate Solid-Phase MicroExtraction fiber, liquid phase extraction with a polydimethylsiloxane patch, and headspace sampling using Carboxen/Polydimethylsiloxane Solid-Phase MicroExtraction (SPME) followed by gas chromatography mass spectrometry were tested for the analysis of volatile fraction. Hydrophilic interaction liquid chromatography and reversed-phase chromatography high performance liquid chromatography mass spectrometry were used for analysis of non-volatile fraction. We found that liquid phase breath condensate extraction was notably superior compared to headspace extraction and differences in employed sorbents manifested altered metabolite coverages. The most

*Correspondence: cedavis@ucdavis.edu.

pronounced effect was substantially enhanced metabolite capture for larger, higher-boiling compounds using polyacrylate SPME liquid phase sampling. The analysis of the non-volatile fraction of breath condensate by hydrophilic and reverse phase high performance liquid chromatography mass spectrometry indicated orthogonal metabolite coverage by these chromatography modes.

We found that the metabolite coverage could be enhanced significantly with the use of organic solvent as a device rinse after breath sampling to collect the non-aqueous fraction as opposed to neat breath condensate sample. Here, we show the detected ranges of compounds in each case and provide a practical guide for methodology selection for optimal detection of specific compounds.

Keywords

Exhaled breath condensate (EBC); metabolites; gas chromatography mass spectrometry (GC/MS); high performance liquid chromatography mass spectrometry (HPLC/MS); hydrophilic interaction liquid chromatography (HILIC); reversed-phase liquid chromatography (RP)

1. Introduction

Breath analysis has been garnering attention as a diagnostic methodology with the potential for broad-scale application [1]. Inherent non-invasive nature of breath sampling and ease of collection make exhaled breath a very attractive matrix [2–5]. In some cases, breath metabolites abundances can be used as a proxy for concentration of certain compounds in blood. The alveoli in the lungs contain a large number of capillary blood vessels in close proximity to the lung surface to facilitate oxygen uptake and carbon dioxide release. This creates an optimal condition for the release of various compounds including volatile organic compounds (VOCs) dissolved from the blood into the exhaled breath. Many of these chemicals may be biomarkers indicative of a specific disease. Indeed, several disorders such as influenza [6, 7], diabetes [8–10], gastrointestinal diseases [11], and pneumonia [12] were reported to be potentially amenable to diagnosis or monitoring based on breath biomarkers. In addition, there was a major effort in identifying breath biomarkers of cancer [3, 13–17]. However, further advances are likely necessary to achieve the full practical utility of that breath analysis methodology.

One perceived disadvantage of using breath for diagnostic purposes is that it contains significantly fewer compounds at trace concentrations [18–20] compared to other matrices such as blood plasma. Another critical impediment that has affected widespread adoption is the lack of standardization of breath collection and analysis. It is challenging to ensure reproducibility and comparability of breath collection and storage methodologies such as to minimize any variations in abundances of compounds of interest. In this regard, exhaled breath condensate (EBC) appears to be more advantageous as opposed to gaseous breath [21]. Although methodologies for gaseous sample storage exist, e.g. Tedlar® (polyvinyl fluoride) bags, it is challenging to ensure sample preservation [22, 23]. Liquid EBC samples have an advantage that they can be stored for extended periods of time under cryogenic conditions without apparent loss of metabolite content [21, 24].

For EBC sample analysis, mass spectrometry (MS) is the method of choice for comprehensive metabolic analysis, most often used in conjunction with gas and liquid chromatography (GC and LC, respectively). In addition to offering a tremendous volume of chemical information, MS offers incredible sensitivity down to attomolar levels [25], and selectivity appropriate for chemical species identification. Depending on circumstances, it is possible to elucidate a metabolomic profile of a sample for “untargeted” metabolomics approaches to seek new biomarkers that associate with a specific condition. It is also possible to specifically measure amounts of selected compounds for “targeted” metabolomics approaches where a biomarker is known *a priori*. Since MS is predominantly a laboratory-based methodology due to high cost and size of MS instrumentation, sample collection occurs prior to analysis at a separate location such as hospitals or other medical facilities in the majority of studies.

The steps of sampling, sample storage, and preparation are critical to ensure that a sample is compatible with the analysis mode of choice (GC/MS or LC/MS). The variations in sampling may introduce biases that obscure biomarker features of interest. For example, selecting an appropriate solid phase extraction (SPE) sorbent can allow for discrimination against abundant matrix compounds while enhancing sensitivity toward compounds of interest. Sorbent choices and elution conditions can induce tremendous effects on metabolite abundances in the breath sample [26]. In another example, derivatization of molecular species that can form hydrogen bonds (such as carbonic acids) is commonly used to promote volatility of these compounds to enhance their detection using GC/MS, but differences in reagent choice and reaction conditions may lead to large differences in resulting metabolite coverages.

EBC is a very low concentration aqueous solution of various organic and inorganic compounds that arise from both biogenic and extraneous sources [27]. Due to the trace nature of EBC biomarkers, we seek appropriate methodologies suitable for analysis of these types of samples. The volatile and semi-volatile fractions of EBC samples are most commonly analyzed using GC/MS in conjunction with Solid-Phase MicroExtraction (SPME) [28] or Stir Bar Sorptive Extraction (SBSE/Twister®) [29]. For the non-volatile fraction, hydrophilic interaction liquid chromatography (HILIC) and reversed-phase (RP) liquid chromatography (LC/MS) are used to analyze very polar and moderately polar/non-polar compounds, respectively [30].

The intent of the present study is to compare these commonly employed analytical methodologies directly for the first time, in order to compare and contrast the detected compounds distributions and outline a set of recommendations to guide the method selection for EBC analysis. In this study, we conducted GC/MS analysis of the volatile and semi-volatile fractions using SPME from liquid and headspace as well as extraction from liquid using polydimethylsiloxane (PDMS) sorbent as a low-cost alternative to SBSE/Twister® methodology. The non-volatile fraction of EBC was analyzed with HILIC and RP HPLC/MS chromatography modes. The residual non-aqueous fraction of EBC was collected from the condenser surface with an organic solvent and was analyzed with HILIC and RP HPLC/MS chromatography modes. To eliminate sample bias at the sampling step, an “averaged” EBC sample aliquoted from individual EBC samples collected from a group of

six volunteers, spanning both genders and a range of ages, was used to represent a “typical” breath sample. The individual EBC samples were collected with a collection device that demonstrated metabolite capture [31]. A systematic metabolite survey is presented in this paper.

2. Materials and Methods

2.1. EBC Collection Device

The engineering design and operational principle of the EBC collection device employed for the EBC collection was recently described elsewhere [31]. The device is portable, suitable for a wide-spread use similar to the RTube®, a common commercially available device for EBC collection: the exhaled breath is passed through a chilled tube where it is condensed and then physically removed. Unlike the RTube®, the employed device uses a glass condenser surface to enhance measurement of metabolites in exhaled breath condensate [32], a flow-controlled saliva filter to reduce sample dilution and contamination with saliva microdroplets originating in the mouth cavity [33–35], and an active thermal mass cooling mechanism to maintain low condenser temperature to avoid physical change of the sample and preserve greater concentrations of VOCs [36]. These design factors contribute to reduced variability in the metabolomic content caused by the collection device [31].

2.2. EBC Sample Collection

The device is comprised of a glass tube placed inside a plastic casing. The space between the glass tube and the outer casing is filled with dry ice pellets that serve as an active thermal mass to keep the condenser surface at low temperature (~ −30 °C). The ice-frozen exhalant is cleared from the condenser tube with the fitted plunger into a glass vial for storage. The physical phase of the sample is preserved in collection and sample transfer steps. The samples are stored in glass cap-sealed vials at −80 °C until further analysis. For a healthy subject of ~80 kg, the described device can typically collect 1.2 ± 0.3 gram of ice-frozen EBC in 10 min [31].

A representative average EBC sample was aliquoted from individual samples collected from a group of six healthy adult volunteers representing three age groups of 20, 30, and 40 years old and two gender groups (3 males and 3 females). All participants were in good health, with no history of smoking. Volunteers restrained from consuming food for 2 h before sampling, and rinsed their mouths with drinking water before sampling. The clean collection device was assembled, charged with dry ice pellets, and allowed to sit for 5 min before sampling for the temperature to equilibrate within the device. Each participant sat in a relaxed, upright position during the collection. All participants were asked to breathe normally (tidal breathing) into the device for a 10 min period. After sampling, the EBC sample was transferred from the condenser tube into a clean borosilicate vial (Sigma-Aldrich, part #: SU860099 SUPELCO), cap sealed (Sigma-Aldrich, part #: SU860101 SUPELCO), and placed into a −80 °C freezer. This process was repeated until ~4 mL of EBC was collected from each participant. All parts of EBC collection device and sample retrieval press were thoroughly cleaned before and after each use. The cleaning protocol

included three rinses: deionized (DI) water rinse, followed by 70% ethanol disinfectant rinse, followed by DI water rinse and drying.

A total of 24 EBC samples (~ 4 mL from each volunteer) were collected randomly during a 6 weeks period. Each volunteer performed 10 min sampling four times; the four EBC samples from the same volunteer were stored in one 10 mL borosilicate vial. The mass of the collected EBC sample was evaluated by measuring mass of the empty vial before sampling and mass of the vial with the sample after collection. After each EBC sampling, the inside surface of the condenser tube was rinsed with 1 mL of HPLC grade ethanol. This organic solvent was used to rinse the non-aqueous EBC fraction residue from the collection tube and stored in a sealed vial for further analysis.

After all individual EBC samples were collected; 3 mL aliquots from each participant were mixed to create an “averaged” sample which represented the combined breath for further analysis. The “averaged” sample was then aliquoted into separate vials: 1 mL for each GC/MS analysis replicate, 0.5 mL for each LC/MS analysis replicate. The vials were capped with 35 (Shore A) PTFE/silicone septum and stored at −80 °C until further analyses, as described below. The sample distribution for analysis is graphically represented on the diagram shown in Figure 1. The UC Davis Institutional Review Board approved all study activities.

2.3. GC/MS Analysis of EBC Volatile Fraction

The GC/MS analysis was conducted with three appropriate sample extraction methodologies that are routinely employed for the analysis of diluted aqueous samples: liquid phase sample extraction with a polyacrylate (PA) d_f 85 μm SPME fiber; liquid phase extraction with a PDMS patch; and headspace sampling using a Carboxen/Polydimethylsiloxane (CAR/PDMS) d_f 75 μm SPME fiber. Polar and semi-volatile compounds (MW 80–300) were targeted with PA SPME [37]. Non-polar compounds of varying molecular weight, both volatile and non-volatile were targeted with PDMS patch extraction [38]. Gases and low molecular weight compounds (MW 30–225) were targeted with CAR/PDMS SPME [37]. Three technical replicate samples were analyzed for each extraction mode.

A Varian 3800 GC (VF-5ms 5% phenol/95% PDMS column, Varian, Walnut Creek, CA) and a 4000 Ion Trap MS (Varian) equipped with Electron Ionization source (EI) was employed for all analyses. The instrument performance was verified before each analysis by injecting a standard Grob DA 280 Column Test Mix (Restek, Bellefonte, PA). GC/MS analysis for all samples was carried out as a single batch, and the samples were randomized during analysis in order to ensure minimal sample-to-sample variation and avoid potential bias. The extraction protocols were optimized individually for each analysis mode.

For the PA SPME analysis, the 3 technical replicates of the EBC samples in borosilicate vials were transferred from the −80 °C freezer onto a 3 °C chilled tray of the GC/MS instrument and allowed to thaw. The PA SPME (Supelco, Bellefonte, PA) tip was automatically inserted by the sampling robot into the liquid EBC, and the sample was agitated at room temperature for 30 min. Upon sample extraction, the SPME was inserted into the GC inlet maintained at 250 °C and the extracted compounds were desorbed for 1

min. The GC protocol for PA SPME analysis included: cryofocusing on the head of the column at −10 °C for 1 min; 50 °C/min oven ramp to 40 °C, 20 °C/min oven ramp to 100 °C, 5 °C/min oven ramp to 180 °C, 10 °C/min oven ramp to 250 °C, 20 °C/min oven ramp to 280 °C, and a 5 min hold period to purge the column for a complete run time of 34.5 min. The helium carrier gas was set to constant 1 mL/min flow. The scanned *m/z* range was 35–250 for all three analysis modes. The DI water blanks (Evoqua, Denver, CO, USA) and empty vial blanks were interspersed with the samples. Quality controls of aqueous D8 naphthalene solution were run along with samples throughout the analysis. In order to eliminate potential systematic bias, the samples were randomized.

For the PDMS patch analysis, 3 technical replicates of the vials with EBC were removed from the −80 °C freezer and allowed to thaw at room temperature. The 1 cm^2, 0.6 mm thick PDMS patches (Goodfellows, Coraopolis, PA) were laser-cut out of a large sheet of material. Before use, the patches were cleaned in HPLC-grade acetonitrile for >72 h, baked in a vacuum oven at 160 °C for 24 h to remove any background contaminants and stored sealed in clean degassed borosilicate vials until use. The clean patches were placed into the thawed EBC by floating each patch on the surface of the liquid. The sample vials were capped and the extraction was carried out for 60 min with periodic vigorous agitation. After sampling, the patches were transferred into clean degassed borosilicate vials and capped with stainless steel screw caps with a 35 (Shore A) PTFE/silicone septum. The vials with PDMS patches were placed on the GC/MS instrument tray. For analysis, each vial was transferred by the auto sampling robot arm from the tray into the heater/agitator station set at 200 °C and heated for 15 min. The desorbed volatiles in the vial headspace were sampled using an automatic headspace injection syringe heated to 80 °C. The syringe needle was inserted through the cap septum and 500 μL of headspace was injected into the GC inlet. The syringe was then purged with helium carrier gas for 30 sec to minimize carryover. The GC protocol for this mode of sample extraction was the same as for the PA SPME analysis. Empty vial blanks, clean baked PDMS blanks and PDMS patches exposed to DI water sampling blanks were randomly interspersed with the samples.

For the analysis with CAR/PDMS SPME, 3 technical replicates of the EBC samples were removed from the −80 °C freezer, thawed at room temperature, and 1 mL of saturated NaCl solution was added into each vial to reduce solubility of dissolved VOCs. The vials were then placed on the GC/MS instrument tray. For analysis, each sample vial was heated to 90 °C and VOCs were sampled from the headspace using a Carboxen/Polydimethylsiloxane SPME fiber for 30 min. The sampled compounds were then desorbed off the fiber in the injector port at 250 °C for 1 min. The GC protocol for this mode of sample extraction was the same as for the PA SPME analysis described above. Empty vial blanks, DI water blanks, and blanks of saturated NaCl solution were randomly interspersed with the samples. Quality controls of aqueous D8 naphthalene solution were also run to verify instrument performance throughout the analysis.

2.4. GC/MS Data Analysis and Interpretation

Collected GC/MS data for all three extraction methods were analyzed offline as described elsewhere [39]. Briefly, the data were visualized using the MS Data Review software v. 6.6

(Varian). Deconvolution was carried out using Automated Mass Spectral Deconvolution and Identification System (AMDIS) software v. 2.64 with medium resolution and high sensitivity and shape requirements. The peaks were aligned using Mass Profiler Professional 13.1 software. Any peaks that appeared in any of the appropriate blanks (blank vials, DI water, clean SPMEs, quality controls) and its ratio (Peak intensity in sample/peak intensity in blanks) is lower than 10 were removed from consideration. Any compounds that are known to be external contaminants such as siloxanes (column, septa bleed) or phthalates (contaminants from plastic) were also disregarded. The peaks which were detected in at least 2 out of 3 technical replicates and when the peak abundance was above 1000 counts were considered.

EI fragmentation matching for chemical identification was performed using MS Data Review v. 6.6 software with the NIST 05 and Wiley 09 databases. The matches with forward and reverse scores of 800 and above were presumed to be correct; those in 300–800 range were considered “tentative”.

2.5. LC/MS Analysis of the EBC Non-volatile Compounds

For LC/MS analysis, 0.5 mL of EBC was lyophilized directly in the vial and then redissolved in 60 μL HPLC grade acetonitrile with sonication. A 20 μL of the re-suspended sample was injected for analysis. Chromatography was performed on an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA). Three replicate samples were analyzed using HILIC and three using RP chromatography. In between extraction and analysis, the samples were stored at −20 °C in capped LC vials with spring inserts (Agilent, Santa Clara, CA). During analysis the samples were housed in an autosampler maintained at 4 °C.

For HILIC analysis, the material was separated on Waters ACQUITY UPLC BEH Amide 130 Å, 1.7 μm, 2.1 mm × 100 mm column (Waters, Milford, MA), held at 40 °C during analysis. Mobile phase A consisted of water. Mobile phase B was 90% acetonitrile in water. Ammonium acetate and acetic acid were added to both A and B to obtain pH 5. Starting mobile phase composition was 100% B, over 20 min the mobile phase B was decreased to 90% and replaced by mobile phase A. The flow rate was held at 0.35 mL/min over this time. Mobile phase B was then reduced to 5% over the next 5 min with the flow rate reduced to 0.2 mL/min. LC eluents were analyzed with an Agilent 6230 accurate mass TOF LC/MS (Agilent Technologies, Santa Clara, CA) in positive ionization mode. The mass range was set to 50–1700 (*m/z*); scan rate 4 spectra/sec; the instrument was operated in high resolution acquisition mode. Fragmentor voltage was 120 V, sheath gas flow was 12 L/min, and sheath gas temp was 325 °C. An internal standard of $TFANH_4$ and purine was infused along with the sample. The blanks of milliQ water used for sample preparation, the acetonitrile solvent and mobile phase B were run along with the samples. Quality controls (QCs) were also run with the samples to verify instrument performance: the Waters 186006963 HILIC QC (Waters, Milford, MA) was run before and after each batch. A custom-made QC was comprised of: carnitine, lysine, adenylputricine, aminocapricoic acid, ornithine, tigonelline, alaninol, acetylcarnitine, 1-(2 pyramidyl) piperazine, methoxychalcone, cholecalciferol, 13-docosenamide and oleamide, all at 5 μM aqueous solution, was run after every 6 samples.

The RP analysis was conducted on an Agilent 1260 HPLC system with the Waters Acquity CSH C18 1.7 μm, UHPLC (2.1 × 100 mm) (Milford, MA USA) column. The samples were separated on the column held at 30 °C during analysis. Mobile phase A consisted of 60% acetonitrile in water. Mobile phase B was 10% acetonitrile in isopropanol. Formic acid and ammonium formate were added to make the final concentration of each mobile phase 10 mM for both formic acid and ammonium formate. Mobile phase composition at time (min) 0 was 5% B, at time 20 was 95% B. The flow rate was 0.3 mL/min. Samples were analyzed by MS using an Agilent 6230 accurate mass TOF LC/MS in positive ionization mode. The mass range was 50–1700 (*m/z*), the scan rate was 4 spectra/sec; the instrument was operated in high resolution acquisition mode. Fragmentor voltage was set at 120 V, sheath gas flow was 11 L/min, and sheath gas temperature was 325 °C. The standard of $TFANH_4$ and purine was infused along with the sample. The Waters 6963 RP QC (Waters, Milford, MA) standard was infused along with the sample. The blanks of milliQ water used for sample preparation, the acetonitrile solvent and mobile phase A were run along with the samples in randomized order.

2.6. LC/MS Data Analysis and Interpretation

The LC/MS raw data files were first processed with the “Find By Molecular” feature in Agilent’s Mass Hunter Qualitative Analysis B.05.00SP1 software in order to deconvolve each peak with the “Match factor” parameters set at 50. The deconvolved chromatograms were then exported to .cef data format, and the peaks were aligned using Mass Profiler Professional 13.1 software. The alignment window was set at 1 min with the match factor of 0.3. The minimum quality score was set at 0.25. A peak was considered as a metabolite if it satisfied three criteria: the peak was detected in at least 2 out of 3 technical replicates, the peak abundance was above 1000 counts and, in case the peak appeared in both sample and blank, the ratio of peak abundance in sample versus blank was greater than 10. The obtained global peak tables for the HILIC and RP data (Tables S4–S6, Supplemental Material) were obtained applying the previous filters.

2.7. Identification of Non-volatile EBC Metabolite Chemical Structures (LC/MS/MS Analysis)

In order to tentatively establish chemical identities of analytes, an additional analysis of 3 technical replicates was carried out using tandem mass spectrometry, both for HILIC and RP chromatography modes. The samples were lyophilized directly in the vial and then re-dissolved as described above, and 3 μL of re-suspended sample was injected for analysis. The CUDA (12– [[(cyclohexylamino)carbonyl]amino]-dodecanoic acid) in methanol:toluene, 9:1 v/v internal standard was used for quality control. Samples were housed in an autosampler maintained at 4 °C; the chromatography was performed using Waters ACQUITY UPLC BEH Amide 130 Å, 1.7 μm, 2.1 mm × 100 mm column (Waters, Milford, MA), held at 40 °C during analysis. Mobile phase A consisted of ultrapure water with 10 mM ammonium formate + 0.125% formic acid, pH 3. Mobile phase B was 95:5 v/v acetonitrile:ultrapure water with 10 mM ammonium formate + 0.125% formic acid, pH 3. The solvent gradient table was as follows: 0 min 100% (B), 0–2 min 100% (B), 2–7 min 70% (B), 7.7–9 min 40% (B), 9.5–10.25 min 30% (B), 10.25–12.75 min 100% (B), 16.75 min 100% (B). The flow rate was held at 0.4 mL/min over this time. LC eluent was analyzed with an Agilent 6530 QTOF MS (Agilent Technologies, Santa Clara, CA) in positive and

negative ionization modes. The mass range was set to 60–1200 (*m/z*). The ESI capillary voltage was set at +4.5 kV for ESI (+). The fragmentation was carried out with collision energy of +45 eV for ESI (+). Untargeted analysis of molecular data was carried out using data-independent acquisition (DIA) as described previously [40]. Device blanks of a milliQ water rinse of clean device inner surface that comes into contact with breath were included along with the EBC samples in a randomized order.

For the reverse phase analysis, the Agilent 1260 high-performance liquid chromatography (HPLC) system with the reverse phase UHPLC Waters Acquity CSH C18 1.7 μm, (2.1 × 100 mm) (Milford, MA USA) column was used. Each sample was dried, and then re-suspended in 100 μL 9:1 methanol:toluene. 3 μL of re-suspended sample was injected for analysis. The CUDA (12 – [[(cyclohexylamino)carbonyl]amino]-dodecanoic acid) in methanol:toluene, 9:1 v/v internal standard was used for quality control and to assess reproducibility. The samples were separated on the column held at 65 °C during analysis. Mobile phase A consisted of 60% acetonitrile in water. Mobile phase B was 10% acetonitrile in isopropanol. Formic acid and ammonium formate were added to make the final concentration of each mobile phase 10 mM for both formic acid and ammonium formate. The flow rate was 0.6 mL/min. The solvent gradient table was set as follows: 0 min 15% (B), 0–2 min 30% (B), 2–2.5 min 48% (B), 2.5–11 min 82% (B), 11–11.5 min 99% (B), 11.5–12 min 99% (B), 12–12.1 min 15% (B), 12.1–15 min 15% (B). To reduce sample carryover for highly lipophilic compounds, an automatic valve switching after each injection was employed using a dual solvent wash: first with a water/acetonitrile mixture (1:1, v/v) and subsequently with a 100% isopropanol. Samples were analyzed with MS using an Agilent 6530 QTOF mass spectrometer in positive and Agilent 6550 QTOF mass spectrometer in negative ionization modes. A “device blank” of milliQ water rinse of clean condenser tube inner surface that comes into contact with breath was included along with the EBC samples.

The HILIC data were processed, collated, and constrained into Agilent’s MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the NIST14/Metlin/MassBank libraries to identify compounds with manual confirmation of adduct ions and spectral scoring accuracy.

For the RP analysis, raw data were processed in an untargeted (qualitative) manner by Agilent’s software MassHunter Qual to find peaks in all chromatograms. The peaks were then collated and constrained into a MassHunter quantification method on the accurate mass precursor ion level, using the MS/MS information and the LipidBlast library to identify lipids with manual confirmation of adduct ions and spectral scoring accuracy. MassHunter enabled back-filling of quantifications for peaks that were missed in the primary peak finding process, hence yielding data sets without missing values.

3. Results and Discussion

3.1. GC/MS analysis of EBC Volatile and Semi-volatile Compounds

Three sample extraction methodologies in conjunction with GC/MS analysis were tested to compare compounds coverages of volatile fraction of an “averaged” EBC sample – a combined EBC sample from 6 healthy individuals spanning both genders and a range of

ages. Two liquid phase extraction methodologies (PA SPME and PDMS patch) and one gas phase headspace sampling (CAR/PDMS SPME) methodology were tested. PDMS sampling is a low-cost alternative to the SBSE (Twister™) sampling technology [41]. The same PDMS sorbent as that used for coating of SBSE beads was tested, with sorbent thickness and surface area selected to be similar to that of sorbent coating of an SBSE bead. Therefore, the performance of this sampling methodology is expected to be representative of Twister™ sampling. The PDMS sorbent by itself, not coated on SBSE, was used for a number of applications as well [41, 42].

The results are shown for a representative replicate for each extraction mode as a Venn diagram (Figure 2). The numbers of detected compounds vary depending on the method of extraction. After removing all of the peaks present in blanks, approximately 80 compounds could be detected using PDMS liquid phase extraction, and up to ~200 compounds with the PA SPME liquid phase extraction. At the same time, only ~40 compounds could be detected in the headspace using CAR/PDMS SPME sampling. Approximately 60 compounds were common for liquid sampling, while only ~20 were common between both liquid sampling methods and headspace sampling (Figure 2). It should be noted that the exact number of compounds is approximate and would change depending on parameters for the peak deconvolution set in the AMDIS software. Adjusting the deconvolution parameters may allow for better indexing of low-abundance compounds, but may also result in the introduction of spurious artifacts. Despite peak counts being dependent on deconvolution and blank subtraction choices, certain trends could nevertheless be clearly observed.

One notable trend is the similarity of compounds coverage achieved with liquid extraction methods, in contracts to a distinct difference in coverage with headspace sampling. The polyacrylate coating of the SPME is conducive to retention of more polar, water-soluble compounds, while PDMS is a broad-range sorbent [37, 38]. Consequently, a large number of peaks detected for both methods overlap, especially for early-eluting compounds (Figure 3). There appears to be a greater number of compounds detected with the PA SPME method. Some losses of high-boiling compounds may be possible for PDMS at the desorption step. Longer sampling time increased may result in altered coverages distributions.

In contrast, headspace sampling results in prominently reduced metabolite capture, both in number and relative abundances of detected compounds (Figure 4). Although the solubility of compounds present in EBC was decreased by adding saturated NaCl solution, the headspace sampling still appears to result in significant loss of detected metabolic content compared to direct liquid phase sampling. The likely reason is very low concentration of the dissolved compounds [18–20] leading to insufficient partition of these compounds into the gas phase. Notably, the compounds detected in the headspace also tend to be detected in liquid, although relative abundances distributions in each case may vary (Figure 4b).

The most notable trend is the differences between methodologies in metabolite coverages, and apparent correlation with size and/or boiling temperature of captured compounds, as reflected by the GC retention times. The extraction of compounds with lower boiling temperatures that elute at earlier retention times appears to be more efficient with the PDMS sampling (Figures 5a, b), while larger compounds are more efficiently extracted by the PA

SPME. Headspace sampling, as expected, produces poor results for high-boiling compounds, and is substantially less efficient than either liquid extraction methodology for lower temperature-boiling compounds. PDMS extraction yields the best results for the compounds eluting in the 80–115 °C range (Figure 5a), while PA SPME extraction was found to be optimal for compounds eluting between 150–280 °C (Figure 5b). Notably, PA SPME extraction is the only method that was found to efficiently retain compounds eluting in this temperature range, and two other tested methods were found to be inadequate.

The molecular species that are sampled preferentially with PDMS and PA SPME extraction of EBC are noted (Tables S1 and S2 in Appendix, respectively). The compounds listed in these tables represent both biogenic compounds and external compounds that may be part of the exposome. Specifically, compounds such as normal and branched hydrocarbons, alcohols, ketones and aldehydes are known to originate from human breath [43, 44]. Compounds such as terpenes might be both endogenous and environmental [43], or could be the result of diet choices. Known environmental contaminants such as phthalates are not included in the tables.

The compounds examples (Tables S1 and S2) illustrate clear tendencies for the two extraction modes. PDMS extraction yielded smaller, more volatile compounds with the boiling temperatures <200 °C and appeared limited to ~11 carbons in the structure for compounds with functional groups and ~16 carbons for hydrocarbons. The compounds (Table S1) span a range of polarities, from polar (e.g. propanal) to non-polar (e.g. 6-methylheptane). Both alkyl and aryl compounds could be detected.

PA SPME extraction method was effective for more structurally complex compounds of over ~11 carbons in the structure for compounds with functional groups and up to 24 carbons for hydrocarbons. Very low volatility and low polarity compounds such as large hydrocarbons or waxy alcohols appear to be especially amenable to this method. Interestingly, detection of aryls seems to be also enhanced, along with cyclic compounds and compounds with branched substituents. Aromatic esters were only detected using this extraction method. It may be possible however, that the range of detected compounds is due to both the chemical composition of EBC coupled with overall propensity of the PA SPME to retain larger molecular weight compounds rather than specific sorption tendencies toward certain structures.

In both liquid extraction modes, we note that small, volatile non-polar compounds were not detected. Since EBC is an aqueous solution, more polar volatile compounds are expected to be captured by the breath collection device due to their higher solubility. Less polar and non-polar volatile and very volatile compounds that are insoluble in water (e.g. methane, isoprene etc.) would be present in EBC in diminished quantities compared to their biogenic abundances due to losses during collection. Finally, in addition to the differences in detection efficiencies depending on metabolite size/boiling temperature, there also exist differences stemming from the metabolite molecular structure (Figure 6).

One factor contributing to these differences is the polarity of the molecule. The polyacrylate sorbent is designed to be used for polar semivolatiles [28], while PDMS is amenable for low

molecular weight or volatile compounds and non-polar semivolatiles or large molecular weight compounds [29]. Another factor is the porocity of the sorbent material that may promote or lead to exclusion of molecules of certain sizes. In some cases, enhanced or diminished adsorption of certain compounds may have resulted from specific interactions with the polymer sorbent (e.g. π-π stacking might greatly enhance adsorption of some aromatic compounds on sorbents with phenyl or similar group in its structure). However, in the case of EBC analysis using PA and PDMS sorbents, the effects of sorbent differences for specific molecular structures is much less pronounced than the effect of molecular size/boiling temperature differences discussed above. Tables S1 and S2 could be used as a methodology selection guide for the liquid phase EBC extraction for GC analysis.

3.2. LC/MS analysis of EBC Non-volatile Compounds

A list of chemicals was identified by tandem MS (Tables S4–S6, Supplemental Materials), as described in the Materials and Methods section. In the non-volatile EBC fraction, hundreds of individual compounds, both very polar (HILIC) and less polar and non-polar (RP) could be detected. In previous studies, the non-volatile compounds content of human [19, 45–48] and animals [39, 49] EBC was characterized. The identified compounds (Tables S3–S6 in Supplemental Materials) group according to the chromatography mode utilized. In HILIC, we detected compounds in positive ion mode such as: amino acids and peptides, various other non-volatile small molecules, small amines, phosphocholine derivatives and heterocyclic compounds, glycans/glycan derivatives, organophosphates, and small lipids. Only minor content was detected in negative ion mode. With RP chromatography, the detected compounds were predominantly lipids such as glycerides in the positive ion mode and long chain fatty acids in the negative ion mode.

A metabolite count was done for compounds observed in the positive ion mode based on average values of three technical replicates for the HPLC/MS analysis (Table 1). There was a significantly greater number of compounds observed in the ethanol rinse of the condenser surface of the device after EBC collection compared to neat EBC samples. Similar to GC/MS analysis, the sheer number of indexed compounds is not exact and depends on settings used for peaks deconvolution and alignment. However, the observed differences are significant and meaningful. Since all samples were processed and analyzed simultaneously as a single batch and utilized the same set of blanks, the relative numbers of detected compounds are presumed to accurately reflect actual metabolic content of the samples.

Since EBC samples are aqueous, many of the lipophilic moieties that are water-insoluble originate as aerosols during exhalation. They may deposit on the surfaces of the breath collection device instead of remaining in the EBC. For these species, the use of organic solvent is essential to promote solubility and ensure consecutive recovery. We found that the metabolite recovery increases nearly 6-fold for the lipid species detected in RP (Table 1) in the device rinse with ethanol compared to EBC samples. The condenser tube rinse with organic solvent after each use was also found beneficial in HILIC where the metabolite coverage increases by nearly 60%, as judged by the number of detected compounds. These results indicate the analysis of non-volatile compounds procured with an organic solvent rinse of the condenser surface is richer in metabolite content than actual EBC, especially

pertaining to lipid species. This may be important when designing methods to detect breath lipid peroxidation products as may be seen in certain lung conditions, such as asthma. Using an organic solvent rinse to collect non-water-soluble compounds from the condenser surface for enhanced metabolite recovery was suggested [26], although this has not been rigorously and quantitatively assessed. Another implication is that any EBC sample manipulation, e.g. transfer or contact with surfaces, especially those that may retain lipophilic substances (such as plastic) would lead to sample compounds distributions alteration and potential loss of content and should be avoided [48].

Due to very low annotation rate for the MS/MS data at the present time (typically less than 2% [50]), it is not possible to draw specific conclusions about possible chemistries that can be detected with each method in more than a general fashion. Also, the numbers of the detected metabolites may not immediately indicate utility of methodology choice if/when no specific knowledge exists whether compounds of interest could be detected. However, the considerations presented here can be used as a general guidance for the experimental design considerations and methodology choices for EBC analysis studies.

4. Conclusions

The results presented in this work provide a practical guide for selection of an appropriate/most suitable analytical methodology for analysis of EBC. We found that liquid phase sampling with two tested methodologies for metabolite extraction, PA SPME and PDMS sorbent patch, yielded notably superior metabolite coverage compared to headspace sampling with CAR/PDMS SPME. The PDMS extraction yielded best results for compounds up to ~11 carbons in the structure (16 for hydrocarbons) with the boiling temperature up to ~200 °C. The PA SPME extraction was found to be highly superior for detection of larger compounds with ~11 carbons and more in the structure. The headspace sampling was found to be less comprehensive in both of the liquid extraction methods across the entire range of compounds sizes. These results are suggested for guidance in methodology selection for the analysis of volatile fraction of EBC using GC.

Analysis of the non-volatile fraction by HILIC and RP HPLC/MS was shown to provide orthogonal metabolite coverage by the two chromatography modes. The comprehensive compound distribution coverage of EBC necessitates the use of both chromatography modes as the EBC compounds span both highly hydrophilic and lipophilic compounds. Importantly, it was found that metabolite coverage could be improved by more than 6-fold for RP and by ~60% for HILIC when organic solvent is used to collect a non-water soluble EBC fraction from the condenser surface. It is highly recommended to employ analysis of this organic-soluble fraction instead or in addition to the neat EBC for most comprehensive compounds coverage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Partial support was provided by: NIH award U01 EB0220003-01 [CED, NJK]; NIH National Center for Advancing Translational Sciences (NCATS) through grant#UL1 TR000002 [CED, NJK]; NIH award 1P30ES023513-01A1 [CED, NJK]; NIH award 1K23HL127185-01A1 [MS]; Office of Naval Research (ONR) grant #N-00014-13-1-0580 [CED, BCW, MS]; and The Hartwell Foundation [CED, NJK]. Student support was provided by NIH award T32 HL07013 [KOZ] and NIH award # P42ES004699 [KOZ]. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the funding agencies. The authors are grateful to lab support personnel for help with sample preparation for the LC/MS analysis and to the UC Davis West Coast Metabolomic Center for the LC/MS/MS analysis.

References

1. Amann, A., Smith, D. Volatile Biomarkers Non-Invasive Diagnosis in Physiology and Medicine. Elsevier; China: 2013. p. 129-154.
2. Modak AS. Breath biomarkers for personalized medicine. Pers Med. 2010; 7(6):643–653.
3. Grob NM, Aytekin M, Dweik RA. Biomarkers in exhaled breath condensate: a review of collection, processing and analysis. J Breath Res. 2008; 2(3)
4. Phillips, M. How to analyze breath and make sense of the data: a personal view. World Scientific Publishing Co. Pte. Ltd; 2005.
5. Risby TH, Solga SF. Current status of clinical breath analysis. Appl Phys B Lasers and Optics. 2006; 85(2):421–426.
6. Aksenov AA, et al. Cellular Scent of Influenza Virus Infection. Chembiochem. 2014; 15(7):1040–1048. [PubMed: 24719290]
7. Phillips M, et al. Effect of influenza vaccination on oxidative stress products in breath. J of Breath Res. 2010; 4(2)
8. Minh TC, Blake DR, Galassetti PR. The clinical potential of exhaled breath analysis for diabetes mellitus. Diabetes Res Clin Pract. 2012; 97(2):195–205. [PubMed: 22410396]
9. Righettoni M, Tricoli A. Toward portable breath acetone analysis for diabetes detection. J Breath Res. 2011; 5(3)
10. Smith D, et al. Can volatile compounds in exhaled breath be used to monitor control in diabetes mellitus? J Breath Res. 2011; 5(2)
11. Markar SR, et al. Exhaled Breath Analysis for the Diagnosis and Assessment of Endoluminal Gastrointestinal Diseases. Journal of Clinical Gastroenterology. 2015; 49(1):1–8. [PubMed: 25319742]
12. Schnabel R, et al. Analysis of volatile organic compounds in exhaled breath to diagnose ventilator-associated pneumonia. Scientific Reports. 2015; 5(17179)
13. Amann A, et al. Lung cancer biomarkers in exhaled breath. Expert Rev Mol Diagn. 2011; 11(2): 207–217. [PubMed: 21405971]
14. Szulejko JE, et al. Evidence for cancer biomarkers in exhaled breath. IEEE Sens J. 2010; 10(1): 185–210.
15. Phillips M, et al. Volatile biomarkers in the breath of women with breast cancer. J Breath Res. 2010; 4(2)
16. Phillips M, et al. Prediction of lung cancer using volatile biomarkers in breath. Cancer Biomarkers. 2007; 3(2):95–109. [PubMed: 17522431]
17. Amann A, et al. Determination of volatile organic compounds in exhaled breath of patients with lung cancer using solid phase microextraction and gas chromatography mass spectrometry. Clinical Chemistry and Laboratory Medicine. 2009; 47(5):550–560. [PubMed: 19397483]
18. Cheng WH, Lee WJ. Technology development in breath microanalysis for clinical diagnosis. Journal of Laboratory and Clinical Medicine. 1999; 133(3):218–228. [PubMed: 10072253]
19. Ahmadzai H, et al. Exhaled breath condensate: a comprehensive update. Clinical Chemistry and Laboratory Medicine. 2013; 51(7):1343–1361. [PubMed: 23420285]
20. Alonso M, Sanchez JM. Analytical challenges in breath analysis and its application to exposure monitoring. TrAC, Trends Anal Chem. 2013; 44:78–89.

21. Kuban P, Foret F. Exhaled breath condensate: Determination of non-volatile compounds and their potential for clinical diagnosis and monitoring. A review. Analytica Chimica Acta. 2013; 805:1–18. [PubMed: 24296139]
22. Steeghs MML, Cristescu SM, Harren FJM. The suitability of Tedlar bags for breath sampling in medical diagnostic research. Physiol Meas. 2007; 28(1):73–84. [PubMed: 17151421]
23. Ghimenti S, et al. Comparison of sampling bags for the analysis of volatile organic compounds in breath. Journal of Breath Research. 2015; 9(4)
24. Horvath I, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. European Respiratory Journal. 2005; 26(3):523–548. [PubMed: 16135737]
25. Gerber SA, et al. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100(12):6940–6945. [PubMed: 12771378]
26. Fernández-Peralbo MA, et al. Study of exhaled breath condensate sample preparation for metabolomics analysis by LC–MS/MS in high resolution mode. Talanta. 2015; 144:1360–1369. [PubMed: 26452970]
27. Pleil JD, Stiegel MA. Evolution of Environmental Exposure Science: Using Breath-Borne Biomarkers for “Discovery” of the Human Exposome. Analytical Chemistry. 2013; 85(21):9984–9990. [PubMed: 24067055]
28. Alonso M, Sanchez JM. Analytical challenges in breath analysis and its application to exposure monitoring. Trac-Trends in Analytical Chemistry. 2013; 44:78–89.
29. Paetznick DJ, et al. Comparison of breath and in-mouth collection for the measurement of oral malodorous compounds by gas chromatography using sulfur chemiluminescence detection. Journal of Breath Research. 2010; 4(1)
30. Cajka T, Fiehn O. Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics. Anal Chem. 2016; 88(1):524–545. [PubMed: 26637011]
31. Zamuruyev KO, et al. Human breath metabolomics using an optimized non-invasive exhaled breath condensate sampler. J Breath Res. 2017; 11(016001)
32. Rosias PP, et al. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. European Respiratory Journal. 2006; 28(5):1036–1041. [PubMed: 16870660]
33. Effros RM, et al. Exhaled Breath Condensates: Analyzing the Expiratory Plume. American Journal of Respiratory and Critical Care Medicine. 2012; 185(8):803–804. [PubMed: 22505753]
34. Holmgren H, et al. Size distribution of exhaled particles in the range from 0.01 to 2.0 mu m. Journal of Aerosol Science. 2010; 41(5):439–446.
35. Morawska L, et al. Size distribution and sites of origin of droplets expelled from the human respiratory tract during expiratory activities. Journal of Aerosol Science. 2009; 40(3):256–269.
36. Loyola BR, et al. Temperature changes in exhaled breath condensate collection devices affect observed acetone concentrations. Journal of Breath Research. 2008; 2(3)
37. SigmaAldrich Co. SPME Applications Guide. 52001. [Available from: http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/General_Information/1/t199925.pdf
38. Baltussen E, et al. Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: theory and principles. J Microcolumn. Sep; 1999 11(10):737–747.
39. Aksenov AA, et al. Metabolite Content Profiling of Bottlenose Dolphin Exhaled Breath. Analytical Chemistry. 2014; 86(21):10616–10624. [PubMed: 25254551]
40. Tsugawa H, et al. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. Nature Methods. 2015; 12(6):523–526. [PubMed: 25938372]
41. Thomas AN, et al. Novel noninvasive identification of biomarkers by analytical profiling of chronic wounds using volatile organic compounds. Wound Repair Regen. 2010; 18(4):391–400. [PubMed: 20492633]
42. Allwood JW, et al. Metabolomics in melon: A new opportunity for aroma analysis. Phytochemistry (Elsevier). 2014; 99:61–72.
43. Mochalski P, et al. Blood and breath levels of selected volatile organic compounds in healthy volunteers. Analyst. 2013; 138(7):2134–2145. [PubMed: 23435188]

44. de Lacy Costello B, et al. A review of the volatiles from the healthy human body. J Breath Res. 2014; 8(1)

45. Davis CE, et al. Analysis of Volatile and Non-Volatile Biomarkers in Human Breath Using Differential Mobility Spectrometry (DMS). Ieee Sensors Journal. 2010; 10(1):114–122.

46. Bajaj P, Ishmael FT. Exhaled breath condensates as a source for biomarkers for characterization of inflammatory lung diseases. J Anal Sci, Methods Instrum. 2013; 3(1):17–29.

47. Griese M, Noss J, Von Bredow C. Protein pattern of exhaled breath condensate and saliva. Proteomics. 2002; 2(6):690–696. [PubMed: 12112850]

48. Nording ML, et al. Endogenous levels of five fatty acid metabolites in exhaled breath condensate to monitor asthma by high-performance liquid chromatography: electrospray tandem mass spectrometry. IEEE Sens J. 2010; 10(1):123–130. [PubMed: 21103452]

49. Reinhold P, Knobloch H. Exhaled breath condensate: lessons learned from veterinary medicine. Journal of Breath Research. 2010; 4(1):017001. [PubMed: 21386203]

50. da Silva RR, Dorrestein PC, Quinn RA. Illuminating the dark matter in metabolomics. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112(41):12549–12550. [PubMed: 26430243]

Highlights

- Exhaled breath condensate metabolites study.
- A practical methodology selection guide for optimal metabolite coverage of breath.
- Comparison of mass spectrometry-based analysis methodologies for broad metabolite coverage.

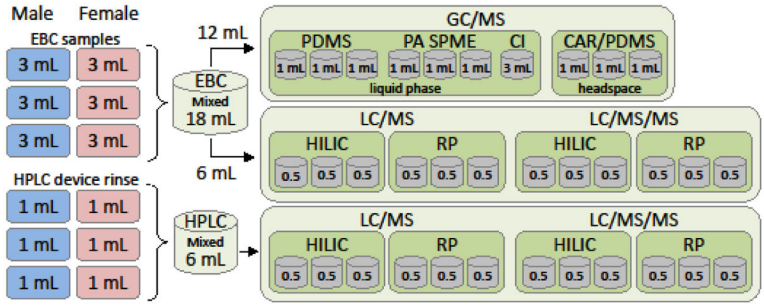

Figure 1.

A diagram of workflow for the collection and usage of an “averaged” exhaled breath condensate sample.

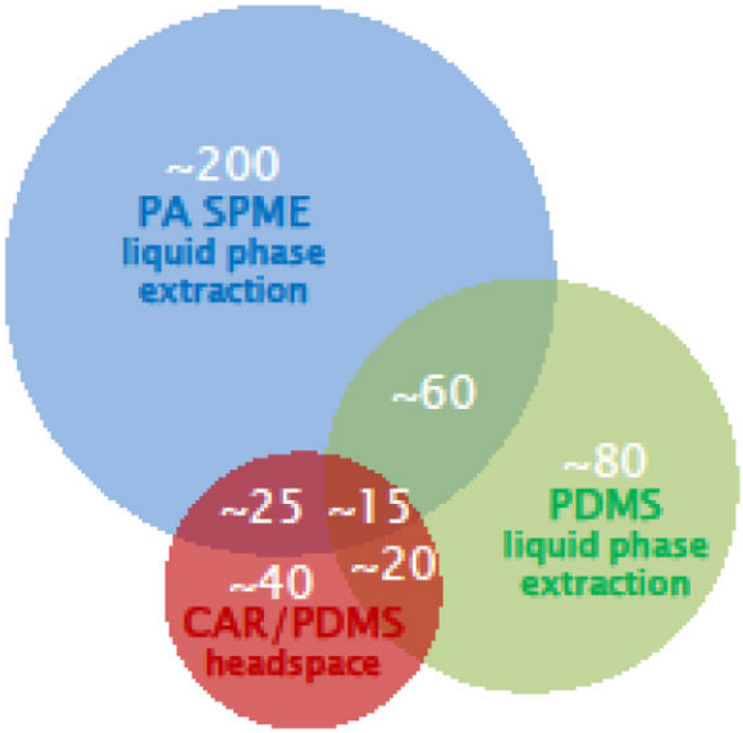

Figure 2.

A Venn diagram of the approximate number of peaks for three sampling methods used for EBC compounds extraction: liquid phase extraction (PA SPME and PDMS patch) and gas phase headspace sampling (CAR/PDMS SPME). The numbers of detected peaks were estimated using data processing approaches described in the Materials and Methods section and would change if alternative approaches and/or deconvolution settings are employed.

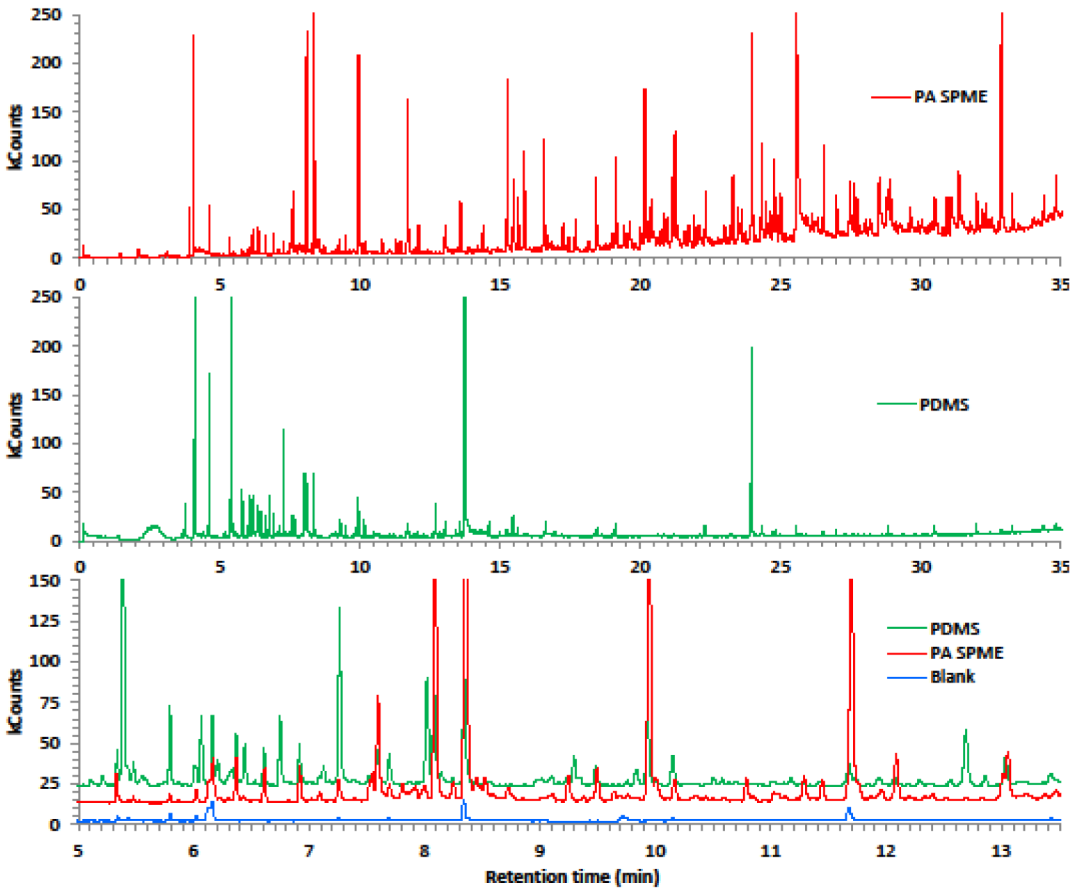

Figure 3.

a) Side-by-side comparison of the liquid phase extraction methods using PA SPME (top panel) and PDMS sorbent (bottom panel). b) Partial chromatograms overlaid for two liquid extraction methodologies (PA SPME red, PDMS green, DI water blank blue); multiple compounds are detected with both methodologies. The chromatograms are offset for clarity.

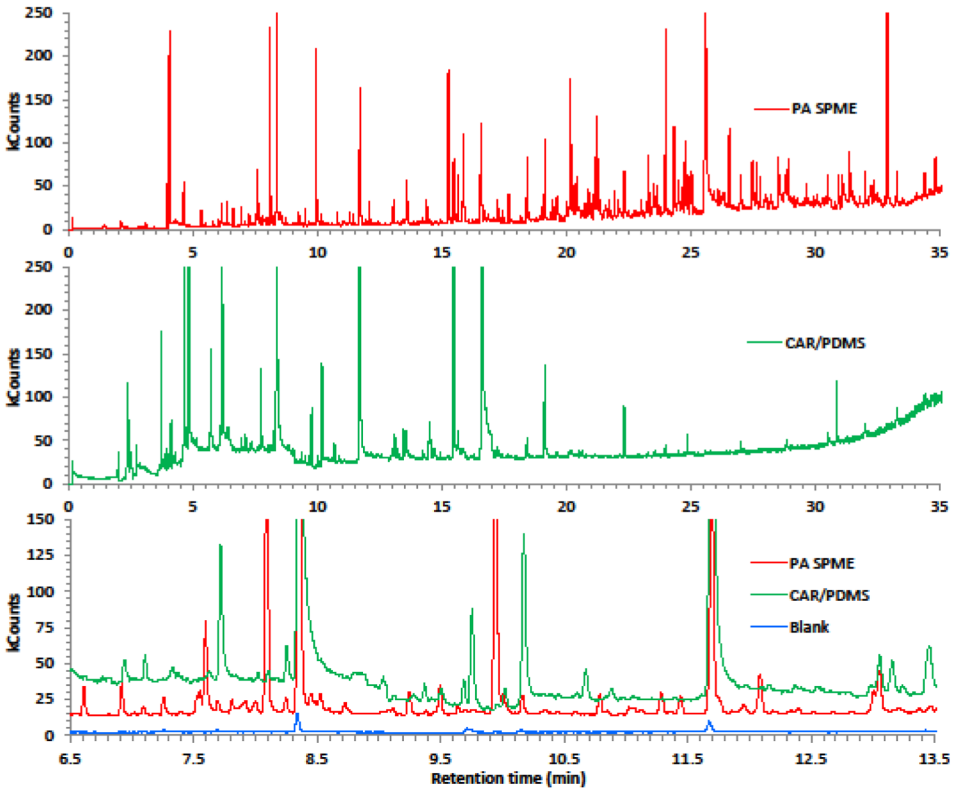

Figure 4.

a) Side-by-side comparison of the liquid phase extraction using PA SPME (top panel) and headspace sampling with CAR/PDMS SPME (bottom panel). b) A fragment of chromatograms overlaid for liquid phase extraction with PA SPME (red) and headspace sampling with CAR/PDMS SPME (green) along with the DI water blank (blue); some compounds are detected with both methodologies, while some are detected only with one of the methods. The chromatograms are offset for clarity.

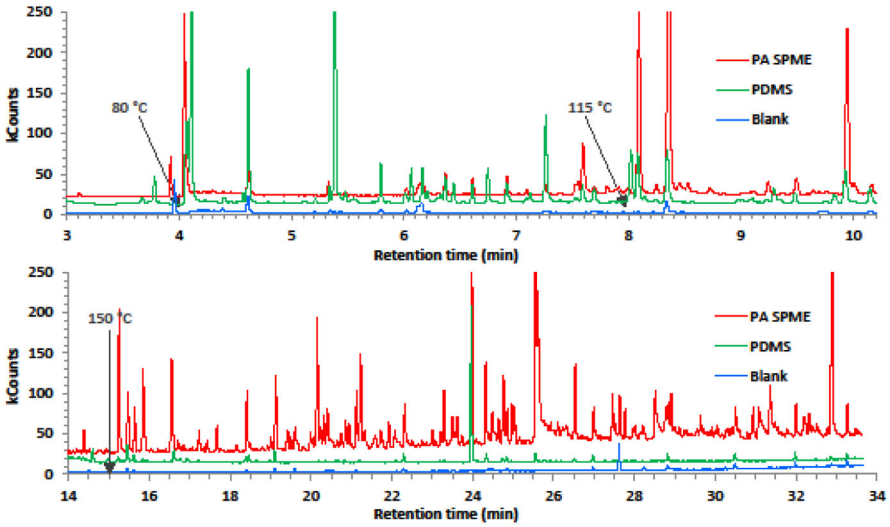

Figure 5.

A comparison of the liquid phase extraction methods using PA SPME and PDMS sorbent for different elution temperature ranges (PA SPME red, PDMS green, DI water blank blue). a) In the 80–115 °C ranges, extraction with PDMS appears advantageous. b) For compounds eluting at 150 °C and above, PA SPME extraction appears advantageous. The chromatograms are offset for clarity.

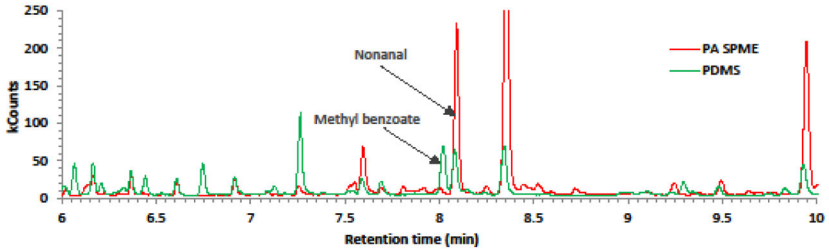

Figure 6.

An example of differences in compounds extraction from EBC with liquid extraction using PA SPME and PDMS sorbent (PA SPME red, PDMS green, DI water blank blue). Greater amount of methyl benzoate appears to be captured by the PDMS sorbent, while nonanal is more efficiently captured by the polyacrylate material of the SPME.

Table 1

The approximate numbers of detected compounds for different chromatography modes of HPLC/MS analysis in positive ion mode. *EBC, neat* refers to analysis of liquid EBC; *Ethanol rinse* refers to the analysis of the collection device glass tube rinse with HPLC-grade ethanol, as described in the Materials and Methods section

	Approximate number of indexed compounds, HILIC	**Approximate number of indexed compounds, RP**
EBC, neat	~770	~530
Ethanol rinse	~1280	~3250