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Investigating the relationship between breath aerosol size and exhaled breath condensate (EBC) metabolomic content

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Abstract

Exhaled breath aerosols contain valuable metabolomic content due to gas exchange with blood at the alveolar capillary interface in the lung. Passive and selective filtering of these aerosols and droplets may reduce the amount of saliva contaminants and serve as an aid to enhance targeted metabolomic content when sampled in EBC. It is currently unknown if breath aerosol size distribution affects the types or abundances of metabolites sampled through EBC. This pilot study uses a previously described hand-held human breath sampler device with varying notch filter geometries to redirect the trajectory of breath aerosols based on size. Ten notch filter lengths were simulated with the device to calculate the effect of filter length on the breath aerosol size distribution and the proportion of aerosols which make their way through to an EBC collection tube. From three notch filter lengths, we investigate metabolite content of various aerosol fractions. We analyzed the non-volatile fraction of breath condensate with high performance liquid chromatography mass spectrometry (LC-MS) for broad metabolite coverage. We hypothesize that: (1) increasing the length of the notch filter in this device will prevent larger aerosols from reaching the collection tube thus altering the breath aerosol size distribution sampled in EBC; and (2) there is not a systematic large-scale difference in EBC metabolomic content that correlates with breath aerosol size. From simulation results, particles typically larger than 10 μm were filtered out. This indicates that a longer notch filter in this device prevents larger particles from reaching the collection tube thus altering the aerosol particle size distribution. Most compounds were commonly present in all three filter lengths tested, and we did not see strong statistical evidence of systematic metabolite differences between breath aerosol size distributions.

1.0 Introduction

Exhaled breath aerosols contain valuable metabolomic content due to gas exchange with blood at the alveolar capillary interface in the lung. Two possible mechanisms of breath

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aerosol generation in the lung are droplet formations at the air-liquid interface due to shear forces in the upper airways, as well as from a reopening of terminal airway structures. There is a tendency for airways to narrow upon expiration and expand during inhalation, which can create tiny aerosols [1]. Exhaled breath is a complex mixture that contains primary respiratory gases, volatile organic compounds (VOCs), and non-volatile compounds from the liquid lining of the lung [2]. Exhaled breath aerosol sampling is a simple and non-invasive medium for public health and occupational exposure biomonitoring [3]. Due to the COVID-19 pandemic, critical attention is warranted on infectious aerosols sourced from respiratory activities [4–6]. The use of respirator surfaces, hospital masks, and ventilators for trapping these aerosols has been discussed [3, 7]. Here, we present novel technology for exhaled breath sampling, non-targeted metabolomic analysis, and sizing of exhaled aerosols.

Exhaled breath condensate (EBC) is a fraction of breath that contains water soluble volatiles and non-volatile compounds [8]. It is a biological matrix in which biomarkers may be identified, similar to saliva and blood and can allow for the discovery of new metabolites and can provide additional health information. Passive and selective filtering of these aerosols and droplets may reduce the amount of saliva contaminants and serve as an aid to enhance targeted metabolomic content when sampled in EBC. The design of a breath sampling device will inevitably affect the particle size range of the collected sample. For instance, long tubing and sharp turns will contribute to losses of larger aerosols. A standardized collection method should thus consider sampling device geometry to account for different aerosol size distributions. Currently, no EBC collection methods count the number of aerosol particles or enable differentiation of their sizes.

It is currently unknown if breath aerosol size distribution affects the types or abundances of metabolites sampled through EBC. In the last few decades, multiple approaches have been carried out to determine the size distribution of exhaled aerosols. Early studies used glass slides and filters with subsequent microscopic analyses which often determined the sizes of droplets above the micron range [9, 10]. In more recent studies, sensitive sampling-based optical particle counters measured aerosols from up to five individuals that were in the submicron size range [11, 12]. An instrument limit of detection for breath aerosol sizes can inadvertently skew a measured size distribution if they are unable to detect smaller aerosol sizes. Another common issue in previous studies is that exhaled aerosol sizes were not measured immediately at the mouth or nose exits. These droplets may go through evaporation, dilution, sampling loss, and other influences from the environment before being measured, which causes error in estimating the original size distribution. Aerosol size distribution during normal tidal breathing has been observed to be similar among up to 16 individuals investigated with diameters primarily in the submicron range [13–15]. Particles expelled from other breathing activities (i.e. coughing and talking) may be much larger (> 10 μm) [9, 16]. These larger particles may also be present during tidal breathing, especially through longer sampling durations (5–15 mins) and when non-tidal episodes can occur [12]. Currently, a category of sampling devices are available to collect breath aerosols with specifically designed polymer filters, and are used for drug monitoring [17].

This pilot study uses a previously described hand-held human breath sampler device with varying notch filter geometries to redirect the trajectory of breath aerosols based on size

[18]. Curved flow profiles have greater inertial effects on larger breath aerosols which cause them to strike the interior walls before they can arrive at a collection site. Shorter notch filter lengths allow larger particles (≳ 10 μm diameter) to pass through while longer notch filter lengths restrict airflow and prevent larger particles from reaching the collection site. In this present work, we investigate metabolite content of various aerosol fractions. We analyzed the non-volatile fraction of breath condensate with high performance liquid chromatography mass spectrometry (LC-MS) for broad metabolite coverage [19]. Additionally, we simulate the trajectories of these aerosols with varying notch filter lengths using COMSOL Multiphysics® software. We hypothesize that: (1) increasing the length of the notch filter in this device will prevent larger aerosols from reaching the collection tube thus altering the breath aerosol size distribution sampled in EBC; and (2) there is not a systematic large-scale difference in EBC metabolomic content that correlates with breath aerosol size.

2.0 Materials and Methods

2.1 Exhaled breath condensate (EBC) sampling hardware

EBC sample collection was achieved using a hand-held human breath sampler described in previous work [18]. Briefly, the outer casing of the device was constructed from polycarbonate tubing and insulated with polyethylene foam. A borosilicate glass tube was used as a condenser surface. Hollow space between the glass condenser tube and insulated housing was filled with dry ice pellets. Computer-aided design (CAD) models of the human breath sampler illustrate these components (Figure 1). Three vertical notch filter lengths were experimentally tested with the device at 23, 28, and 33 mm (Figure 1D). An airway chamber contains a pair of asynchronous valves designed to promote unidirectional breath flow and keep the condenser chamber closed for condensation from the ambient air. This device features a saliva trap to allow selective filtering of breath aerosols by capturing heavy droplets (≳ 100 μm) and allowing small aerosols (≲ 20 μm) which originate in the deep lungs to pass through, and this was demonstrated earlier using an amylase assay [18]. The housing is constructed out of polytetrafluoroethylene (PTFE), a chemically inert material used to reduce chemical absorbance. The mouthpiece used has an inner diameter of 22 mm and is made of polystyrene butadiene (BE 120–22D; Instrument Industries, Inc. Pittsburg PA, USA). Further details on the inner dimensions of the device are annotated in Supplemental Figure 1.

2.2 Simulated passive droplet filtering

Ten notch filter lengths were simulated with the device to calculate the effect of filter length on the breath aerosol size distribution and the proportion of aerosols which make their way through to the EBC collection tube. Additionally, three notch filter lengths were experimentally tested with the device to determine if there are variations of breath metabolomic profiles.

Aerosol particle flight paths inside the Teflon™ housing were estimated with a particle tracing application in COMSOL Multiphysics® simulation software with the assumption that particles were at thermal equilibrium with the carrier fluid and underwent no phase change (no evaporation or condensation) in flight. This is reconciled with a ‘freeze’ wall

boundary condition, so when particles strike a wall they no longer move. Particles pass through the sampling device into a chilled glass tube (−30 ºC), the collection site, and are counted at t_{final} = 0.5 s. The fluid properties at the inlet were approximated with those of saturated moist air mixture at body core temperature (36.6 ºC), taken from previous work and literature. A set of simulations consisted of a uniform distribution of particles with 1000 evenly spaced diameter values in a range of 0.01 to 20 μm to demonstrate what may happen to larger particles that originate from tidal breathing and other breathing activities. Presumably, particles generated in situ and then exhaled are liquid spheres. The principal component of EBC is condensed water vapor which represents nearly all the volume (>99%) of fluid collected in EBC [20, 21]. Nonvolatile and water-soluble molecules inside respiratory droplets may increase or decrease the density of these droplets. The density of the droplets also changes as a function of temperature. We assume these alterations are negligible and we assume that the average density of these exhaled breath aerosols to have the density of water (1 g/cm^3, at 4 ºC). The breath aerosols were modeled in these simulations as spheres and with a density of 1 g/cm^3.

The number of exhaled particles per exhalation has been found to vary among subjects by orders of magnitude, ranging widely from 10^2 to 10^5 particles per exhalation [14, 22–26]. Based on these results, the number of particles released from the inlet is set to an upper estimate of $N_{inlet} = 10^5$ for the simulations. All particles are released with a velocity of 2 m/s at time t = 0 s at 100 different locations the inlet surface. The inlet flow rate corresponds to an average tidal breathing rate (12–20 breaths min^{-1}, tidal volume 0.5 L, exhaled in 1s) [27, 28]. We assume an initial velocity of 2 m/s to be a generalized value of breath aerosol velocity from tidal breathing in healthy adults [29–31]. Ten vertical notch filter sizes (0, 3, 8, 13, 18, 23, 28, 33, 38, and 43 mm) were iterated with the same uniform distribution.

2.3 EBC sample collection

Collection of EBC was performed with the device from one healthy volunteer to standardize the aerosol emission source (male, age 27, no history of smoking). The sampling was time controlled at 10 min with normal tidal breathing. To reduce the effect of food related confounders, the volunteer restrained from food consumption one hour before EBC collection and rinsed the mouth with water prior to sampling. All parts of the device were thoroughly cleaned with 70% ethanol disinfectant spray and deionized (DI) water and air-dried after each use. After sampling, the frozen EBC condensate was removed and transferred to a clean borosilicate glass vial (Sigma-Aldrich, SU860099 SUPELCO), immediately sealed with a stainless-steel threaded cap with PTFE fluorosilicone rubber septum (Sigma-Aldrich, SU860101 SUPELCO) and placed in a laboratory freezer at −80 °C until mass spectrometry analysis. A total of 6 EBC samples were collected, 2 replicates for each filter length tested (23, 28, and 33 mm).

2.4 EBC Sample Analysis

EBC samples were directly lyophilized and the obtained dried extract was reconstituted in mobile phase (5% acetonitrile in water) to obtain a concentration factor of 20. Samples were analyzed using an Agilent 1290 series HPLC system coupled with an Agilent 6530 quadrupole -time of flight (qTOF) mass spectrometer (Agilent Technologies, Santa Clara,

CA, USA). 20 μL of each sample were injected through an InfinityLab Poroshell 120 EC-C18 column (2.7 μm, 3.0 mm × 50 mm; Agilent Technologies, Palo Alto, CA, USA). The mobile phases consisted of water (A) and acetonitrile (B), both with 0.1% formic acid. The solvent flow rate was set to 0.6 ml min^{-1}, the column temperature to 35 °C and the autosampler to 8 °C to increase sample stability. An electrospray ionization (ESI) source with an Agilent Jet Stream nebulizer was used in positive and negative mode with the following operating parameters: capillary voltage, 4000(−)/3500(+) V; nebulizer pressure, 25 psi; drying gas, 10 L min^{-1}; gas temperature, 250 °C; fragment voltage, 130 V. Mass spectra were acquired at MS resolution level at a scan rate of 2 spectra/s over a range of m/z 100-950.

2.5 Data processing

The LC-MS data were initially checked for qualitative purposes using Agilent's Mass Hunter Qualitative Analysis B.06.00 software. For the untargeted analysis, data mining was performed using an automated algorithm for peak finding, alignment, and integration in Agilent's Mass Hunter Profinder B.09.00 software. A Bach Recursive Feature Extraction method was used with mass tolerance and window of 20 ppm and 0.025 Da, RT window of 0.3 min, with minimum absolute abundance of 1000 counts. The obtained dataset was exported into a *.pfa* format and imported to Agilent's Mass Profiler Professional (MPP, V13.0) software for identification, and initial statistical analysis. Afterwards, a tentative identification of the obtained molecular features (markers), described as *mass@retention time*, was performed using ID browser, an integrated software in MPP. Based on matching experimental and theoretical isotope pattern of the markers, the software proposed formulas and names with scores above 70%, using the METLIN database. The dataset was filtered by removing compounds that appear in blank samples with signals higher than 10 (peak sample/blank ratio). Final data were normalized using probabilistic quotient normalization with median values per sample to correct the bias between sample collection and preparation [32].

3.0 Results and Discussion

3.1 Simulated passive droplet filtering

Figure 2 (a) shows the numerical solutions for breath aerosols with different diameters passing through the device. The data were grouped into 25 evenly distributed bins. The absence of a notch filter (designated as length 0 mm) corresponds to nearly 5.5% of particles trapped which are greater than 10 μm in diameter. The trapping of larger particles increases with a greater length of the notch filter, which supports our first hypothesis. Notch filter lengths of 18, 23, 28, 33, and 38 all appear to have the same effect on the particle size distribution, all correlating to an averaged 43.5% trapping of particles, mostly larger than 10 μm in diameter. Increasing the notch length from 13 to 18 mm as well as from 38 to 43 mm appear to have much greater changes on the resulting particle size distribution in the collection tube. A notch length of 43 mm corresponds to nearly 70.8% of particles trapped, mostly larger than 5 μm in diameter. The percentage of particles trapped is defined to be the number of breath aerosols which pass through to the chilled glass tube divided by the total particles in the device, multiplied by one hundred. These counts of particles which made it

to the collection tube compared to the ones remaining at the trapping site are plotted (Figure 2B).

The velocity profiles are modeled in steady state of the sampling device (Supplemental Figure 2). It is evident the notch filter length has a direct impact on the velocity flow profile of the breath aerosols. The ambient pressure of the device was set to 1 atm and the resulting pressure profiles inside the device for each simulation (Supplemental Figure 3). Mols et al. concluded that a pressure drop of 950 Pa across an endotracheal tube resulted in excessive tidal volumes and airflow, which was perceived as discomfort [33]. A notch filter length of 43 mm could cause respiratory discomfort with a pressure difference of ~140 Pa which may depend upon one's health condition. The aerosol particle trajectories in the exhaled breath condensate sampling device are animated (Supplemental Figure 4).

3.2 Metabolomic content of the EBC

A total of 6,225 metabolites were obtained from the LC-MS chromatograms in negative and positive ionization modes. Data were previously aligned and filtered, as described in section 2.4. Metabolite identification of untargeted data were performed based on the MS and MS/MS spectra and the accurate masses obtained using METLIN database. The putatively identified biomarkers are listed with their exact molecular mass and retention time (Table 1). It lists the 50 highest abundant metabolites (highest to lowest) detected by untargeted LC-MS analysis among all six EBC samples. Molecular formula and compound identification are described together with their identification (ID) score, calculated with the average values from molecular formula extraction and database ID scores.

The number of common compounds found from exhaled breath condensate samples using different vertical notch filter lengths are listed (Table 2). Table 2 lists the number of compounds common for filter length combinations in LC-MS negative and positive electrospray ionization modes. Most compounds were present in all EBC samples collected from the device using each filter length. Compounds were considered unique to a filter length if they were present in at least one of the two replicate samples per filter length. Approximately 11% of compounds were unique to a filter length, both for negative and positive ionization modes. The notch filter with length 28 mm had a higher number of unique compounds in both negative and positive ionization modes in comparison to the 23 mm notch filter. The notch filter with length 33 mm had no unique compounds in either ionization mode. A higher number of compounds were present in both 23 and 28 mm filter lengths in comparison to the two other sets (23/33 and 28/33 mm). We do not believe these differences can be determined as statistically significant given the samples were from only one person and the extremely limited sample numbers involved (n = 6 total). We do not observe striking data at this time that breath aerosol size affects metabolite profiles.

4.0 Conclusion

Our human exhaled breath condensate sampler described was modified with varying notch filter lengths to determine if breath aerosol size affects EBC metabolite content. From simulation results, particles typically larger than 10 μm were filtered out for notches longer than 18 mm. This indicates that a longer notch filter in this device prevents larger particles

from reaching the collection tube thus altering the aerosol particle size distribution. Three notch lengths were experimentally tested with the sampling device. Most compounds were commonly present in all three filter lengths, and we did not see strong statistical evidence of systematic metabolite differences between breath aerosol size distributions. If there are differences in metabolomic content based on breath aerosol size, it is possible that they are not significant for practical sampling if the sampling device design has already been optimized for proper saliva filtering, cooling temperature, and air flow rates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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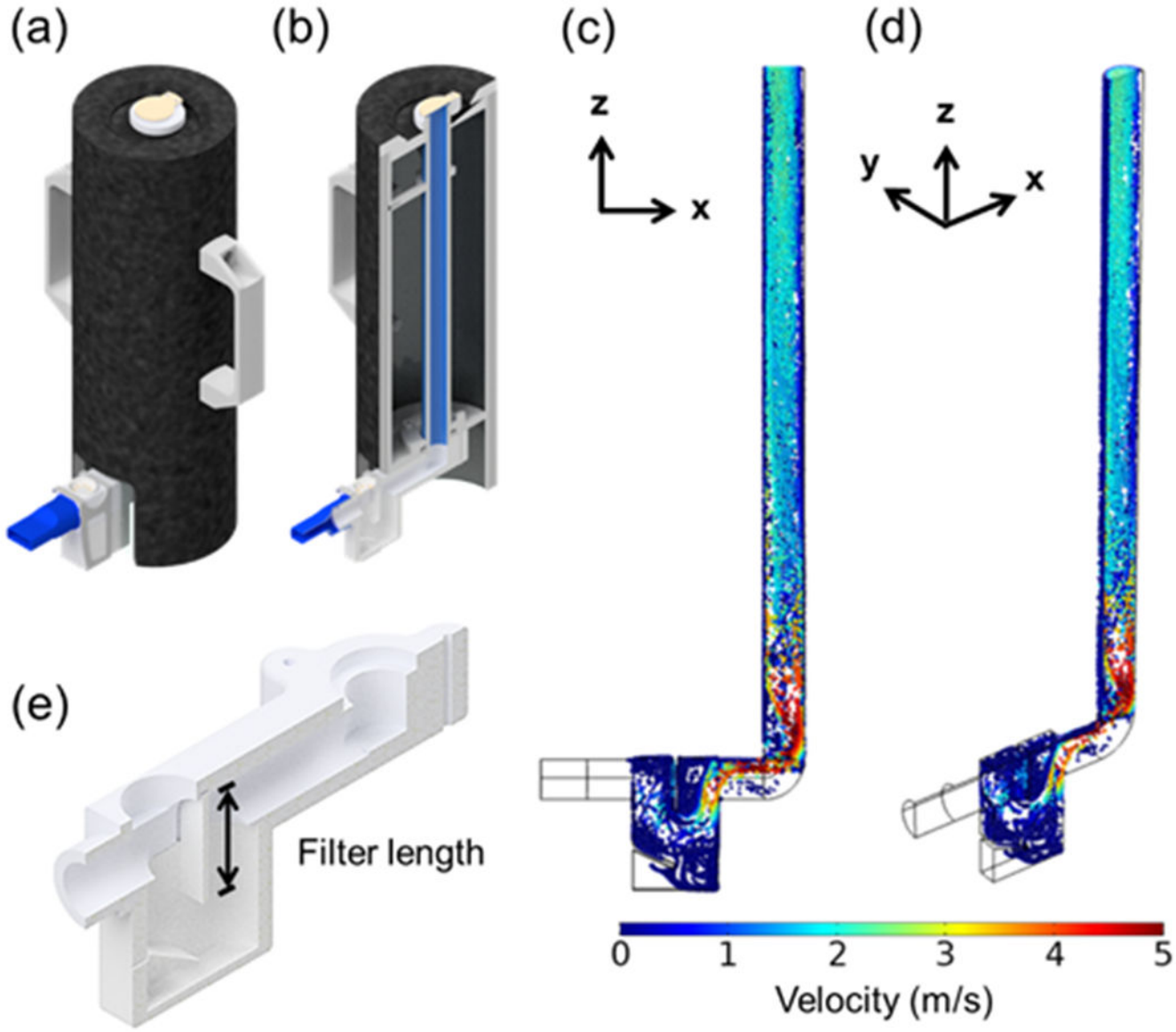

Figure 1.

CAD models of the human exhaled breath condensate (EBC) sampler. **(a)** Model of the whole device and **(b)** Sectioned in half. **(c)** A simulation snapshot illustrating the velocity of exhaled aerosols. **(d)** Isometric view of simulation snapshot. **(e)** The vertical notch filter length variable for experiments in this study were 23, 28 and 33 mm.

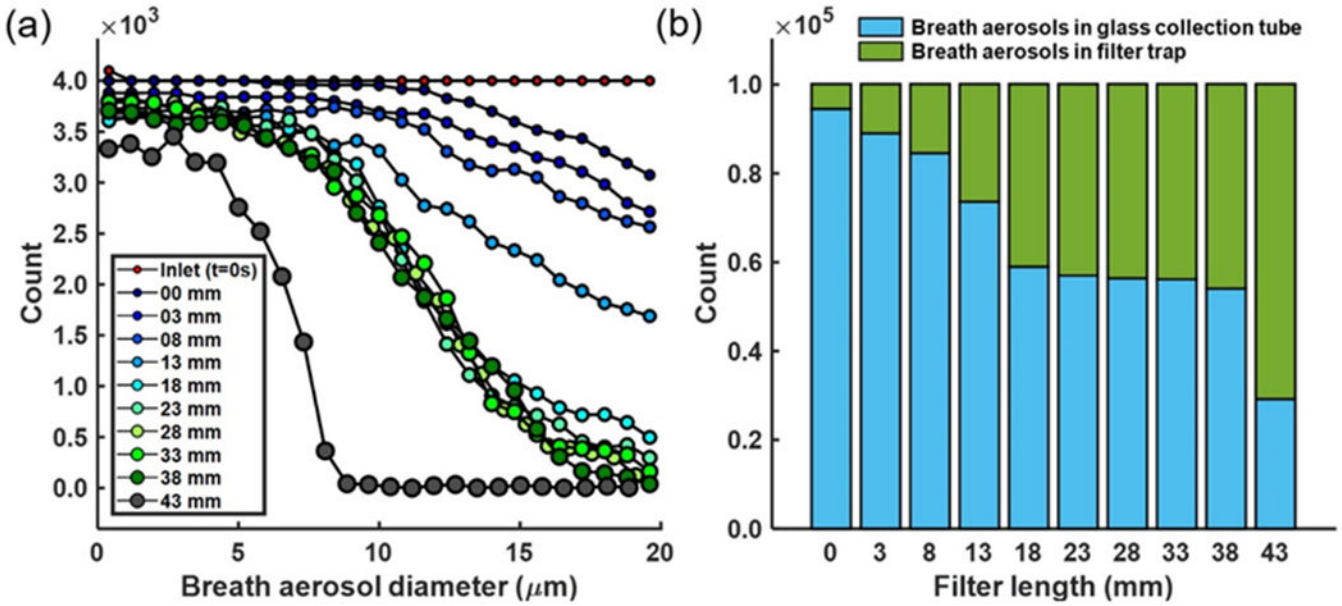

Figure 2.

COMSOL Multiphysics® software simulations of breath aerosol particles which passed through the exhaled breath condensate (EBC) sampling device into a chilled glass tube, the collection site at time t_{final} = 0.5 s. Ten vertical notch filters were iterated. **(a)** A uniform diameter distribution of particulates enters the inlet with 1000 evenly spaced values in a range of 0.01 to 20 μm at 100 different locations. Data are grouped together into 25 evenly distributed bins. **(b)** Total count of breath aerosols which pass through to the chilled glass tube compared to the count of these aerosol particles trapped in a reservoir by the filter.

Table 1.

The list of highest abundant metabolites (highest to lowest) putatively identified by untargeted liquid chromatography-mass spectrometry (LC-MS) analysis among all exhaled breath condensate (EBC) samples.

Variable number	Mass (m/z)	RT (min)	Formula	Compound Name	ID Scores	Description
1	199.200	4.11	C12 H24 O2	3-Methyl-undecanoic acid	73.35	Fatty acid
2	324.200	4.70	C18 H28 O5	Cibaric acid	80.21	Fatty acid
3	266.162	8.64	C12 H27 O4 P	Tributyl phosphate	94.70	Extractant and a plasticizer
4	326.200	2.31	C14 H28 N6 O4	Arg Gly Ile	94.34	Amino acid
5	282.200	2.09	C19 H28 N O3	Glycopyrrolate	94.92	Anticholinergic
6	238.100	1.80	C9 H18 O7	(x)-1,2-Propanediol 1-O-b-D-glucopyranoside	84.95	Found in herbs and spices
7	370.200	2.49	C20 H28 N4 O4	Ile Ala Trp	94.19	Amino acid
8	294.190	7.16	C16 H28 N2 O4	Oseltamivir	95.60	Antiviral
9	255.300	7.38	C17 H36 O	14-Methyl-1-hexadecanol	89.69	Fatty alcohol
10	206.200	3.74	C15 H28 O	7-Ethyl-4-tridecen-6-one	75.48	–
11	209.100	2.82	C13 H11 N3	Proflavine	74.70	Disinfectant
12	452.300	3.06	C29 H42 O5	(3beta,17alpha,23S)-17,23-Epoxy-3,29-dihydroxy-27-norlanosta-7,9(11)-diene-15,24-dione	91.75	Oxosteroid
13	154.100	3.00	C9 H14 O2	Allyl hexenoate	77.28	Flavouring ingredient
14	211.100	3.00	C14 H13 N O	2-Hydroxyiminodibenzyl	86.16	Aromatic compound
15	424.300	4.94	C21 H45 O6 P	1-Octadecyl Lysophosphatidic Acid	94.17	Glycerophospholipid
16	422.300	6.62	C25 H45 O4 P	Dolichyl phosphate	92.93	Lipid
17	310.200	4.34	C20 H26 N2 O	Astrocasine	82.05	Alkaloid
18	540.400	6.51	C30 H59 N O7 P	PC(22:2(13Z,16Z)/0:0)	95.73	Lecithin
19	193.100	3.00	C6 H15 N5 O2	NG-amino-L-Arginine	85.72	Inhibitorofnitric oxide synthase
20	286.100	2.53	C12 H17 N O7	3-Hydroxy-N-methylpyridinium glucuronide	87.09	O-glucuronide
21	546.300	3.02	C29 H59 N O7 P	PC(O-18:0/3:1(2E))[S]	94.04	–
22	557.400	6.51	C37 H48 O3	1?,25-dihydroxy-25,25-diphenyl-26,27-dinorvitamin D3 / 1?,25-dihydroxy-25,25-diphenyl-26,27-dinorcholecalciferol	95.82	Secosteroid
23	366.300	4.37	C26 H40 O2	26:6(8Z,11Z,14Z,17Z,20Z,23Z)	91.13	Omega-3 fatty acid
24	590.400	3.13	C31 H59 O8 P	PA(13:0/15:1(9Z))	88.52	–
25	222.100	2.63	C11 H14 N2 O3	Phe Gly	83.07	Dipeptide
26	138.000	0.59	C3 H8 O5 S	(R)-2,3-Dihydroxypropane-1-sulfonate	72.20	Alkanesulfonic acid
27	187.127	2.84	C9 H19 N O4	Dexpanthenol	95.19	Cholinergic agent
28	348.200	2.31	C18 H32 O6	2,3-dinor Thromboxane B1	74.19	Eicosanoid
29	108.100	2.99	C6 H14	hexane	77.14	Neutotoxin
30	229.200	4.53	C13 H27 N O2	2-amino-tridecanoic acid	91.43	–
31	288.100	8.64	C13 H21 O3 P S	Iprobenfos	91.66	Rice fungicide

Variable number	Mass (m/z)	RT (min)	Formula	Compound Name	ID Scores	Description
32	298.170	8.63	C12 H24 N6 O4	Ala Arg Ala	71.12	Amino acid
33	148.000	6.91	C4 H7 O5 P	Deamino-?-keto-demethylphosphinothricin	80.02	–
34	90.0334	0.57	C3 H8 O4	2,2-Dihydroperoxypropane	99.37	Added to foods as a bleaching agent
35	656.500	8.02	C36 H71 N2 O7 P	PE-Cer(d16:2(4E,6E)/18:0(2OH))	75.35	–
36	243.185	5.12	C13 H26 N O4	L-Hexanoylcarnitine	90.85	Human metabolite
37	250.200	3.95	C15 H26 N2 O	Retamine	88.54	Pain reliever
38	156.001	4.85	C7 H5 Cl O2	4-Chlorobenzoate	97.77	Bacterial xenobiotic metabolite
39	250.160	11.69	C14 H22 N2 O2	Rivastigmine	96.64	Acetylcholinesterase inhibitor
40	293.200	4.34	C17 H27 N O3	Pramoxine	87.47	Topical medication to relieve pain
41	651.400	3.22	C32 H62 N O10 P	PS(12:0/14:0)	82.23	–
42	517.400	5.04	C35 H53 N O3	dl-alpha-Tocopherol nicotinate	91.39	Ester of vitamin E
43	176.000	6.91	C5 H11 N3 O4	O-Ureidohomoserine	88.48	–
44	673.500	8.02	C38 H71 N O8	GlcCer(d14:2(4E,6E)/18:0)	91.92	–
45	208.100	2.03	C9 H14 N4 O3	Carnosine	87.13	Synthesized *in vivo*
46	731.500	8.77	C40 H81 N2 O7 P	PE-Cer(d14:1(4E)/24:0(2OH))	89.71	Lipid
47	310.185	9.73	C16 H31 Cl O	2-chloropalmitaldehyde	80.31	Lipid
48	222.200	2.50	C15 H28 O2	2,5-dimethyl-2E-tridecenoic acid	91.22	Fatty acid
49	340.200	3.76	C17 H28 N2 O5	Perindoprilat	72.99	ACE inhibitor
50	610.356	7.16	C31 H52 N2 O5 S	Valnemulin	92.42	Pleuomutilin antibiotic

Table 2.

The number of common compounds found from exhaled breath condensate samples using different vertical notch filter lengths. The table lists the number of compounds common for filter length combinations in liquid chromatography-mass spectrometry (LC-MS) negative and positive electrospray ionization modes.

	LC-MS ESI negative mode count	LC-MS ESI positive mode count	LC-MS ESI total count
Total	744	5481	6225
Common all filters	458	3439	3897
Common in 2 filters	148	1111	1259
Unique	68	575	643
23 and 28 mm	113	540	653
23 and 33 mm	35	336	371
28 and 33 mm	0	235	235
23 mm	29	179	208
28 mm	39	396	435
33 mm	0	0	0