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Headspace sorptive extraction-gas chromatography–mass spectrometry method to measure volatile emissions from human airway cell cultures

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Abstract

The human respiratory tract releases volatile metabolites into exhaled breath that can be utilized for noninvasive health diagnostics. To understand the origin of this metabolic process, our group has previously analyzed the headspace above human epithelial cell cultures using solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS). In the present work, we improve our model by employing sorbent-covered magnetic stir bars for headspace sorptive extraction (HSSE). Sorbent-coated stir bar analyte recovery increased by 52 times and captured 97 more compounds than SPME. Our data show that HSSE is preferred over liquid extraction via stir bar sorptive extraction (SBSE), which failed to distinguish volatiles unique to the cell samples compared against media controls. Two different cellular media were also compared, and we found that Opti-MEM® is preferred for volatile analysis. We optimized HSSE analytical parameters such as extraction time (24 h), desorption temperature (300 °C) and desorption time (7 min). Finally, we developed an internal standard for cell culture VOC studies by introducing 842 ng of deuterated decane per 5 mL of cell medium to account for error from extraction, desorption, chromatography and detection. This improved model will serve as a platform for future metabolic cell culture studies to examine changes in epithelial VOCs caused by perturbations such as viral or bacterial infections, opening opportunities for improved, noninvasive pulmonary diagnostics.

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Conflict of interest disclosure

The authors have no conflicts to declare.

1. Introduction

Human respiratory tract infections are a leading cause of chronic lung disease exacerbations [1, 2] and they contribute to a substantial cost of treatment for chronic lung diseases [3]. The ability to noninvasively detect respiratory tract infections, including viruses [4, 5] and bacteria [6], may aid in prompt diagnosis and treatment, leading to improved clinical outcomes. Thus, noninvasive disease detection is an attractive concept, but current platforms are limited in the number of studies and the methods used [6].

Noninvasive respiratory analysis relies on the idea that the human respiratory tract emits both local- and systemically-derived volatile and semi-volatile metabolic compounds that can be captured, analyzed and correlated to disease states [7]. The human respiratory tract has many cell types that reside in both air and liquid environments, complicating the ability to identify the exact cellular source of chemical compound production. This has prompted *in vitro* cell culture studies designed to identify the site(s) of compound production and correlate changes in production as cells are infected or otherwise perturbed. We have previously shown that cultured human airway and immune cells emit metabolic compounds into air at baseline [8] and that VOC levels change when cells are infected with human rhinovirus [4] or influenza virus [5].

Other prior studies for airway relevant cell culture volatiles have used solid-phase microextraction (SPME) fibers, with methods developed by Arthur and Pawliszen [9]. The SPME fiber is coated with a sorbent, typically polydimethylsiloxane (PDMS), which absorbs volatile organic compounds (VOCs) such as lightweight metabolites. After extraction, the SPME fiber is placed directlyinto the inlet of a gas chromatogram (GC), thermally desorbing the VOCs and allowing for chromatography and detection. While generally a good laboratory technique, SPME does have potential limitations including a reduced capacity of analyte detection due to the small volume of sorbent on the fibers. Consequently, cellular VOCs may have been present in concentrations below the limit of detection for SPME concentration, as volatile emission rates from epithelial cells are not yet understood. In untargeted metabolomic screening studies, it is necessary to extract as many metabolites as possible to explore potential correlations of metabolites to cell pertubations.

Baltussen et al. developed magnetic stir bars coated with PDMS sorbent [10]. The volume of sorbent is larger relative to SPME, increasing the recovery of analytes and thus enhancing sensitivity. Tienpont et al. [11] and Bicchi et al. [12] established headspace sorptive extraction (HSSE), where the stir bars are suspended above a liquid matrix to extract volatile analytes. The stir bars can also be placed directly into the liquid matrix, known as stir bar sorptive extraction (SBSE), to extract analytes in liquid [10]. After sampling, analytes are recovered from the PDMS by thermal desorption and are analyzed, commonly with gas chromatography-mass spectrometry (GC-MS).

In this study, we present a modifiied HSSE collection system for metabolites from a human airway cell culture system. We compare the metabolite recovery using the HSSE method to our previous SPME method and, for the first time, compare HSSE and SBSE methods for epithelial cell VOC extraction. The goal was to determine which method results in increased

cell culture metabolite detection. We also compared cellular growth media effects and optimized analytical parameters such as extraction time, desorption time, and temperature. Finally, we developed an internal standard to account for analytical error in extraction and chromatography. Our long term goal is to incorporate this optimized HSSE method into future *in vitro* human airway culture system experiments as a superior metabolite recovery platform. This methodology will advance the field of *in vitro* metabolite analysis from cell cultures, potentially even those with non-epithelia cultures.

2. Materials and methods

2.1 Cell line

All media additives were purchased from Sigma Aldrich (St. Louis, MO). The immortalized NHBE cell line, HBE1[13], was plated on Transwell (Coring Costar, Corning, NY) chambers (12 mm) at 1–2 × 10^4 cells/cm^2 in the growth medium; LHC Basal Medium (Life Technologies, Carlsbad, CA) supplemented with insulin (5 μg/mL), transferrin (10 μg/mL), epidermal growth factor (24 ng/mL), hydrocortisone (0.1 μM), triiodothyronine (0.01 μM), bovine hypothalamus extract (10 μg/mL), bovine serum albumin (0.5 mg/mL), epinephrine (0.6 μg/mL), phosphorylethanolamine (0.5 μM), ethanolamine (0.5 μM), zinc sulfate (3 μM), ferrous sulfate (1.5 μM), magnesium chloride (0.6 mM), calcium chloride (0.11 mM) and trace elements (selenium, manganese, silicone, molybdenum, vanadium, nickel sulfate and tin) [14]. Once HBE1 cultures were confluent, they were transferred to ALI culture conditions in Pneumacult ALI medium (StemCell Technologies, Vancouver, BC) for 5–7 days.

2.2 VOC extraction

The transwells containing confluent cells were placed into sterile borosilicate glass jars filled with 5 mL of Opti-MEM® (OPT) medium (ThermoFisher Scientific) and capped with screw on lids containing a Teflon insert. Cells grown on the transwells are oriented so the apical side is exposed to the headspace in the jar and the basolateral side is bathed in OPT medium. The internal standard was introduced by dissolving 1 μL of the standard solution (either 0.1 mL/L or 10 mL/L of decane-D22 in ethanol) per 5 mL of cell media. Headspace sorptive extraction (HSSE) and stirbar sorptive extraction (SBSE) were performed with Twisters® (Part 011222-001-00, Gerstel, Inc., Linthicum, MD), which were 10 mm long and coated with a 0.5 mm thick polydimethylsiloxane layer. Beforehand, Twisters® were cleaned per manufacturer recommendations and sterilized in 70% aqueous ethanol prior to introduction to cultures. For HSSE, Twisters® were held in place with a magnet on the outside of the Teflon insert of the lid. For SBSE, Twisters® were placed directly into the liquid medium. SPME fibers coated with 100 μm of PDMS (Part 57300, Millipore Sigma, St. Louis, MO) were introduced by piercing the fibers through the Teflon insert of the lids. All of the sampling was performed inside enclosed jars placed in an incubator at 37 °C.

Within one set of experiments, extractions started at t=0 h. Six hours extractions were removed at t=6 h, etc. Cells were confluent at t=0 but were also undergoing normal senescent during the duration of each experiment. During the sampling period, the lids were left ajar for 3 min every 12 h to allow for normal cellular respiration.

2.3 Cell viability

To monitor metabolic activity of the cells, an alamarBlue® cell viability reagent was used per manufacturer recommendations (ThermoFisher). This colormetric assay allows resazurin to permeate cells. Living cells reduce resazurin into resorufin, which is a red color. Then, absorbance is measured with a spectrophotometer (Powerwave, Biotek) at 570 nm with normalization at 600 nm. Higher absorbance readings correlate with higher metabolic activity of viable cells.

2.4 Experimental design

The number of cell culture and media control jars used for each optimization varied. Furthermore, the number of technical replicates, or the number of Twisters® per jar, also changed. We summarize these details in Table 1. A visual representation of the cell culture jar and HSSE bars is shown in Figure 1.

2.5 GC-MS analysis

PDMS stir bars are marketed under the name of Twister® (Gerstel, Mülheima/d Ruhr, Germany). Twisters® were loaded into desorption tubes. As an internal standard, a 0.5 μL aliquot of a 50 mg/mL naphthalene-D8 in 100% ethanol was added to every desorption tube to monitor performance of the instruments. This standard was used in every sample; even those with the additional decane-D22 standard. Twisters® were desorbed with a thermal desorption unit (TDU) and cooled injection system (CIS, Gerstel, Inc.). As part of the methodology development, parameters were optimized in the order they appear in **Results and Discussion**; once a parameter was optimized, that value was used throughout the remainder of the study. Initial values were as follows: Twister® desorption occurred in the TDU under a flow of helium. The TDU was initially set to 30 °C for 0.5 min then ramped at 60 °C/min to 250 °C and held for 1 min. Desorbed VOCs were transported via helium flow into the CIS, which remained at −80 °C. After desorption, the VOCs were splitlessly injected into the GC column by heating the CIS at 12 °C/s to 260 °C, holding for 3 min.

Chromatography occurred on a 7890B GC (Agilent Technologies Inc., Santa Clara, CA) with a DB-5ms column, 30 m x 250 μm x 0.25 μm (Part 122-5532, Agilent Technologies Inc., Santa Clara, CA) with a constant 1.5 mL/min flow of helium. The oven was programmed as follows: initial temperature 35 °C for 3 min, then a ramp of 5 °C/min to 75 °C, then 2 °C/min to 220 °C and a final ramp of 30 °C/min to 300 °C, holding for 3 min. A transfer line set at 280 °C lead to a 5977A mass spectrometer (Agilent Technologies Inc., Santa Clara, CA) with a solvent delay of 5 min. The MS scanned the range from 33 to 520 m/z. The MS source was set to 230 °C and the MS quad set to 150 °C. With every batch of samples, a standard mix of C_8-C_{20} alkanes was analyzed to calculate the Kovats Retention Indices and also to monitor the performance of the TDU-CIS-GC-MS system.

For the Twister® and SPME comparison, SPMEs were desorbed using the same GC-MS system with the column swapped from the CIS to a normal GC inlet. The inlet was set to the same desorption temperature as the Twisters® and SPMEs were splitlessly desorbed for the same length of time. An identical GC-MS method (oven ramp rates, MS parameters, etc.) was used to analyze desorbed SPME volatiles.

2.6 Data analysis workflow

Data files were deconvoluted, integrated and aligned using MassHunter Profinder B.08.00 (Agilent Technologies Inc.). Peaks with amplitudes of less than 1000 counts were ignored. Compounds must have been present in at least 60% of replicates from one treatment to be included in statistical analyses. Peak areas were normalized to the naphthalene-D8 internal standard. Statistical analysis was performed using GeneSpring MPP (Agilent Technologies, Inc.), where $p \leq 0.05$ was used throughout to test for statistical significance. T-tests and ANOVA included the Benjamin-Hochberg false discovery rate corrections. Tukey's honest significance difference was applied to ANOVAs *post hoc.* Prior to principal component analysis (PCA), the data was mean centered and scaled to unit standard deviation within each compound, across all samples. Putative compound identification was performed by comparing mass spectra to the NIST 2014 Library and by a comparison of calculated Kovats Indices to reported values, when available.

2.7 Theoretical analyte recovery

Pawliszyn et al. estimated the mass recovery of an analyte through sorbent extraction by the following equation:

$$n = \frac{K_{fs} V_f C_o V_s}{K_{fs} V_f + V_s} \quad (1)$$

Where n is the mass of analyte sorbed by the sorbent; C_o is the original concentration of the analyte in the sample matrix; V_f is the volume of the sorbent coating; V_s is the volume of the liquid sample matrix; and K_{fs} is the partition coefficient of the analyte between the coating and the sample. For most determinations, K_{fs} is relatively insignificant compared to the phase ratio of the sample matrix to coating volume ($V_f \ll V_s$). In this case, the capacity of the sample is much larger compared to the capacity of the fiber, resulting in a simple relationship [15]:

$$n = K_{fs} V_f C_o \quad (2)$$

When both a SPME fiber and Twister® bar with the same type of sorbent are introduced to the same sample matrix, the only theoretical variables that will differ in Equation 2 for a particular analyte will be the mass recovered, n, and V_f, which equals 24 μL for the Twisters® and 0.5 μL for the SPME fibers used herein. Thus, a ratio can be derived (Equation 3) to calculate the theoretical improvement for mass of analyte recovered when using Twister® versus SPME, which is equal to the ratio of sorbent volumes: 48 times greater for Twister®.

$$\frac{n_{Tw}}{n_{SPME}} = \frac{V_{fTw}}{V_{fSPME}} \quad (3)$$

To calculate the actual recovery ratio from Equation 3, the internal standard was used. Direct injections of decane-D22 were made into the GC-MS to produce a 6 point mass calibration curve ranging from 84.2 to 0.0760 ng, with duplicates taken at each mass point. SPME and Twister® sorbents extracted VOCs from the same cell culture jar that was spiked with decane-D22 in the media. Using the mass calibration curve, extracted decane-D22 peak areas from Twister® and SPME samples were converted into mass recovered. These mass values were used to calculate the analyte recovery ratio.

3. Results and discussion

In the present work we developed and optimized a HSSE-GC-MS method using Twisters® to extract volatile organic compounds emitted by cultured human epithelial cells.

3.1 Media Selection

We initially sought a cell culture medium that would support normal cell growth but not overwhelm the volatile background of our model. Two types of media were compared: OPT and phosphate buffered saline (PBS). OPT is a chemically defined, low protein, minimal essential medium that contains insulin, transferrin, hypoxanthine, thymidine and trace elements. PBS is a phosphate buffered saline, which only contains minimal salts to maintain osmolarity (NaCl, KCl, Na_2HPO_4 and KH_2PO_4). From a cellular perspective, OPT is the preferred medium as it provides the epithelial cells with essential nutrients that are not found in PBS. Providing cells with essential nutrients is important to maintain the health of the cells as we make comparisons between infected and uninfected cells. Otherwise, healthy but starved cells (using PBS as the sole growth media) may introduce artefacts that could overwhelm other perturbations such as infection.

From a volatile perspective, OPT and PBS generated similar volatile backgrounds in media controls that did not contain cells (Figure 2). A t-test resulted in 27 compounds with different abundances between the two media controls (Table 2). Of these, 17 compounds were either unique or in higher abundances to the PBS medium and only 10 were unique to OPT, suggesting that PBS media generates slightly more VOC noise.

Cell samples grown in the two types of media were also compared. A t-test found 21 different compounds between the media treatments, with more compounds being either in higher abundance or unique to cell samples grown in PBS. These differences likely arise from metabolic differences caused by the nutrient availability in the different media. Still, a PCA of that data was unable to distinguish PBS and OPT cell samples (data not shown).

Because of the similar VOC backgrounds from media controls and similarities in cell samples, we considered the health of the cells to be the most crucial factor in media selection. We concluded that OPT is the appropriate medium for our cell culture model. While differences may not have been apparent from our VOC techniques with a 24 h extraction, long term effects could arise during studies of cellular infections on the scale of days.

3.2 Headspace versus stir bar sorptive extraction (HSSE versus SBSE)

Airway epithelial cells line the respiratory tract in a confluent layer and act as a protective barrier. They are anchored to a basement membrane (basolateral) from which they receive nourishment, and they are exposed to the air on the apical side. In our cell culture model, cells grow in a monolayer on a permeable membrane that allows for the cells to orient themselves similarly to in the airway. They are bathed in the medium below and are exposed to the jar headspace above. Thus, there is a possibility that these cells could channel VOCs into either the liquid medium or into the air. We compared the two volatile signatures from the liquid below cells (SBSE) and the headspace above cells (HSSE) in cell culture samples and in media controls.

The HSSE measurements yielded far better results with regards to distinguishing cell culture samples from media controls. There were 59 compounds with statistically different abundances with HSSE between media and cell samples. Of these, 55 compounds were found in higher abundances in the cell samples. For SBSE, there were only 9 compounds found to be statistically different between media controls and cells, with 8 of these compounds were in higher abundances in the cell samples. Comparing HSSE cell samples to SBSE cell samples, there were 80 compounds with statistically different abundances and 77 of these compounds were in higher abundances in the HSSE samples.

PCAs of the HSSE and SBSE extractions were generated (Figure 3). Liquid extractions were not successful at differentiating medium samples to cell culture samples. In fact, two SBSE media samples were more like two of the SBSE cell culture samples than to the third media sample. Headspace extractions distinguished medium controls from cell samples along the first principal component. These results suggest that epithelial cells send more VOC signals into the airway than into the basement membrane. Thus, HSSE was chosen as the preferred VOC extraction method for sampling epithelial cell analyte production.

3.3 Extraction time

Extraction time is well-known to affect analyte recovery. In non-changing, static sampling environments, minimum extraction times of 8 h have been reported to allow the liquid, air and sorbent phases to reach equilibrium [16]. Our system is dynamic, as VOCs are continuously generated by the cell cultures. The rates at which epithelial cells emit VOCs have not yet been studied, and so it is also unknown how quickly the headspace VOC concentration would accumulate to allow volatiles to partition into the PDMS sorbent. Three extraction times were compared: 6, 24 and 48 h.

Longer extraction times from cell samples resulted in more compounds and higher intensities of peaks. Of the 86 different compounds between cell samples extracted for 6 and 24 h, only 5 of these compounds were in lower abundances in the 24 h extraction. In other words, 79 compounds increased in intensity as extraction time increased from 6 to 24 h. Similarly, of the 194 statistically different compounds from cell samples at 6 and 48 h, only 5 had lower abundances in the 48 h extraction. At this point, it is unclear if these VOC differences were unique to longer extraction times or were in conjunction with cellular metabolism. These metabolic processes are not yet understood and may have introduced

diurnal or other natural artefacts that influenced the number and intensities of VOCs captured by the Twisters®; however, we saw a similar trend with the media controls.

Media samples also saw increases to the number of peaks and their intensities with longer extraction times. This affected the ability to distinguish media controls from cell samples. Comparing compounds found in both cell samples to media controls within one extraction time point, the 24 h extraction yielded the largest number (97) of statistically different compounds (Table 3). In the 6 h extraction, only 67 compounds were different between cell samples and media controls and, smaller yet, the 48 h extraction only yielded 22 different compounds. Most of these distinguishing compounds were found in higher abundances in cell samples: comparisons of media controls and cell samples extracted at 6, 24 and 48 h had only 4, 7 and 7 compounds, respectively, in higher abundances from the media controls.

Looking at compounds unique to cell cultures and not found in media, the 48 h extraction only had 2 unique compounds. The 6 and 24 h extractions had 64 and 62 unique cell compounds, respectively, in cell culture samples (Figure 4). Thus, we concluded that 24 h was the most appropriate extraction time. It captured the largest number of unique cellular VOCs while still maintaining a distinction from the media control samples.

We putatively identified cell volatiles by matching mass spectra to the NIST 14 library. Half of the compounds were classified as either an alkane or an alkene. Also identified were alcohols (17%), aromatics (7%), aldehydes (6%), ketones (6%), acids (4%) and esters (2%) in addition to others (8%). Example compounds are presented in Table 4. This VOC profile is in general agreement with other studies of human respiratory cell cultures [4, 5, 17, 18]. While using PDMS Twisters® biased our results towards apolar compounds, a study of human laryngeal cancer cells using divinylbenzene/carboxen/polydimethylsiloxane sorbents, which extract both polar and apolar compounds, also reported a high number of apolar compounds, [19] As the intent of our work was to optimize the sampling method, we did not make further attempts to identify cell VOCs. We will further identify metabolites in future studies of epithelial cell cultures.

3.4 Desorption time

Following analyte extraction, TDU desorption times of 5, 7 and 10 min were tested. Three jars were used in this experiment: one jar of cell media and two jars of cells. Each jar had 9 Twisters® and 3 each were analyzed with each desorption time. As a result, there were no clear separation among desorption times per a PCA (**Supplemental 1**). Cell culture samples were distinguished from media samples in all three desorption times. However, 7 and 10 min desorption times decreased carryover for heavier volatile compounds compared to 5 min (data not shown). A t-test between 7 and 10 min cell culture samples yielded zero compounds of significantly different abundances. Therefore, a 7 min desorption time was chosen for the rest of the experiment.

3.5 Desorption temperature

TDU desorption temperatures of 250, 275 and 300 °C were compared. Three jars were used in this experiment: one jar of cell media and two jars with cells. Each jar had 9 Twisters®

and 3 each were analyzed with each desorption time. There was a clear tendency towards desorption temperature separation among the jars (Figure 5) along the first principal component, which explained 19.74% of the variance. Along PC2, which explained 11.52% of the variance, media controls separated from cell culture samples with great success among Twisters® desorbed at 300 °C. Raw chromatograms, while not shown here, showed that 300 °C desorbed more chemicals with higher intensities compared to other desorption temperatures. This is confirmed by an ANOVA: increasing desorption temperature from 250 to 275 °C significantly affected 24 cell culture compounds, with 23 having higher intensities with the higher desorption temperature. Similarly, from 275 to 300 °C, 49 cellular VOCs were affected, with 43 having higher intensities with higher desorption temperatures. A desorption temperature of 300 °C was chosen for the rest of the experiment.

3.6 Selection of internal standard

In this study, all samples included a naphthalene-D8 internal standard that was added after extraction into desorption tubes. This accounted for error from desorption, chromatography and detection. To account for error from extraction, a second internal standard was used. As many cell VOCs were putatively identified as a type of alkane, deuterated decane (decane-D22) was chosen. We initially tested a concentration of 0.1 mL/L decane-D22 in ethanol but this concentration was too low to be measured by HSSE-GC-MS. A concentration of 10 mL/L decane-D22 could be measured and had an appropriate peak height, between ~1 and 3 $\times 10^6$ counts. Furthermore, the base peak for deuterated decane, 66.1 m/z, was only seen from the internal standard and did not appear in the rest of the entire chromatogram.

Cell viability, as measured with a spectrometer, was not considered to be significantly different per a student's t-test between samples spiked with deuterated decane and control samples (**Supplemental 2**). Cell volatiles were also not affected by the addition of the internal standard. An unpaired t-test did not find any statistically different VOCs between the control and deuterated samples, with the exception of decane-D22. A PCA analysis did not distinguish the treatments (**Supplemental 2**). Thus, we concluded that an addition of 842 ng of decane-D22 to 5 mL of media per jar was an appropriate amount for our cell culture model.

3.7 Twister® Versus SPME

The Twister® versus SPME experiment was conducted in a single cell culture jar. Twisters® captured a total of 143 compounds while 46 compounds were captured with SPME. Thirty-two compounds were captured in both HSSE methods, while 111 compounds were unique to Twister® extraction (Table 5). Raw chromatograms showed that the Twister® captured higher intensities of compounds that were also extracted by SPME (Figure 6).

Theoretically, Twister® should improve mass recovery by 48 times relative to SPME, per Equation 3. The internal standard was used to compare extraction capabilities. A 6 point mass calibration curve of decane-D22 was used to calculate the mass recovered from Twister® and SPME samples. Twister® improved recovery from cell culture jars by 52 times compared to SPME. Our actual recovery improvement was similar to the theoretical improvement. From the results, we concluded that in the case of detecting low concentration

VOCs in the headspace, such as in cell culture headspace extraction experimental system, Twister® has a better recovery compared to SPME due to the larger volume of PDMS sorbent.

4. Conclusions

We have presented an improved method for extracting volatile compounds from human epithelial cell cultures. Various analytical parameters were tested and results are summarized in Table 6. By employing Twisters®, we have vastly enhanced VOC detection from these cell culture models compared to SPME.

As we strive to improve noninvasive diagnostic platforms for human breath, optimizing VOC detection methods is a critical step. Human airway cells, a principle cell type involved in respiratory tract disease, are biologically complex and emit VOCs at low concentrations. The HSSE-GC-MS method presented here is an improvement over prior methods in terms of increased VOCs recovered. This method addresses the low-abundance VOCs from human airway cells, and it is relatively easy to perform. Future applications will include assessing metabolites from airway cells after viral or bacterial infection, after allergic sensitization and allergen challenge, and after exposure to a host of topical medications. Our method will help advance the general field of volatile analytics and the focused field of human airway cell biology.

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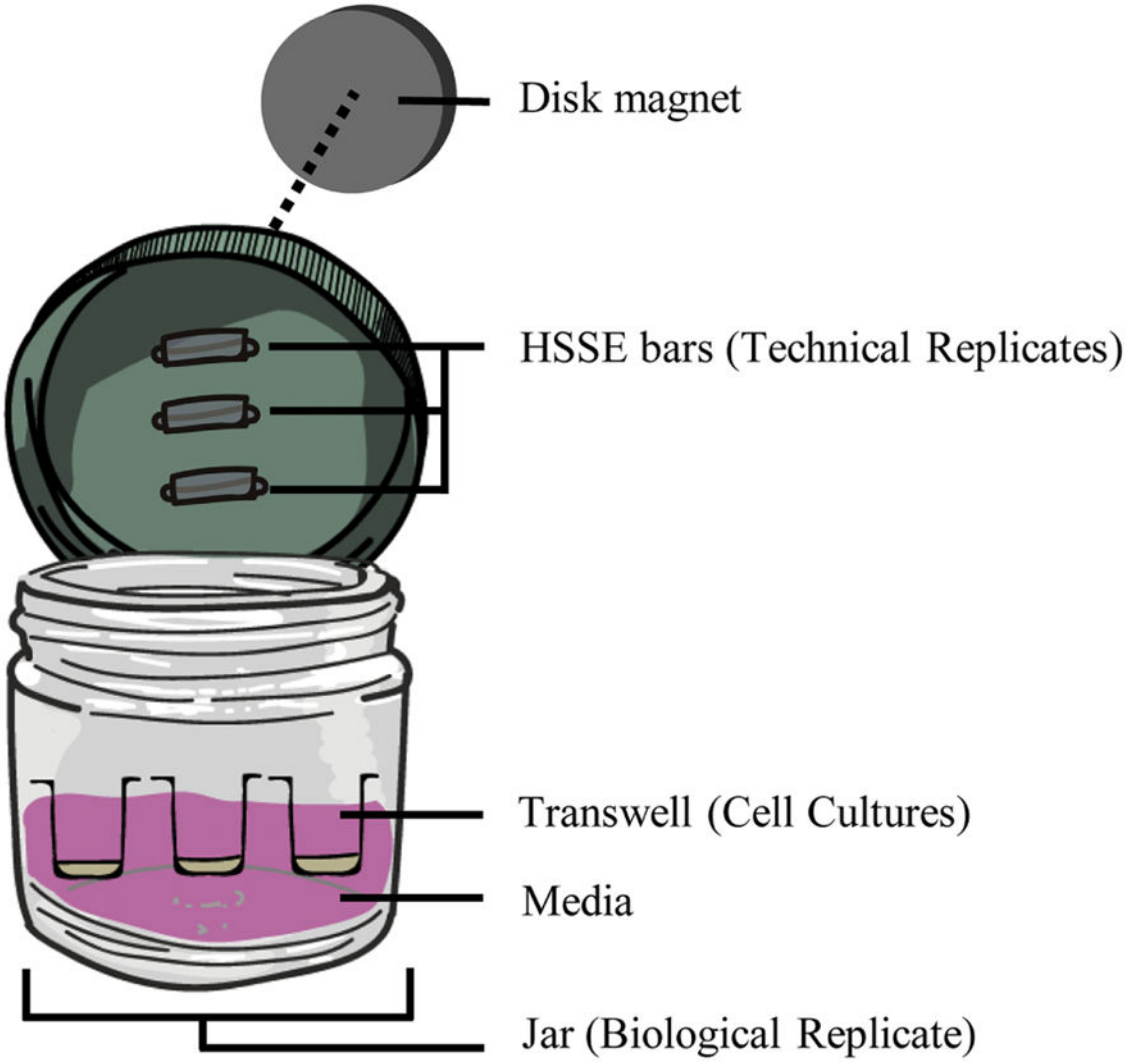

Figure 1.
A typical cell culture jar. Magnets hold PDMS-coated stir bars in place for headspace extraction. Transwells contain an immortalized epithelial cell line, which lives as a confluent layer on top of the media.

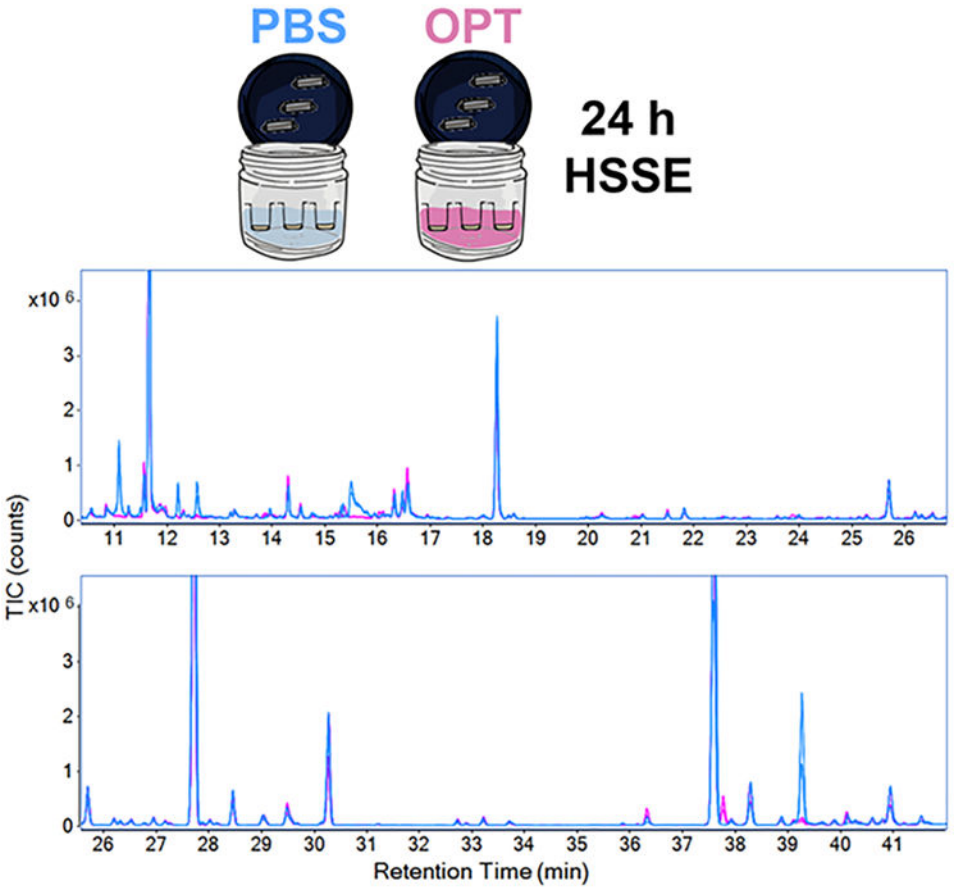

Figure 2.

Raw chromatograms of media control samples. Background VOCs from the two media types were very similar; Opt-MEMS (OPT) was chosen as the optimum medium as it provides more nutrients to cells compared to the phosphate-buffer solution (PBS).

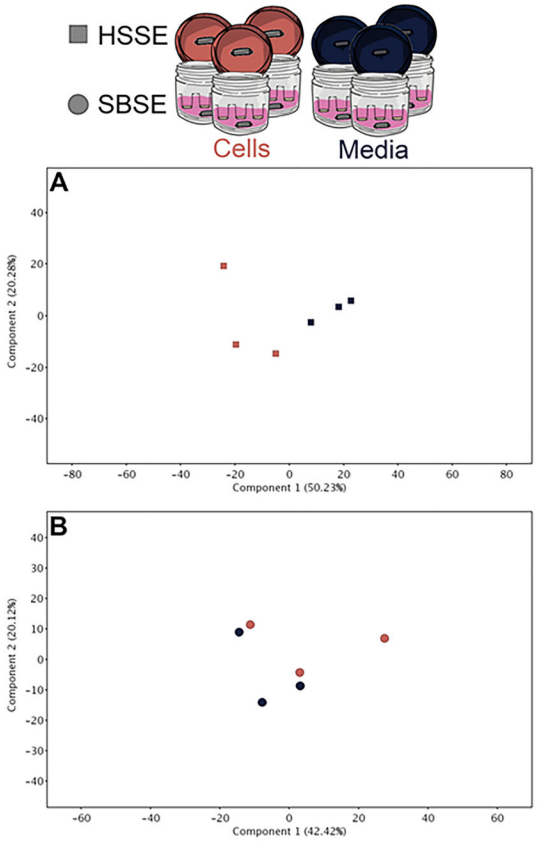

Figure 3.

PCAs of headspace extraction (HSSE, A) and liquid extraction (SBSE, B) for cell cultures and media control samples. HSSE was able to distinguish epithelial VOCs from the media along the first principal component whereas SBSE was not.

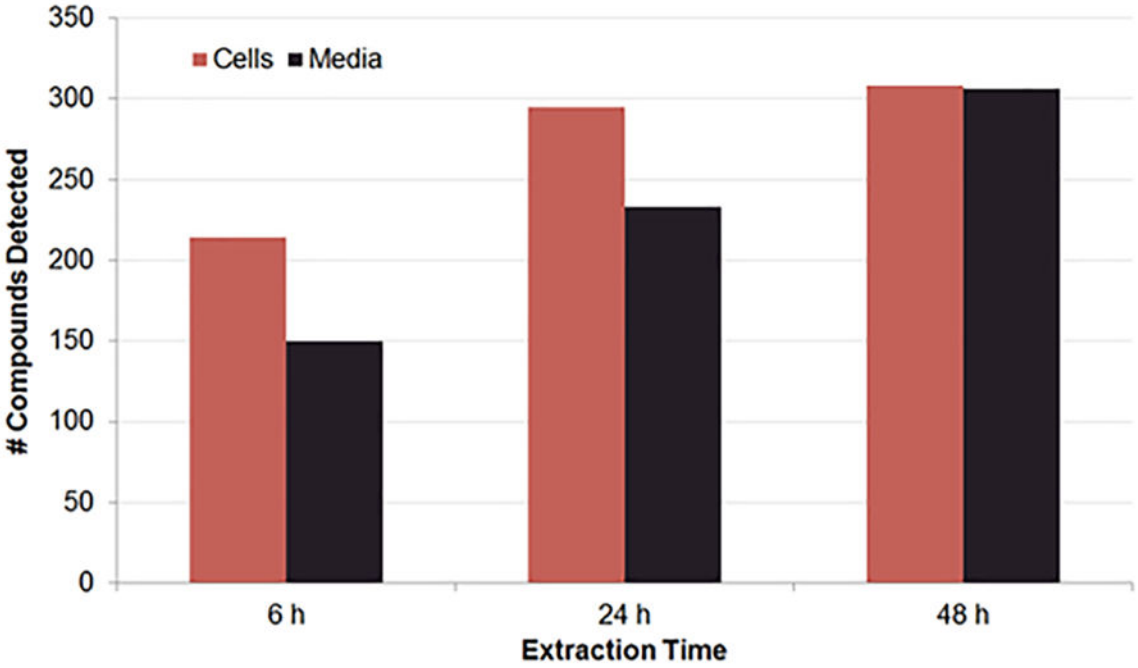

Figure 4.

Number of discrete compounds detected from epithelial cells and media controls at three HSSE extraction times. The 24 h samples extracted the highest number of cellular VOCs while still being distinguishable from media controls.

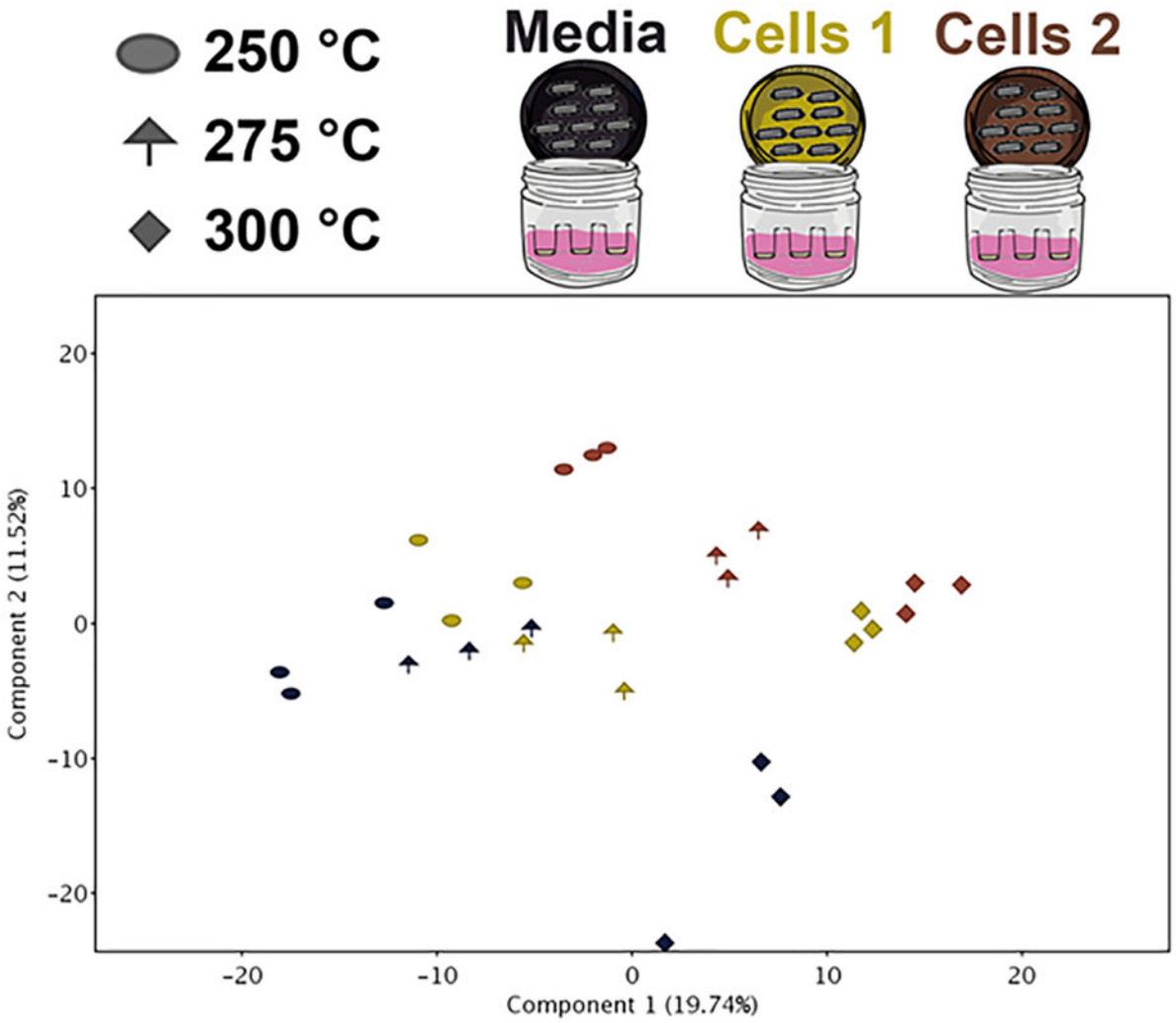

Figure 5.

PCA comparing desorption temperatures. Nine samples were taken per jar and were split into three desorption temperatures. The highest, 300 °C, gave the best separation between media and cell culture samples.

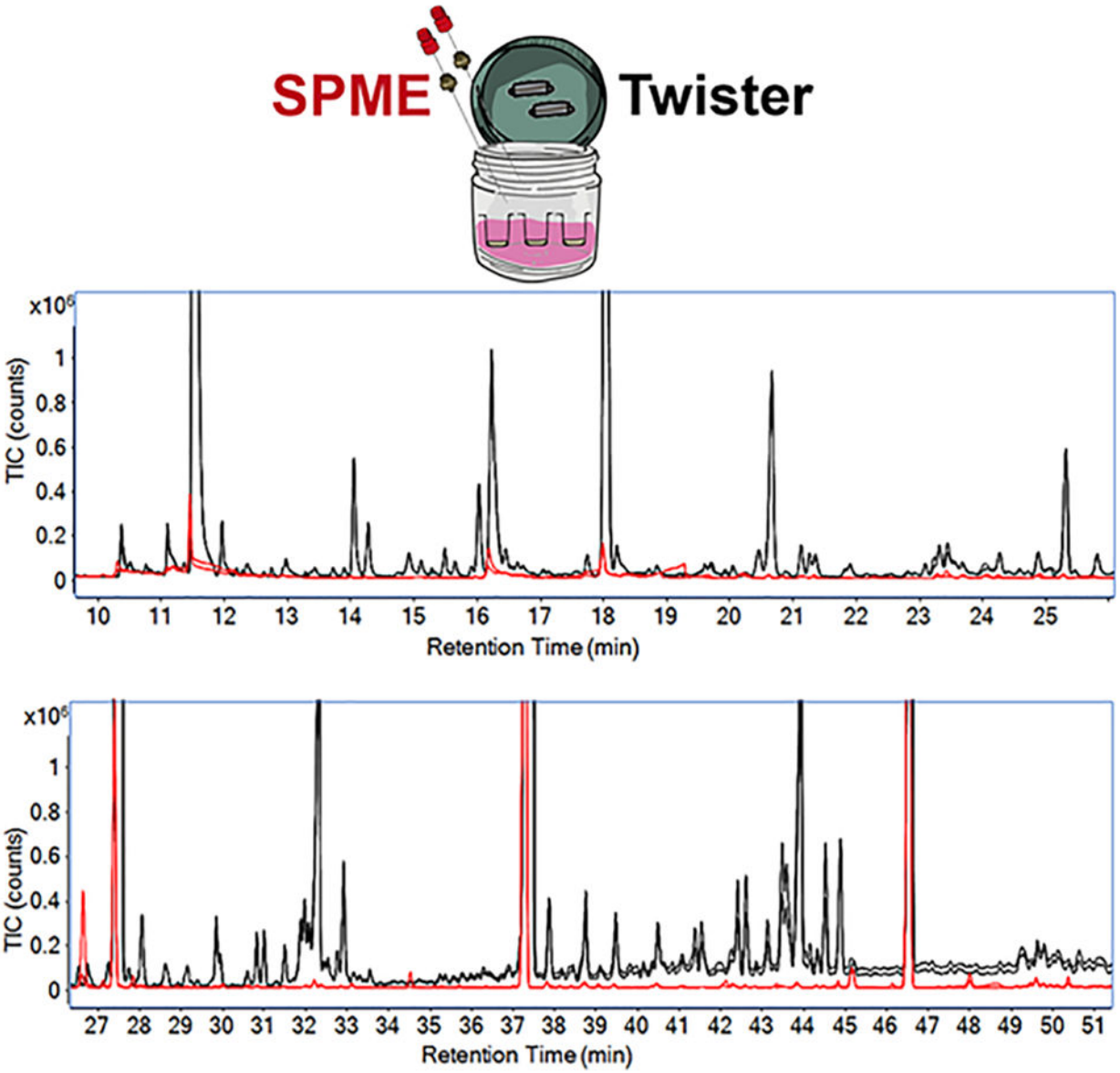

Figure 6.

Raw chromatograms of SPME versus Twister® samples from the same jar of cells. Twisters® extracted more cellular VOCs due to the larger amount of sorbent compared to the SPME fibers.

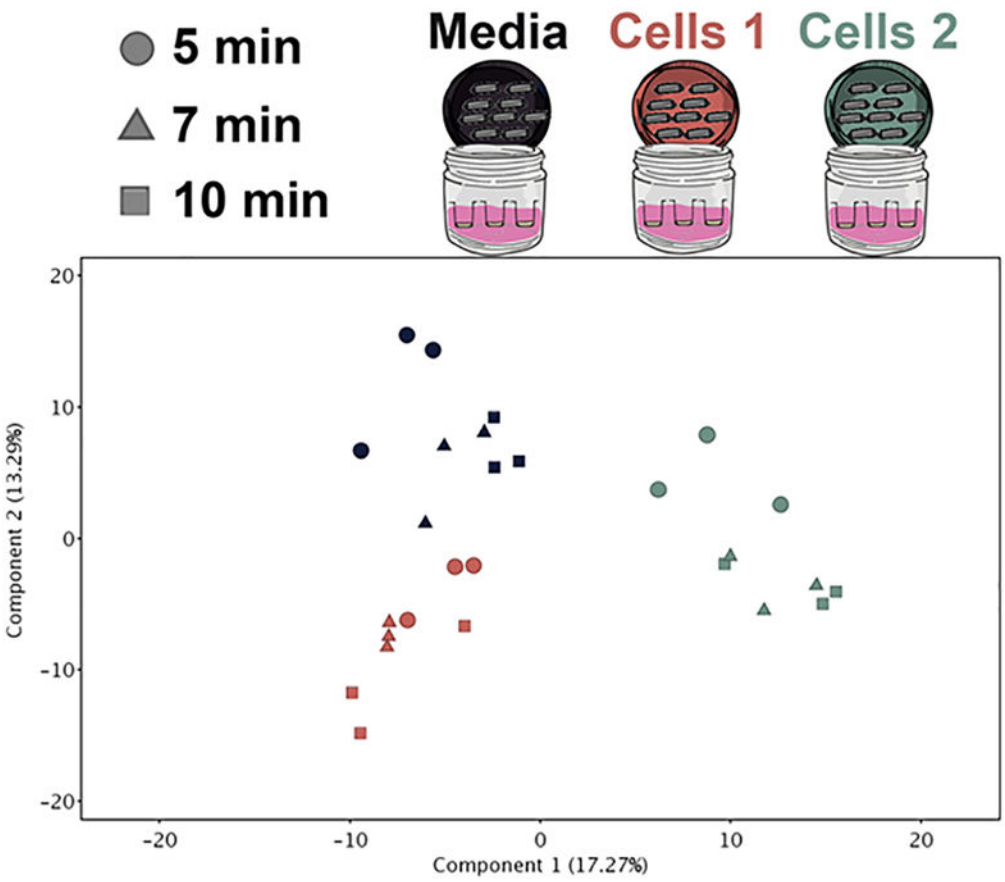

Figure 7.

A

	Medium	Deuterated-1	Deuterated-2	Deuterated-3	Control-1	Control-2	Control-3
Well 1							
Rep1	-0.051	0.105	0.120	0.113	0.100	0.107	0.106
Rep2	-0.050	0.094	0.113	0.116	0.109	0.110	0.109
Rep3	-0.057	0.112	0.111	0.113	0.110	0.109	0.106
AVERAGE	**-0.053**	**0.104**	**0.115**	**0.114**	**0.106**	**0.109**	**0.107**
STDEV	**0.004**	**0.009**	**0.005**	**0.002**	**0.006**	**0.002**	**0.002**
Well 2							
Rep1		0.113	0.122	0.110	0.109	0.111	0.109
Rep2		0.115	0.119	0.119	0.113	0.113	0.114
Rep3		0.117	0.114	0.125	0.117	0.109	0.113
AVERAGE		**0.115**	**0.118**	**0.118**	**0.113**	**0.111**	**0.112**
STDEV		**0.002**	**0.004**	**0.008**	**0.004**	**0.002**	**0.003**
Well 3							
Rep1		0.111	0.117	0.122	0.112	0.113	0.109
Rep2		0.115	0.118	0.122	0.113	0.113	0.111
Rep3		0.115	0.119	0.121	0.117	0.114	0.109
AVERAGE		**0.114**	**0.118**	**0.122**	**0.114**	**0.113**	**0.110**
STDEV		**0.002**	**0.001**	**0.001**	**0.003**	**0.001**	**0.001**
TOTALS							
AVERAGE		**0.111**	**0.117**	**0.118**	**0.111**	**0.111**	**0.110**
STDEV		**0.007**	**0.004**	**0.005**	**0.005**	**0.002**	**0.003**

B

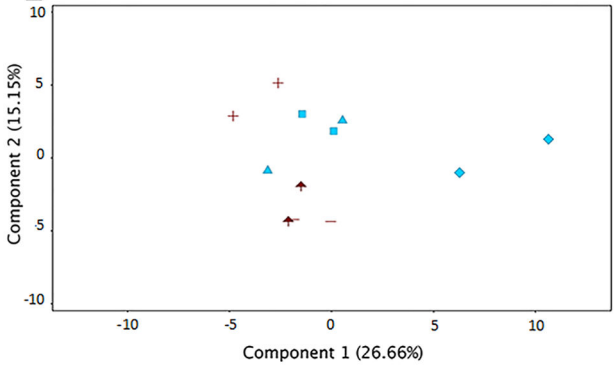

Figure 8.

Table 1

Experimental design. Visualization of a typical cell culture jar is show in Figure 1. The columns represent the 7 experiments performed.

	Media	Extraction	Extraction Time	Desorption Time	Desorption Temperature	Internal Standard	Technology
Values	OPT, PBS	HSSE, SBSE	6, 24, 48 h	5, 7, 10 min	250, 275, 300 °C	0.842, 842 ng of decane-D22	Twister®, SPME
# Biological Replicates (Jars with cell cultures)	6 total 3 OPT, 3 PBS	3 total	9 total 3 per extraction time	2 total	2 total	6 total 3 with internal standard, 3 without	1 total
# Media Control Replicates (Jars without cell cultures)	2 total 1 OPT, 1 PBS	3 total	9 total 3 per extraction time	1 total	1 total	None used	None used
# Technical Replicates per jar	3 HSSE per jar	1 HSSE and 1 SBSE per jar	1 HSSE per jar	3 HSSE replicates per desorption time per jar 9 HSSE replicates total per jar	3 HSSE replicates per desorption temperature per jar 9 HSSE replicates total per jar	2 HSSE per jar	2 HSSE and 2 SPME per jar

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Table 2

Number of statistically different compounds per an unpaired t-test ($p<0.05$). Media controls were only compared to each other and cell samples were only compared to each other. No t-tests were performed between media controls and cell samples.

	Media Controls	Cell Samples
Unique to OPT	10	1
Higher in OPT	0	4
Higher in PBS	2	10
Unique to PBS	15	6
Total Statistically Different Compounds	**27**	**21**

Table 3

Number of compounds found in statistically different abundances (ANOVA) between cell culture and media control samples, extracted for 6, 24 and 48 h ($p<0.05$).

	6 h Media	6 h Cell	24 h Media	24 h Cell	48 h Media	48 h Cell
6 h Media	--	67	112	31	127	117
6 h Cell	67	--	202	86	208	194
24 h Media	112	202	--	97	14	27
24 h Cell	31	86	97	--	115	84
48 h Media	127	208	14	115	--	22
48 h Cell	117	194	27	84	22	--

Table 4

Brief list of putatively identified epithelial cell volatiles.

Chemical Group	Example compounds
Acid	Octanoic acid; Hexadecanoic acid
Alcohol	7-Tetradecanol; 3,7,11-trimethyl-1-dodecanol
Aldehyde	Nonanal; Decanal
Alkane	Decane; Undecane; Heptadecane
Alkene	1-Hexadecene; 2-Methyl-1-nonadecene
Ketone	2-Hydroxy-2-cyclopenten-1-one; 2-Undecanone

Table 5

Number of VOCs detected with Twister® and SPME from the cell culture jars.

	Number of VOCs captured
Only in SPME	14
Twister® and SPME	32
Only in Twister®	111

Table 6

Optimized parameters for HSSE-GC-MS measurements of epithelial cell culture VOCs.

Parameter	Optimal Value	Considered Alternatives
Medium	Opti-MEM®	Phosphate-buffer saline
Extraction Technique	HSSE	SBSE, SPME
Extraction Time	24 h	6, 48 h
Desorption Time	7 min	5, 10 min
Desorption Temperature	300 °C	250, 275 °C
Internal Standard per 5 mL Media	842 ng	0.842 ng