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Headspace stir bar sorptive extraction–gas chromatography/mass spectrometry characterization of the diluted vapor phase of cigarette smoke delivered to an *in vitro* cell exposure chamber

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ABSTRACT

Advanced smoke generation systems, such as the Borgwaldt RM20S® smoking machine used in combination with the BAT exposure chamber, allow for the generation, dilution and delivery of fresh cigarette smoke to cell or tissue cultures for *in vitro* cell culture analyses. Recently, our group confirmed that the Borgwaldt RM20S® is a reliable tool to generate and deliver repeatable and reproducible exposure concentrations of whole smoke to *in vitro* cultures [1]. However, the relationship between dose and diluted smoke components found within the exposure chamber has not been characterized. The current study focused on the development of a headspace stir bar sorptive extraction (HSSE) method to chemically characterize some of the vapor phase components of cigarette smoke generated by the Borgwaldt RM20S® and collected within a cell culture exposure chamber. The method was based on passive sampling within the chamber by HSSE using a Twister™ stir bar. Following exposure, sorbed analytes were recovered using a thermal desorption unit and a cooled injection system coupled to gas chromatograph/mass spectrometry for identification and quantification. Using the HSSE method, sixteen compounds were identified. The desorption parameters were assessed using ten reference compounds and the following conditions led to the maximal response: desorption temperature of 200 °C for 2 min with cryofocussing temperature of −75 °C. During transfer of the stir bars to the thermal desorption system, significant losses of analytes were observed as a function of time; therefore, the exposure-to-desorption time interval was kept at the minimum of 10 ± 0.5 min. Repeatability of the HSSE method was assessed by monitoring five reference compounds present in the vapor phase (10.1–12.9% RSD) and n-butyl acetate, the internal standard (18.5% RSD). The smoke dilution precision was found to be 17.2, 6.2 and 11.7% RSD for exposure concentrations of 1, 2 and 5% (v/v) cigarette vapor phase in air, respectively. A linear response of analyte abundance was observed as a function of dilution. Extrapolation to 100% (v/v) cigarette vapor phase, *i.e.*, undiluted smoke, gave yields for the five compounds ranging from 6 to 450 ng for 10 min exposure.

1. Introduction

Recent advancements have led to the development of several smoke generation systems such as the Borgwaldt RM20S® [2], the Burghart Mimic Smoker-01® [3,4] and the Vitrocell Smoking Robot VC 10® [5]. Also, this has led to the development of novel *in vitro* exposure systems such as British American Tobacco's (BAT) exposure chamber [2] and the CULTEX system [5]. These systems generate fresh cigarette smoke over a wide range of dilutions (*i.e.*, exposure concentrations) required for *in vitro* cell culture investigations.

The Borgwaldt RM20S® in combination with BAT's exposure chamber using Transwell® inserts enables direct exposure of *in vitro* cellular cultures to whole cigarette smoke at the air–liquid interface (Fig. 1) [2,6,7]. This smoking machine, first commercialized in 2005, can smoke up to four cigarettes simultaneously with the smoke collected into four independent syringes. Each syringe can dilute the cigarette smoke with air in ratios ranging from 1:1.14 to 1:4000 (smoke volume:air volume), which corresponds to a range of 87–0.025% (v/v) cigarette smoke in air. For biological exposures, doses tend to be in the range of 0.4–5% (v/v) cigarette smoke in air [2]. The dose at this range of whole smoke dilution has only been correlated to the mass of total particulate matter (TPM) deposited on a Cambridge filter pad (CFP) placed either before the exposure chamber or within it, on a Transwell® insert [2,6].

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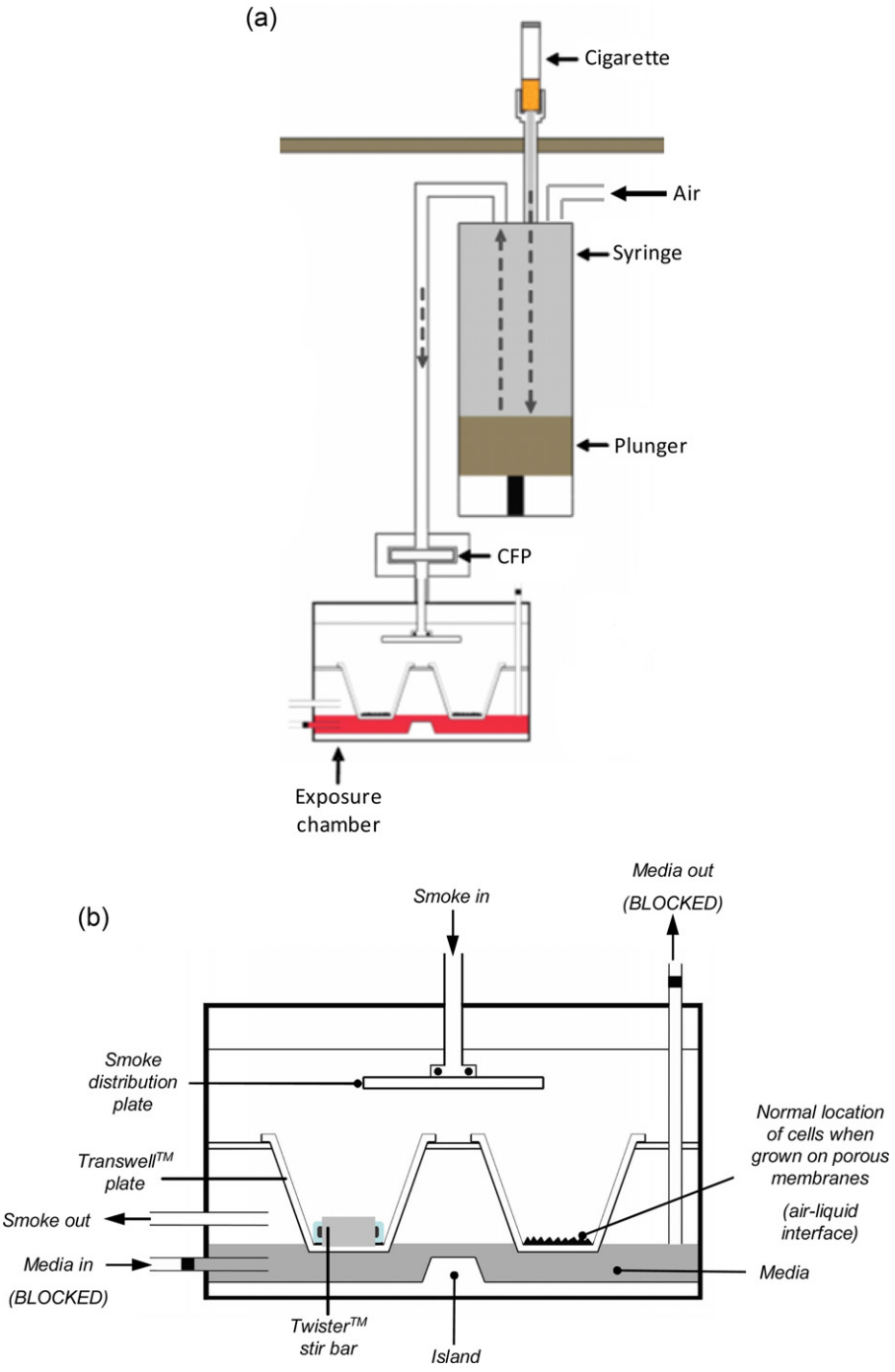

Fig. 1. (a) Schematic of the Borgwaldt RM20S® in combination with the BAT exposure chamber showing one of the four smoking ports connected to a dilution syringe. The machine smokes the cigarette, dilutes the smoke and delivers it to the exposure chamber [7]. Insertion of a Cambridge filter pad (CFP) to trap particulate matter downstream of the syringe allows exposure of cell or tissue culture to the diluted vapor phase only. (b) Cross-section of the exposure chamber [7] showing the location for the Twister™ stir bar for HSSE experiments. For *in vitro* cell culture assays, medium flows in and out of the chamber. In this work, no cells and no culture medium were used.

Our group recently carried out a study to determine the precision and accuracy of dilution of the smoke dose generated by the Borgwaldt RM20S® and delivered to the exposure chamber by measuring two reference standard gases (CH_4 and CO) introduced at the smoking port and a cigarette particulate phase marker (solanesol) from whole smoke [1]. The repeatability of vapor phase dilution was ≤4.5% RSD for dilutions of 0.1–0.52% (v/v) CH_4 in air and was ≤3.7% RSD for dilutions in air of 1–10% (v/v) CO. The accuracy of CO measurements was 5.8–6.4% error for the dilution range studied. The repeatability of dilution of the particulate phase in air ranged from 8.8 to 12% RSD when quantifying solanesol. Overall, the findings suggested that the Borgwaldt RM20S® is a reliable tool to generate and deliver repeatable and reproducible doses of whole smoke to *in vitro* cultures [1]. Scian et al. [4] measured in detail the chemical constituents of the particulate phase and reported recoveries at the exposure chamber of <40% in the Burghart smoking system for most of the compounds monitored, with repeatability of the measurements reaching over 35% RSD for smoke diluted to 50% (v/v) in air. To date, no studies have reported the chemical characterization of the vapor phase smoke components within the exposure chamber

itself. Evaluation of the dosimetry linearity of gaseous compounds present in the cigarette smoke following dilution and transfer to the exposure chamber is important to complete the characterization of this type of *in vitro* cell system.

Cigarette smoke is an extremely complex aerosol mixture composed of over 5000 chemical compounds [8,9] found distributed between the particulate and vapor phases. The vapor phase of cigarette smoke contains volatiles and semi-volatiles that play a major role in the *in vitro* toxicological responses [10–14]. In this study, only the vapor phase of cigarette smoke was targeted. Techniques used for the collection and extraction of vapor phase constituents include vapor–liquid extraction, simultaneous distillation extraction (SDE), solid phase microextraction (SPME) and stir bar sorptive extraction (SBSE). The first two techniques tend to be time consuming and have resulted in poor repeatability [15] compared to SPME and SBSE. SPME using poly(dimethylsiloxane) (PDMS) as the absorptive phase has been used for the analysis of the volatile phase of tobacco flavors [15]. Among other problems with SPME is the reduced sensitivity due to limiting volumes of PDMS that can be used. SBSE, which also relies on PDMS as the sorptive phase, may resolve some of the issues faced when using SPME.

SBSE, which was introduced in 1999 [16], is based on the theory of SPME and has been used extensively for environmental, food and biological applications [17]. SBSE was explored for extraction of cigarette vapor phase components due to its higher mass loading compared to traditional SPME, as it uses a thicker film of adsorbent together with an increased surface area [18]. For the moment, PDMS is the only commercially available SBSE coating. It provides low detection limits; in the sub-ppb level [16,19]. Typical SBSE devices, such as the Twister™ from Gerstel, consist of a magnetic stir bar encased in glass and coated with a $\leq$1 mm film of PDMS, which can contain between 55 and 219 μL of PDMS (compared to $\leq$0.5 μL in SPME) depending on the length of the stir bar. SBSE used to sample the vapor phase is referred to as headspace stir bar sorptive extraction (HSSE) [20–23]. Following exposure to a liquid or gaseous sample, the stir bar is placed within a thermal desorption unit coupled to a GC for separation and analysis. Advantages of HSSE and SBSE include robustness, ease of handling volatile compounds, automation, stability, good reproducibility and application to many types of analytes, heterogeneous samples and vapor, liquid and solid samples. A major drawback associated with both techniques is the difficulty to perform true quantitation even when using an internal standard, particularly in HSSE when only liquid phase standards are available.

The objective of the current study was to develop a solvent-free method to chemically characterize the vapor phase components (volatiles and semi-volatiles) of diluted 3R4F cigarette smoke, generated by the Borgwaldt RM20S® and collected within an exposure chamber. The method chosen is based on passive sampling within the chamber (where cells or tissue cultures would be placed) by HSSE using a Twister™ stir bar. Following exposure to diluted cigarette smoke, sorbed analytes are recovered using a thermal desorption unit (TDS) and a cooled injection system (CIS) coupled to GC/MS for identification and quantification. A central composite experimental design based on three factors was proposed to determine the optimal desorption temperature, desorption time and CIS cryofocussing temperature needed to maximize the peak areas of ten selected reference compounds. Each factor was assessed at three levels. Using the experimental center-point, the repeatability of the HSSE method for diluted cigarette smoke was assessed for five of the vapor phase reference compounds. Recovery of the vapor phase components was measured as a function of time. Lastly, the dilution precision was measured for a range of smoke dilutions typically used for cell culture assays (range of 1–5% (v/v) cigarette smoke in air).

2. Materials and methods

2.1. Whole smoke exposure system: the Borgwaldt RM20S® smoking machine and BAT's exposure chamber

The Borgwaldt RM20S® (Borgwaldt KC GmbH, Hamburg, Germany) is an automatic smoking machine that generates and dilutes cigarette smoke for *in vitro* cell culture investigations [24]. It has a rotary based engine that can simultaneously smoke four types of cigarettes for several hours, depending on the smoking regime used. The instrument has an incorporated anemometer allowing for correct air flow, as well as electrical lighter, butt detector and butt extractor. The Borgwaldt RM20S® was designed in collaboration with BAT (Southampton, UK) and can be used with BAT's exposure chamber to enable cells or tissues to be exposed to the diluted smoke generated by the Borgwaldt RM20S® (Fig. 1a). Within the chamber, the cells or tissues lie on a 24 mm diameter Transwell® clear insert (Corning, NY, USA), which is a microscopically transparent porous polyester membrane, and are exposed to smoke at the air–liquid interface [2,7]. However, no cells/tissues were used in the current study. At the beginning of each day and following each usage, the machine was run through a thorough maintenance routine, as this can affect the performance of the instrument [1]. The cigarettes used in this study were Kentucky Reference cigarettes (3R4F) (University of Kentucky, USA) and were conditioned at 22 °C and 60% relative humidity for 48 h prior to smoking. Cigarettes were smoked in compliance to International Standard Organization (ISO) puffing profiles, consisting of 35 mL puff volume over a 2 s puff duration and 60 s puff interval [25] for a total of 10 min unless otherwise indicated. Despite having four dilution syringes available for use, the same syringe (syringe C) was used throughout this study to eliminate any potential bias that could have been generated by the effect of a given syringe [1]. Cigarette smoke was generated with the Borgwaldt RM20S® and a 44 mm diameter CFP was placed at the inlet of the exposure chamber to capture the particulate phase allowing only the vapor phase of the smoke to enter the exposure chamber. Some semi-volatiles are distributed between both the particulate and vapor phases (*i.e.*, phenols), and a portion of these compounds can be retained on the CFP [26,27]. Within the chamber, the PDMS-coated Twister™ stir bar was placed on a 24 mm diameter Transwell™ plate (Corning, NY, USA) for the duration of the smoke run (Fig. 1b). During exposure, no liquid/medium was used within the exposure chamber, where it would normally be if cells were present. Following exposure to the diluted smoke vapor phase (ranging from 1 to 10% (v/v) in air), the stir bar was removed and transferred to the TDS.

2.2. HSSE sampling and collection

Commercially available Twister™ stir bars from Gerstel (Mülheim an der Ruhr, Germany) were 2 cm in length coated with a 0.5 mm thick PDMS film, which corresponds to 47 μL of PDMS. Conditioning was carried out according to the manufacturer's directions as follows: stir bars were placed in HPLC grade acetonitrile (Fisher Scientific, Whitby, Ontario, Canada) for 2 days and transferred into a specialized thermal conditioning unit for stir bars (Gerstel) at 280 °C for 4 h with a helium flow rate of 40 mL/min. This procedure provided for removal of residual acetonitrile and other impurities present in the PDMS phase. After conditioning, then cooling to room temperature, the stir bars were either exposed to a sample or stored in sealed glass tubes to prevent contamination from the surrounding environment. Following exposure, the stir bar was removed using a piece of stainless steel wire and transferred into an empty glass thermal tube of 4 mm ID and 177 mm length, blocked at both ends (Gerstel). The minimum transfer time between the end of smoke exposure and insertion into the TDS

was 10 ± 0.5 min. Using the optimized desorption method, the stir bar was immediately ready for the next extraction. According to the manufacturer, stir bars may be used for hundreds of extractions with little or no deterioration, as long as temperatures are below 325 °C and solvents are not used for extraction of compounds. Multiple carry-over tests were performed to confirm that stir bars would not contribute to carry-over if re-used. During the experiments involving the use of higher than normal thermal desorption temperatures, stir bars were not re-used if deterioration of the PDMS phase was observed; *e.g.*, if siloxane background peaks were seen in the chromatogram, corresponding to hexamethylcyclotrisiloxane (*m*/*z* 207), octamethylcyclotetrasiloxane (*m*/*z* 281) or decamethylcyclopentasiloxane (*m*/*z* 73, 267 and/or 355).

2.3. Thermal desorption and GC/MS analysis

Analyte determination by GC/MS was carried out with an Agilent 6890N/5973N System (Agilent Technologies, Waldbronn, Germany) equipped with a TDS (Gerstel) connected to a programmable temperature vaporization cooled injection system (CIS-4 model, Gerstel) using a transfer line heated at 300 °C. Desorption was carried out at 200, 250 or 300 °C for 1, 2 or 3 min under a helium flow of 60 mL/min. On the CIS-4 injector, the cryofocussing temperature was set to −100, −75 or −50 °C using liquid nitrogen (Praxair, Danbury, CT, USA). Following cryofocussing, the temperature of the CIS-4 was ramped up to 300 °C at 10 °C/min and held for 2 min during which time the analytes were transferred to the GC column with a split ratio of 37.3:1, unless otherwise indicated. Deactivated quartz wool liners were used (Gerstel). Separation was carried out in a DB-5MS column (60 m × 0.25 mm ID, 0.25 μm film thickness, from Agilent Technologies) with a constant column head pressure of 25 psi helium as the carrier gas. The oven temperature was set to 60 °C for 0 min and increased by 2 °C/min to 108 °C, then held for 1 min, for a total run time of 25 min. Finally, the analytes were detected by the mass selective detector (MSD) in scan mode from 41 to 300 *m*/*z* at 5.29 scans/s, in positive ion mode. For all quantitative work, peak areas were obtained from integration of peaks in the extracted ion chromatograms (XICs).

2.4. Reference compounds

From the compounds identified in the vapor phase of cigarette smoke using the methods described above, five were chosen for use as reference compounds based on their abundances, their wide distribution across the chromatographic elution window and their peak resolution: benzene, 2,5-dimethyl furan, toluene, ethylbenzene and limonene (Thermo Fisher Scientific, ≥95%).

2.5. Preparation of n-butyl acetate internal standard (IS)

A 7.6 μM standard solution of n-butyl acetate (Thermo Fisher Scientific) was prepared in HPLC grade methanol (Thermo Fisher Scientific). The IS concentration was selected based on the abundance range of the analyte ions in our sample run. The standard solution was aliquotted into amber GC vials with crimped aluminum caps (Chromatographic Specialties, Brockville, ON, Canada) for single use and stored at 4 °C until use.

In each case, 1 μL of the IS was transferred directly onto the 2 cm stir bar using a micropipette and left in a closed dish for 15 min to partition into the bulk PDMS. To assess the repeatability of the internal standardization procedure, the stir bar was placed in the center of a TDS tube and transferred to the TD system using the center point conditions (*i.e.*, desorption at 250 °C for 2 min and cryofocussing at −75 °C) of the experimental design as described in the next section. The repeatability was calculated as the percent relative standard deviation (RSD) obtained for the mean XIC peak area of the IS (n = 12, using twelve different stir bars) by GC/MS analysis.

For smoke exposure experiments, stir bars spiked with IS were exposed to diluted vapor phase (10% (v/v) cigarette smoke in air). Following exposure, the stir bar was transferred to the TDS within 10 ± 0.5 min. Repeatability was calculated as the percent RSD (n = 6, using six different stir bars) in mean XIC peak areas of the IS, of each of the five reference compounds listed above, as well as for the ratio reference peak-to-IS.

2.6. Optimization of desorption parameters and statistical analysis

Three factors (TDS desorption temperature, desorption time and CIS-4 cryofocussing temperature) were selected for maximization of the GC/MS peak area response using a face-centered composite experimental design. Three different levels of each factor were selected: desorption temperatures of 200, 250 and 300 °C; desorption times of 1, 2 and 3 min; cryofocussing temperatures of −50, −75 and −100 °C. Other than these parameters, all other conditions were the same as those previously described for exposure of the stir bar to 5% (v/v) vapor phase in air for HSSE–GC/MS experiments. The XIC peak areas from the five reference compounds and an additional five analytes (2-methyl-1,3-butadiene, 3-methyl-2-butanone, p-xylene, styrene and 1-methyl-4-(1-methylethylidene)cyclohexane), were analyzed using a quadratic regression with second order interactions to determine the maximal response for the three experimental factors.

2.7. Stability of sorbed compounds on the stir bar

Following exposure to 5% (v/v) vapor phase in air, the stir bar (*i.e.*, with sorbed sample) was transferred to the TDS at various time intervals to assess the effect of the delay time between vapor phase exposure and thermal desorption on recovery of the sorbed analytes. The times selected for this study were 10, 40, 160 and 1440 min (24 h), where 10 min represented the fastest possible transfer from the exposure chamber to the TDS due to instrumental constraints. For stir bars not immediately analyzed, the desorption tubes were placed in individual plastic containers with caps (having inert inserts), wrapped in foil and stored at 4 °C. Using the optimized TDS and cryofocussing parameters, the XIC peak areas of the five reference compounds were used to assess their sorption persistence on the stir bar after exposure. Each time point was analyzed in triplicate (*i.e.*, n = 3, using three different stir bars).

2.8. Measurement of dilution precision

Stir bars were exposed to various smoke dilution levels typically used for cell culture assays (1, 2 and 5% (v/v) cigarette vapor phase in air) for a 10 min smoking period. The optimized HSSE method and TDS parameters were used for GC/MS measurement of the XIC peak areas of the five reference compounds for each dilution level. Each dilution was analyzed in triplicate (*i.e.*, n = 3, using three different stir bars).

2.9. Semi-quantitative analysis of diluted smoke vapor phase

Standard solutions of the five reference compounds were prepared in HPLC grade hexane (Thermo Fisher Scientific) and calibration curves comprising 4–7 points covering the following ranges were prepared: benzene, 0–250 μM; 2,5-dimethyl furan, 0–50 μM; toluene, 0–100 μM; ethylbenzene, 0–10 μM; limonene, 0–10 μM. These concentration ranges were selected to match the XIC peak areas obtained in the smoke vapor samples. In each case, 1 μL of

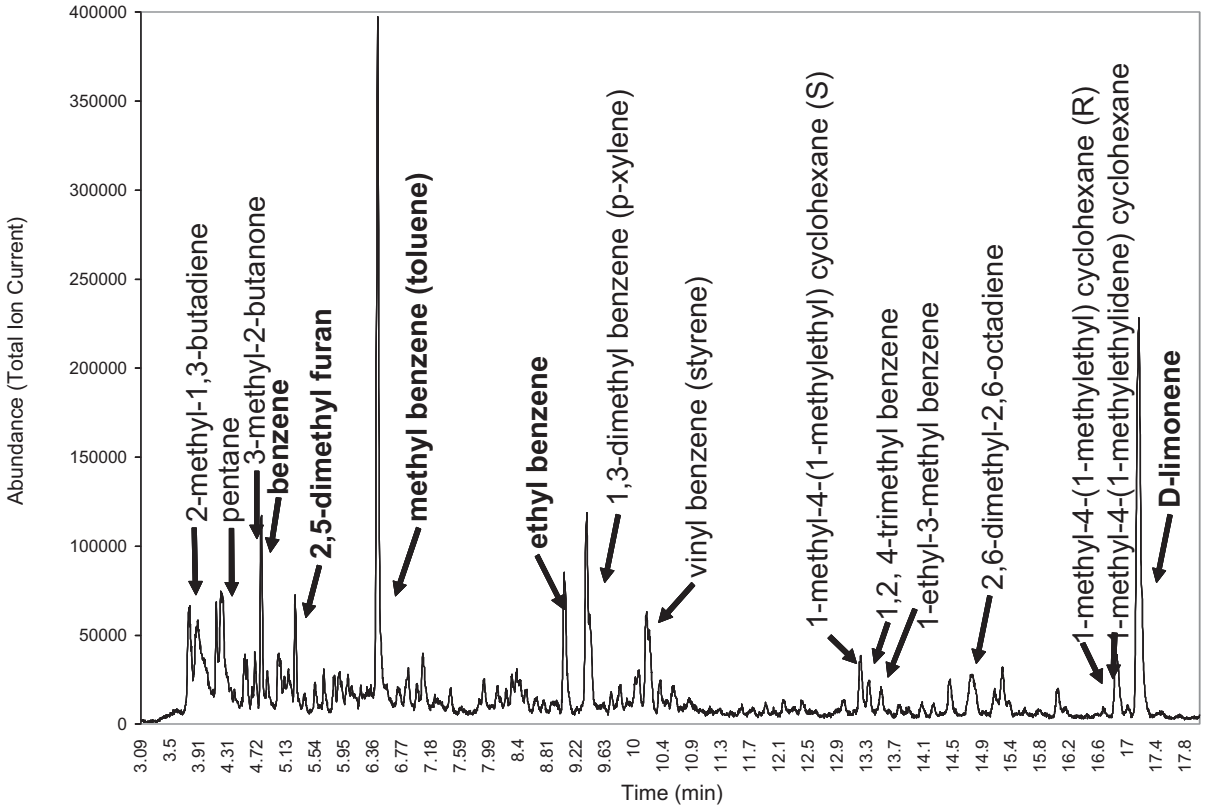

Fig. 2. HSSE–GC/MS chromatogram showing the identification of 16 compounds found in the vapor phase sample. Sorption was carried out using a 2 cm PDMS-coated stir bar (Twister™) exposed to 10% (v/v) smoke vapor phase dilution in air for a smoking period of 30 min. The five compounds chosen for reference purposes, shown in bold type, were benzene, 2,5-dimethyl furan, toluene, ethylbenzene and limonene.

diluted standard was directly transferred onto the 2 cm stir bar using a micropipette and left in a closed dish for 10 min (same duration as smoke exposure). Following exposure, the stir bar was transferred to the TDS within 10 ± 0.5 min. This quantification procedure was used so that the sorption/desorption processes were taken into account. The optimized HSSE method and TDS parameters were used for GC/MS measurement of the XIC peak areas of each standard. Each concentration was analyzed once (*i.e.*, $n = 1$). The linear equations obtained from the calibration curves (Table 3) were used to estimate the minimum quantities of each reference compound present after exposure to 1, 2 and 5% (v/v) cigarette vapor phase in air (Table 3).

3. Results and discussion

3.1. Vapor phase characterization using HSSE, thermal desorption and GC/MS analysis

Our long term goal is to correlate the chemical composition with the toxicological response of cells or tissue exposed to diluted whole cigarette smoke. These experiments complement previous work carried out on the reliability of the smoke generation and dilution in the Borgwaldt RM20S® where repeatability, reproducibility and accuracy for solanesol, a particulate phase marker, and the standard reference gases CH_4 and CO were determined [1]. Following a 30 min exposure of the stir bar to 10% (v/v) vapor phase in air, 16 compounds were identified by thermal desorption–GC/MS using the NIST Scientific and Technical Database Library with a match criterion of ≥80% (Fig. 2). All were confirmed to be present in cigarette smoke based on open literature [8,27–35]. Of the compounds identified, which included primarily esters, ketones, aldehydes or hydrocarbons, five were chosen as reference compounds for further study and method optimization (benzene, 2,5-dimethyl furan, toluene, ethylbenzene and limonene) based on their distribution across the elution window. The remaining eleven compounds detected in the sample are identified in the GC/MS chromatogram (Fig. 2).

Prior to assessing the repeatability of the method, the use of an IS was explored. Ideally, the IS should be a volatile species exposed in a similar way as the vapor phase to the stir bar during the smoke exposure process and should not interfere with the analytes in the chromatogram. However, due to limitations of the sample type, smoking machine and health and safety concerns, the IS had to be applied directly to the stir bar as a liquid prior to exposure. n-Butyl acetate was chosen for its volatility, which was similar to the reference compounds, its absence in the cigarette vapor phase sample and its elution time, which was well separated from the most abundant analytes. Its purpose was to eliminate response variability due to the thermal desorption–GC/MS analysis steps, but not due to the smoke generation, dilution, or volatile sorption processes. The IS peak areas obtained from XICs were compared following a 15 min sorption period in a closed vessel. The peak area repeatability for the IS was 25.8% RSD ($n = 12$). Despite its poor precision, the IS was used during collection of data following smoke vapor phase exposure. Sources of precision error associated with the use of the IS may include pipetting and transferring at the 1 μL level, volatility of the IS over the sorption period, variability in the stir bar retention capacity and/or TDS and GC/MS steps.

The repeatability of the HSSE method was assessed by comparing the peak areas of the five reference compounds and the spiked IS after exposure to 5% (v/v) cigarette vapor phase in air. The repeatability of the IS following exposure improved to 18.5% RSD ($n = 6$) (Table 1), which was surprising because it included additional handling steps and a longer total exposure period compared to the

Table 1
Repeatability data for exposure of a PDMS-coated stir bar (Twister™) to 5% (v/v) vapor phase in air from 3R4F cigarettes for a 10 min smoking period. Integration results were obtained from the peak areas of the 5 reference compounds and the IS by GC/MS using the XICs.

Peak #	Peak ID	Mean peak area	RSD (%) ($n = 6$)	Ratio $A_{analyte}/A_{IS}$	RSD (%) of ratio
1	Benzene	56816.0	11.2	0.939	17.6
2	2,5-Dimethyl furan	20759.7	12.9	0.342	17.3
3	Toluene	235373.8	12.2	3.900	19.7
4	Ethylbenzene	35956.2	10.1	0.596	19.1
5	Limonene	34796.5	12.2	0.578	20.9
IS	n-Butyl acetate	61921.2	18.5	–	–

closed vessel experiment. The repeatability of the five reference compounds varied from 10.1 to 12.9% RSD ($n = 6$) (Table 1, column 4), which represents errors associated with the combined steps of smoke generation, smoke dilution, sorption by the stir bar, desorption by the TDS, cryofocussing and analysis by GC/MS. The poorer precision for the IS compared to the five reference compounds could be due to it not having been sorbed in the same way (*i.e.*, applied to the stir bar from the liquid versus vapor phase) as well as due to errors associated with pipetting only 1 μL at each replicate. Application of the IS to correction of analyte peak areas resulted in higher RSDs (Table 1, column 6) than for the analytes alone and thus was not used for quantitative purposes. As a result, peak areas for the reference compounds without IS correction were compared in subsequent studies. Due to the complexity of the sample, XICs were used for integration of the peak areas to avoid measurement of co-eluting peaks and for accurate quantification.

3.2. Optimization of desorption parameters and statistical analysis

A face-centered composite experimental design was used to obtain the maximal response for 10 analytes as a function of three

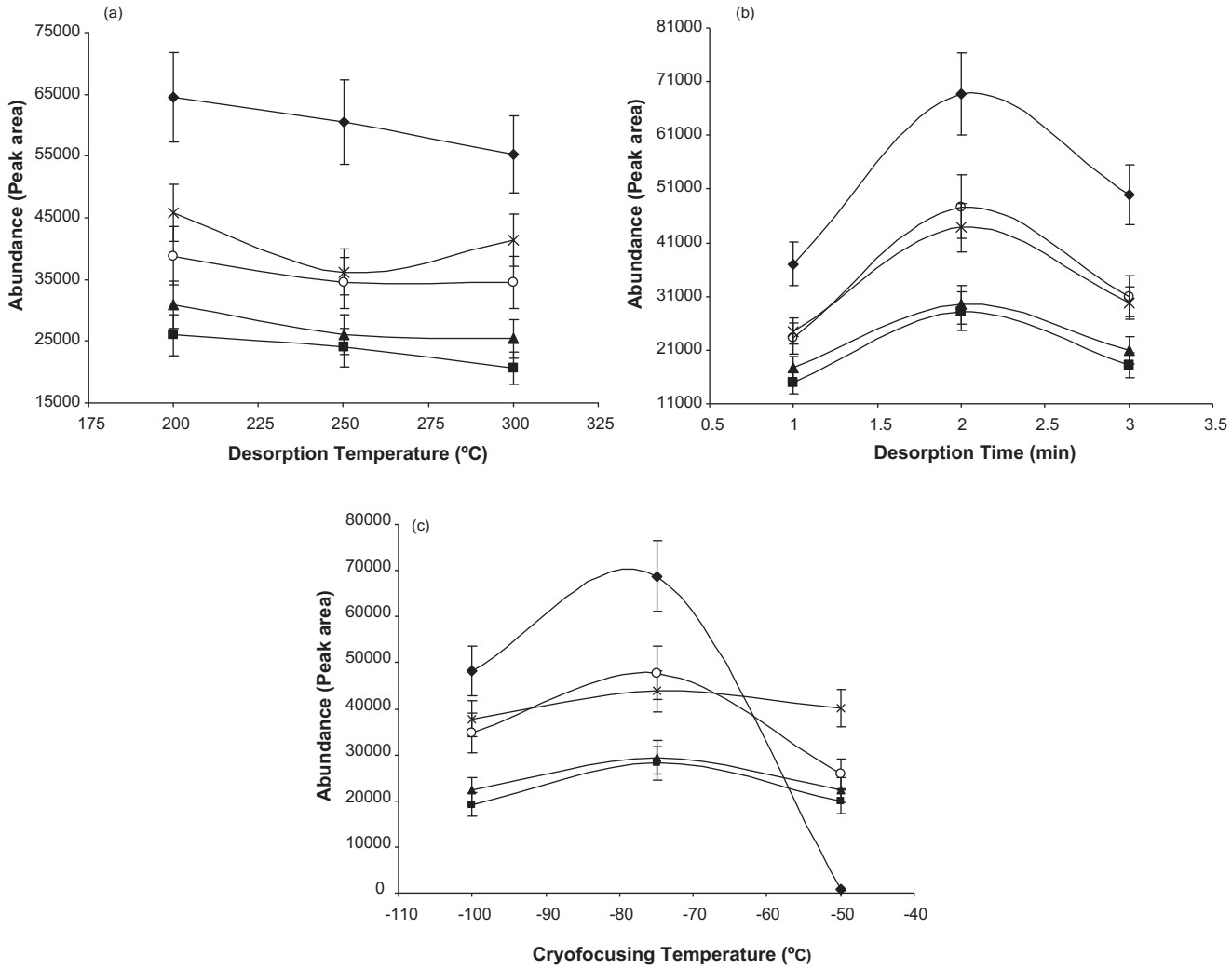

Fig. 3. Effect of the three optimization factors on the abundances of five reference compounds exposed to 5% (v/v) vapor phase in air. (a) Peak area as a function of stir bar desorption temperature for a 2 min desorption time and cryofocussing at −75 °C. (b) Peak area as a function of stir bar desorption time for desorption at 250 °C and cryofocussing at −75 °C. (c) Peak area as a function of cryofocussing temperature when desorption was held at 250 °C for 2 min. Integrated peak areas of the reference compounds were obtained from the GC/MS XICs: benzene, —◆—; 2,5-dimethyl furan, —■—; toluene (peak area/10 to aid plotting), —▲—; ethylbenzene, —✕—; limonene, —○—. Each data point represents an average of three individual runs using three different stir bars and error bars indicate relative standard deviation.

desorption parameters. The 3-factor, face-centered design can be visualized as a cube with a star centered inside having its six points at the center of each face of the cube. Fifteen combinations of the three levels of each factor—desorption temperature, desorption time and cryofocussing temperature—were used, corresponding to those at the 8 "corners" of the cube, at the centers of the 6 faces (*i.e.*, the star points) and at the single, center point. The order of the runs was randomized to reduce bias and the center point condition (desorption at 250 °C for 2 min, cryofocussing at −75 °C) was repeated at the beginning, the end and throughout the series of runs (*e.g.*, runs 1, 9, 11, 15, 16 and 20), which is typical in face-centered composite design. Quadratic regression analysis of the XIC peak area responses for each analyte did not yield any significant factor effects or interactions in the parameters assessed for the measured analytes. Low precision—in excess of 10 % RSD in peak areas (Table 1)—may have been responsible for this result. However, a slight trend in two of the factors could be seen graphically for the five main reference compounds (Fig. 3b and c) with the best responses occurring at a desorption temperature of 250 °C for 2 min with a cryofocussing temperature of −75 °C. It is important to note that at higher desorption temperatures (>250 °C), deterioration of the PDMS phase was observed by the presence of siloxane background peaks. Therefore, 200 °C was deemed the best desorption temperature to avoid damage to the stir bar.

Arrangement of the runs in decreasing order of cryofocussing temperature followed by increasing desorption time (Table 2) also showed that the highest peak area responses for six of the ten analytes occurred at the desorption temperature of 200 °C for a 2 min desorption time and cryofocussing at −75 °C, and thus these maximal conditions were used for all further studies. These desorption conditions are similar to those used in another tobacco flavor study of volatile and semi-volatile components [22]. Some analytes were not efficiently cryofocussed, and thus absent, when using −50 °C (Table 2).

3.3. Stability of sorbed compounds on the stir bar

Using the optimized desorption conditions, the stability of the vapor phase components sorbed on the stir bar (*i.e.*, their resistance to spontaneous desorption) was studied. Four time intervals representing transfer of the stir bar from the exposure chamber to the TDS were assessed: 10 min, which was the minimum transfer time, 40, 160 and 1440 min (24 h). The stability was estimated by comparing the peak areas of the five reference compounds as a function of transfer time following exposure to 5% (v/v) cigarette vapor phase in air (Fig. 4). A 19–31% loss in the reference compounds was observed within 40 min followed by only a 0–9% loss over the next 2 h. The losses were independent of the compounds' volatility. As predicted, these results indicate the importance of considering the volatile, or semi-volatile, nature of the sample in such studies. Therefore, for all further studies, stir bars were consistently transferred to the TD system as soon as possible (*e.g.*, 10 ± 0.5 min) to maintain consistency between runs and to maximize sensitivity. This study did not take into account potential degradation of vapor phase compounds, or reactions and interactions between various smoke components [36].

3.4. Measurement of dilution precision

Using the optimized HSSE–GC/MS method, the dilution precision of the vapor phase was measured by monitoring the peak areas of the five reference compounds as a function of the exposure concentration (*i.e.*, dose equivalent): 1, 2 and 5% (v/v) cigarette vapor phase in air (Fig. 5). The average RSDs associated with the vapor phase generated and diluted by the Borgwaldt RM20S® were 17.2, 6.2 and 11.7% (n = 3) at 1, 2 and 5% (v/v) smoke, respectively. The

Table 2
Optimization of the HSSE desorption parameters representing 10 vapor phase components found on a stir bar following exposure to 5% (v/v) cigarette vapor phase in air. The 10 compounds identified by GC/MS are listed in order of retention time, t_r. Relative peak areas were calculated from the XIC peak areas for each analyte.

Run #	Desorption parameters			Relative peak area[a]									
	Desorption temperature (°C)	Desorption time (min)	Cryofocussing temperature (°C)	2-Methyl-1,3-butadiene t_r = 3.77 min b.p. = 34 °C	3-Methyl-2-butanone t_r =4.72 min b.p. = 94 °C	Benzene t_r =4.78 min b.p. = 80 °C	2,5-Dimethyl furan t_r =5.27 min b.p. = 93 °C	Toluene t_r =6.42 min b.p. = 110 °C	Ethylbenzene t_r =9.06 min b.p. = 136 °C	p-Xylene t_r =9.36 min b.p. = 138 °C	Styrene t_r =10.20 min b.p. = 145 °C	1-Methy-4-(1-methyl-ethylidene) cyclohexane t_r = 16.80 min b.p. = 172 °C[b]	Limonene t_r = 17.14 min b.p. = 176 °C
12	200	1	−50	0	0	0	0.80	0.70	0.75	0.66	0.63	0.54	0.58
8	300	1	−50	0.04	0	0.09	0.88	0.90	0.96	0.94	0.95	0.88	0.80
5	250	2	−50	0.02	0	0.01	0.76	0.72	0.88	0.84	0.77	0.50	0.52
19	200	3	−50	0	0	0	0.69	0.68	0.77	0.29	0.70	0.78	0.84
6	300	3	−50	0	0	0.06	0.92	0.94	0.91	0.92	**1.00**	**1.00**	**1.00**
4	250	1	−75	0.74	0.73	0.57	0.57	0.57	0.53	0.54	0.54	0.35	0.47
7	200	2	−75	0.94	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	0.90	0.74	0.78
Mean[c]	250	2	−75	0.90	0.86	0.91	0.79	0.73	0.80	0.70	0.74	0.63	0.67
10	300	2	−75	0.91	0.76	0.86	0.79	0.82	0.90	0.87	0.75	0.58	0.70
14	250	3	−75	0.82	0.75	0.78	0.70	0.68	0.65	0.30	0.62	0.60	0.63
3	200	1	−100	0.82	0.68	0.82	0.65	0.56	0.73	0.74	0.77	0.60	0.62
2	300	1	−100	**1.00**	0.81	0.86	0.72	0.65	0.68	0.67	0.69	0.57	0.62
13	250	2	−100	0.86	0.74	0.75	0.74	0.72	0.83	0.37	0.77	0.62	0.70
17	200	3	−100	0.88	0.87	0.82	0.62	0.64	0.84	0.39	0.78	0.71	0.67
18	300	3	−100	0.76	0.65	0.56	0.63	0.55	0.61	0.59	0.58	0.47	0.50

[a] Per compound, *i.e.*, data were normalized vertically with respect to the highest abundance (peak area) in a given column, which is shown in bold type.
[b] Boiling point estimated based on relative retention time.
[c] Mean response of six center point runs: 1, 9, 11, 15, 16 and 20.

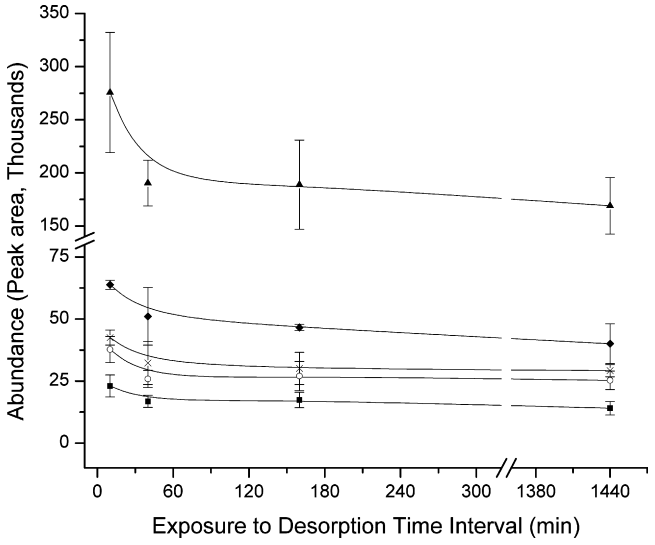

Fig. 4. Stability measurements of five vapor phase reference compounds desorbed from a Twister™ stir bar, following exposure to 5% (v/v) cigarette vapor phase in air for HSSE experiments. The transfer time intervals (*i.e.*, between exposure and desorption) selected for the analysis were 10, 40, 160 and 1440 min (24 h). Integration results were obtained from the XIC peak areas of the 5 reference compounds by GC/MS: benzene, ◆; 2,5-dimethyl furan, ■; toluene, ▲; ethylbenzene, ✳; limonene, ○. Each data point represents an average of 3 individual runs using 3 different stir bars and error bars indicate standard deviation.

linearity was good with correlation coefficients (r^2) of >0.99 for each compound. Previous work in our laboratory indicated that the repeatability of the dilution of reference standard gases (CH_4 and CO) was between 0.7 and 4.5% RSD with $r^2 > 0.99$ for a similar dilution range [1]. From the same study, it was also shown that the precision of the smoke generation itself was only 12% RSD based on measuring solanesol in the particulate phase over the same smoke dilution range: 1–5% (v/v) in air. The lower precision seen in the current study at 1% smoke dilution (*i.e.*, 17.2% average RSD for 5 compounds) is likely due to high variability in the minute quantities of analyte sorbed on the stir bar at this dilution level since all other factors affecting the mass sorbed, *i.e.*, PDMS film thick-

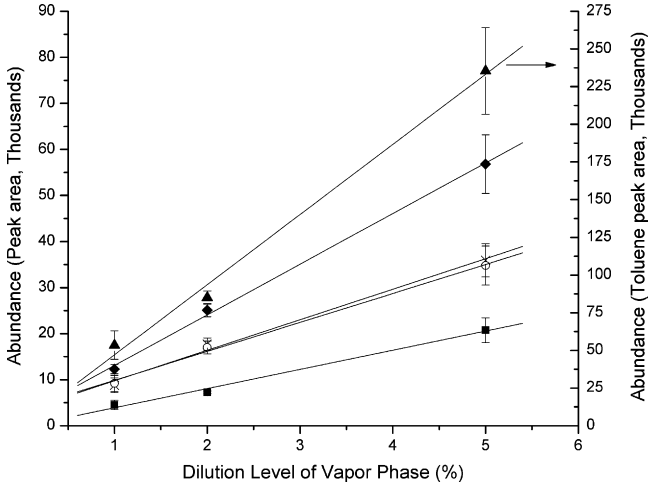

Fig. 5. Dilution precision data representing the vapor phase components found on a PDMS coated stir bar (Twister™), following exposure to 1, 2 and 5% (v/v) cigarette vapor phase in air from 3R4F cigarettes for HSSE experiments. Integration results were obtained from the peak areas of the 5 reference compounds by GC/MS using the XICs: benzene, ◆; 2,5-dimethyl furan, ■; toluene, ▲ (abundance shown on right vertical axis); ethylbenzene, ✳; limonene, ○. Each data point represents an average of 3 individual runs using 3 different stir bars and error bars indicate standard deviation.

Table 3
Semi-quantitative analysis of the five reference vapor phase compounds found on a stir bar following 10 min exposure to 1, 2 and 5% (v/v) cigarette vapor phase in air for HSSE experiments.

Reference compound	t_r of standard (min)	t_r of sample (min)	Relative response factor[a]	Calibration curve[b]	Estimated minimum amount for 10 min exposure to diluted smoke vapor phase (ng)[c]			Extrapolation to undiluted smoke vapor phase (ng)
					1%	2%	5%	(100%)
Benzene	4.79	4.78	1.0	$y = (189 \pm 30)x + (3401 \pm 4013)$ $r^2 = 0.9529$	3.7	8.9	22.0	450
2,5-Dimethyl furan	5.26	5.27	1.9	$y = (364 \pm 65)x + (1626 \pm 1660)$ $r^2 = 0.9405$	0.76	1.5	5.1	110
Toluene	6.41	6.42	8.5	$y = (2257 \pm 121)x + (1300 \pm 5145)$ $r^2 = 0.9858$	2.1	3.4	9.6	190
Ethylbenzene	9.03	9.06	27	$y = (11896 \pm 634)x - (3712 \pm 3252)$ $r^2 = 0.9915$	0.11	0.19	0.35	6
Limonene	17.1	17.1	15	$y = (48004 \pm 2200)x - (1006 \pm 1538)$ $r^2 = 0.9937$	0.21	0.38	0.75	13

[a] Based on XIC peak areas for standards at 10 μM.
[b] Linear regression curves have slope units of peak area/μM; r^2, correlation coefficient.
[c] Calculated from average peak areas (Fig. 5) then converted to ng based on 1 μL standard applied to stir bar. Exposure of 10 min = 10 puffs. Error is estimated to be on the order of 18–25% RSD based on results for the IS, n-butyl acetate.

ness, sample volume, partition coefficient, etc., were unchanged. Taking this into account, the results obtained in the current study corroborate the conclusion that the Borgwaldt RM20S® presents acceptable limits for repeatability measurements across dilutions.

3.5. Semi-Quantitative Analysis of Diluted Smoke Vapor Phase

External calibration was achieved using standard solutions in hexane applied to the stir bar. The same TDS-GC/MS parameters as with the vapor phase measurements were used. Relative response factors calculated from the XIC peak areas for each standard at 10 μM showed that ethylbenzene and limonene gave large responses (Table 3), presumably because of their stronger partition into the PDMS phase on the stir bar. Based on the smoke vapor phase peak areas at each dilution level (*e.g.*, Fig. 5 data), yields ranging from 0.1 to 22 ng were estimated (Table 3). It should be noted that the values in Table 3 represent minimum amounts as it is unlikely that equilibrium was reached for each puff. Quantitative HSSE is typically carried out in a closed system whereas in this experiment, each puff remained in the exposure chamber for 1 min and was then pushed out by a subsequent puff of fresh cigarette vapor phase. In addition, 100% recovery was likely not attained for the sorption/desorption process with the PDMS phase, which is compound dependent. Extrapolation to 100% vapor phase, *i.e.*, undiluted smoke, gives estimated yields in the range of 6 to 450 ng for the reference compounds (Table 3), which is 2 – 3 orders of magnitude less than reported amounts per cigarette (one full cigarette = 7 – 10 puffs). Other studies on the vapor phase components of cigarette smoke have reported quantities of benzene ranging from 23 to < 70 μg/cig [27–29,31,33], 2,5-dimethylfuran at 58 μg/cig [33], toluene ranging from 57 to < 200 μg/cig [27–29,31,33], ethylbenzene ranging from 4.4 – 5.5 μg/cig [29,31] and D-limonene at 64 μg/cig [33]. The fact that previous studies were carried out on different tobacco blends (*e.g.*, 1R4F, 2R4F reference cigarettes, etc.) with different smoking apparatuses and assessed using different sampling methods (*e.g.*, cold or cryo traps [29,31,33]; collection bags [29,31]; solvent trap [31]; on-line analysis [27,33]) does not fully account for the large differences in quantities obtained compared to the present study. Besides the HSSE factors mentioned above, underestimation of the yields may also result from the procedure used to introduce the liquid-phase standards (*i.e.*, error in pipetting 1 μL; differences between the PDMS/liquid versus PDMS/gas phase sorption processes), the known dilution error of up to 6.4% for the smoking machine [1] and the large error in the calibration curve intercept values (Table 3).

4. Conclusions

The HSSE method was successfully applied to the characterization of the vapor phase of diluted cigarette smoke collected in an exposure chamber. This procedure allowed for the components sorbed on the stir bar to be desorbed, re-focused and analyzed in one integrated/automated experimental step, without the use of extraction solvents. This technique, when coupled to GC/MS, allowed for the rapid and direct qualitative analysis of volatile and semi-volatile smoke vapor phase components in the exposure chamber of the Borgwaldt RM20S® and provided a linear response across smoke dilutions. The IS, n-butyl acetate, did not prove useful for improving precision associated with the method. Semi-quantitative analysis of five smoke vapor phase components showed systematic underestimation of the yields compared to previously published values. Improvements in quantification and precision might be achieved by using deuterated standards or a gas-phase internal standard (*i.e.*, hexane) introduced at each puff via a switching valve inserted before the dilution syringe. Nonetheless, the results obtained in this study were within acceptable limits for repeatability measurements between dilutions.

The HSSE technique is simple, cost effective, can be easily implemented in most laboratories and can be applied to a wide range of analyses, *e.g.*, environmental, food and automotive. Moreover, these results show that although there are a wide range of volatiles and semi-volatiles with different physical properties, dilution and delivery in the Borgwaldt RM20S® are achieved through a non-selective manner. In addition, this study provided additional knowledge about a whole smoke exposure system to give us a more complete picture of the exposure concentration applied to cell cultures for future toxicology studies.

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