HOW LONG MAY A BREATH SAMPLE BE STORED FOR AT -80°C? A STUDY OF THE STABILITY OF VOLATILE ORGANIC COMPOUNDS TRAPPED ONTO A MIXED TENAX: CARBOGRAPH TRAP ADSORBENT BED FROM EXHALED BREATH

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ABSTRACT

Thermal desorption is used extensively in exhaled breath volatile organic compound (VOC) analysis, and it is often necessary to store the adsorbent tube samples before analysis. The possible introduction of storage artefacts is an important potential confounding factor in the development of standard methodologies for breath sampling and analysis.

The stability of VOCs trapped from breath samples onto a dual bed Tenax® TA:Carbograph adsorbent tube and stored -80°C was studied over 12.5 month. 25 samples were collected from a single male participant over 3 hr and then stored at -80°C. Randomly selected adsorbent tubes were subsequent analysed by thermal desorption-gas chromatography-mass spectrometry at 5 times points throughout the 12.5 month of the study. Toluene-d_8, decane-d_{22} and hexadecane-d_{34} internal standards were used to manage the instrument variability throughout the duration of the study. A breath-matrix consisting of 161 endogenous and 423 exogenous VOC was created . Iterative orthogonal partial least squared discriminant analysis (OPLS-DA) and principal components analysis (PCA) indicated that it was not possible to detect storage artefacts at 1.5 month storage. By 6 month storage artefacts were discernible with significant changes observed for 27% of the recovered VOC. Endogenous VOC were observed to be more susceptible to storage. A paired two-tailed t-test on the endogenous compounds indicated that the maximum storage duration under these conditions was 1.5 month with 94% of the VOCs stable. This study indicates that a prudent approach is best adopted for the storage of adsorbent samples; storage times should be minimised, and storage time examined as a possible discriminatory factor in multivariate analysis.

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INTRODUCTION

Endeavours to discover exhaled breath VOC biomarkers for the observation of biochemical processes, and the ultimate development diagnostic and/or prognostic techniques continue apace. The prospect of non-invasive, safe, rapid and low-cost in-clinic, or at patient, diagnostic health-screens and tests continues to be the motivation for much of this research, and examples include studies of: lung cancer [1]; chronic obstructive pulmonary disease (COPD) [2], tuberculosis [3], and asthma [4]. Exhaled breath research is not confined to disease of the lung [5] and an authoritative introduction and overview of the instrumentation and methods used for exhaled breath analysis as well as putative breath biomarkers has been provided in a significantly useful text book [6] as well as a land-mark monograph [7].

Thermal desorption has been widely used as a VOC analysis methodology due to its versatility and the low-limits of detection possible from the high enrichment ratios generated by the cold-trapping of the recovered VOC prior to injection onto a gas chromatography (GC) column by ballistic heating. The stability of VOCs trapped from a breath sample onto adsorbents for thermal desorption is an important aspect of the methodology. Despite the desirability of immediate analyse, it is often impracticable to achieve this, particularly if samples are taken at multiple locations for analysis at a central laboratory. Further there are often operational factors (instrumentation compliance for example) that necessitate a delay between taking the sample and its subsequent analysis. The adsorbent sample storage conditions depend on the type of adsorbent tubes used. Single-bed adsorbent sampling tubes are held to have better storage stability [8,9] with benzene, xylene and toluene observed to be stable at room temperature conditions for up to 1 year when trapped on a single-bed Tenax TA adsorbent tube [10]. Chlorinated hydrocarbons have been observed as stable for up to 2 years under the same conditions [11]. However, multi-adsorbent bed sampling tubes are used to sample VOCs in breath to account for the wide volatility range of the compounds present, and the fact that sampled breath is saturated with water. The recommendation for the storage of multi-adsorbent tubes is that they are stored under refrigerated conditions (4°C) for no-longer than 30 days for targeted compounds [8, 9]. The reduced storage time is invoked to account for the diffusion of lower volatility VOCs from the weaker adsorbent into the stronger adsorbent resulting in irreversible adsorption and potential losses during the subsequent analysis. This phenomenon may be minimised by

increasing the amount of the weaker adsorbent in the mixed bed, or adding a further medium strength adsorbent. Additionally, the refrigeration at a lower temperature reduces the volatility and hence diffusion of trapped VOCs reducing the effect of migration. Importantly the stability of trapped VOCs under storage conditions has been reported to be compound dependent [9].

What has yet to be established is the maximum time sampled breath VOCs may be stored at low temperature (-80°C) on a dual-bed adsorbent sample without the introduction of artefacts into the subsequent analysis. Such knowledge would be useful in the planning and preparation of metabolomic studies that seek to be applied to larger cohorts spread across many different regions and territories that encompass humanity's cultural and phenotypic diversity. The costs associated with the storage and international shipment of many batches of breath samples over the course of such study are high enough to impede and limit such research; longer term storage with a single shipment would resolve such constraints. Also, the production of reliable composite quality assurance samples is not feasible without validated longer-term storage methods. Consequently this study examined the effect of sampled breath VOC storage at -80°C on the recovered breath VOC profile over a 12.5 month period. The hypothesis was that VOC profiles trapped in a dual-bed adsorbent tubes stored at -80°C would be stable. 25 breath samples obtained from a single participant over a 3 hr period were analysed in five batches by thermal desorption-gas chromatography-mass spectrometry at five time points throughout a 12.5 month storage period to test this hypothesis.

METHODS

Breath sample collection

The study was conducted in accordance with the ethical principles of good lab practice and the Declaration of Helsinki. The research methodology was reviewed and received a favourable opinion from the local ethics advisory committee, ref G09-P5 Loughborough University. A single participant recruited to the study gave written consent and was screened by health questionnaire.

A single participant with no signs or symptoms of illness or knowledge of underlying medical conditions was selected in an attempt to minimise the inter-sample variation in this study (Age: 25 yr, BMI: 25.5 $kg.m^{-2}$, non-smoker). 25 breath samples were collected over 3 hr from the participant in a designated breath-sampling room within a laboratory

setting. The participant was encouraged to sip water and remain hydrated throughout the sampling campaign. In order to minimise stress, any discomfort or boredom, short breaks were introduced in-between every two samples and the participant was distracted with a selection of TV programmes provided by a tablet PC. In addition to the breath samples, 10 background room-air samples and 5 respirable-air samples were also collected.

The sampling method and analysis workflow has been described previously [12, 13]. The participant was fitted with a non-vented full-face continuous positive airway pressure face mask (ResMed, Mirage Full Mask Series 2) supplied with highly-purified air at a flow of 30 $dm^3.min^{-1}$. The purified air provided a constant low concentration background of exogenous contaminants in the breath samples and protected the experiment from disruption by short time-scale changes in the immediate environment of volatile compounds. (The ventilation rate of the sampling facility generates about 10 air changes hr^{-1}) This feature of the experiment along with the randomisation of the allocation of the samples to different storage times reduced the potential impact of transient changes in exogenous concentrations on the experiment. The potential effect of washout of the exogenous compounds from exposure prior to sampling over the sampling period was also addressed through the randomisation of the allocation of the samples to different storage times. The participant was encouraged to rest and familiarise themselves with the arrangement while breathing normally in a relaxed manner for 10 min; the participant's normal breathing was observed to be through their nose with their mouth closed. A pressure sensor fitted to one of the face-mask's inlets enabled continuous tracking of the participant's breathing profile. The output from the pressure sensor was processed with a virtual instrument (LabviewTM) that also controlled the micro-valves that switched between the sampling and vent modes enabling the distal portion of each breath to be sampled in a reproducible manner. An adapted Leur-lock fitting enabled a silco-steel capillary tube to be inserted into the mask into the respiratory zone to enable the sampled breath to be pumped onto a mixed adsorbent trap (100 mg to 200 mg Tenax® TA / 100 mg to 200 mg Carbograph 1 TD, Part number: C2-AXXX-5032, Markes International, UK). An Elf Escort air sampling pump (MSA, USA) was used to draw sampled breath at a flow rate of 0.8 $dm^3.min^{-1}$ through the silco-steel capillary and into the adsorbent sampler. The average exhaled breath sample volume was 2.52 dm^3 ± 0.01 dm^3 and was taken from the distal portion of 88 ± 17 breaths with a breathing rate of 17 ± 2 $breath.min^{-1}$, over 4.97 min ± 0.56 min. The

average breath amplitude was monitored via the pressure sensor signal and recorded as 3.33 V ± 0.01 V. An example breath profile is shown in Figure 1. It is important to note that the participant was supported and encouraged to remain in a quiet and relaxed state throughout the whole sampling procedure.

Heart-rate, blood-pressure and room temperature.

The Participant's blood pressure and pulse rate were measured every 15 min throughout the sampling campaign (Blood Pressure Monitor HPL-300), Figure S1. The systolic and diastolic blood pressures were recorded as 148 mmHg ± 3.82 mmHg at the 95 % confidence limit (n=9) and 103 mmHg ± 4.33 mmHg at the 95 % confidence limit (n=9) respectively, while the pulse rate was observed to be 69 min^{-1} ± 3.82 min^{-1} at the 95 % confidence limit (n=9). Room temperature is also an important factor in breath sampling affecting metabolism and environmental background contamination levels and the room temperature remained at 17.4°C ± 0.1 min^{-1} at the 95 % confidence limit (n=9); see Figure S1.

These data indicate that the participant was physiologically stable throughout the sampling procedure with none of the parameters exceeding ± 2σ from the overall mean values. The participant reported some level of psychological and, or, physical stress associated with a prolonged intervention in an unfamiliar environment and this was reflected in the blood pressure and heart rate levels after ca. 1 hr; he was bored.

Breath sample storage at -80°C

All the exhaled breath samples were sealed with air-tight storage caps immediately on cessation of sampling and 20 were randomly allocated into four batches of five adsorbent tubes that were to be analysed after different intervals of storage. These 20 adsorbent tubes were placed in an air-tight box made from polypropylene (0.55 dm^3) that had been cleaned and conditioned under vacuum prior to use (Really Useful Boxes, UK) [**14**] and transferred to a freezer and cooled to -80 °C as soon as the last sample has been taken. After the tubes had been chilled at -80°C for approximately 30 min the sealing caps were retightened to account for any possible cap loosening from thermal contraction. The remaining five tubes were placed in a different air tight box and transferred to a refrigerator at 4 °C and analysed as soon as possible after sampling had been completed.

The storage intervals were selected on the basis of a central composite design and were set at zero storage (blank), 1.5 month, 6 month, 10.5 month and 12 month. The last time point 12 month was delayed to 12.5 month as the instrument required servicing and was unavailable at the 12 month point. In addition to the breath samples four samples of room air and four samples of the air supply were included in the storage study and one of each of these samples was analysed along with the breath samples over the four time points.

Sample analysis by thermal desorption gas chromatography mass spectrometry (TD-GC-MS)

The breath VOCs collected were analysed using thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS). A Varian 3800 GC was interfaced to Varian Saturn 4000 ion-trap mass spectrometer (GC-MS). The sampled VOCs were recovered, enriched and injected by a two-stage Markes Unity Series 1 thermal desorption unit using a general purpose hydrophobic cold trap. The instrumentation parameters are summarised in Table 1.

The adsorbent traps were removed from -80°C storage and equilibrated to room temperature for 3 hours before analysis, allowing frozen condensate to melt and evaporate. Before each batch was analysed an adsorbent tube with a mixture of standards (see below) was analysed and two thermal desorption blanks were run between each sample to eliminate any contamination carry over between samples.

Quality control

Before sample analysis 0.2 µl of a mixture of 16 hydrocarbon compounds (approximately 1.4ng each) dissolved in dichloromethane was spiked onto an adsorbent tube and analysed to track retention time drift on the column and validate and standardise the instrumentation's performance. The hydrocarbon standard (retention index standard) test was augmented by infusing test atmospheres containing deuterated internal standards (toluene-d_8, decane-d_{22} and hexadecane-d_{34}) into the adsorbent tubes immediately before they were loaded into the thermal desorption unit for analysis. These three compounds were selected to provide internal standard responses at 5.2 min, 10.6 min and 26.7 min respectively.

The test atmosphere was generated using permeation sources (calibrated gravimetrically) fitted into a gas tight housing that was maintained at 40°C and ventilated with, high

purity nitrogen at flow rate of 50 $cm^3.min^{-1}$. The adsorbent sample tubes were connected to the test atmosphere generator for 30 s prior to analysis and 19.5 ng, 2.7 ng and 1.3 ng of toluene-d_8, decane-d_{22} and hexadecane-d_{34} respectively were loaded onto the adsorbent trap. Figure 2 summarises the results from the internal standards across the whole experiment.

The data processing workflow adopted has been described previously [**15**]. Briefly, the TD-GC-MS data were deconvoluted (AnalyzerPro Spectral Works, UK), and a typical breath sample was found to contain approximately 500 resolvable compounds. All compounds were catalogued by their retention index derived from the primary retention index scale, generated from retention index standards and a secondary retention index ladder based on five ubiquitous siloxane components present in all the breath samples. Each resolved breath component was given a unique breath-library reference code of the form (BRI-RI-< m/z_1>-< m/z_2>-< m/z_3>-< m/z_4>-< m/z_5>). BRI, signifying a VOC feature extracted from a breath sample and catalogued by retention index, was followed by the retention index value that was then proceeded by a list of the ion fragment masses identified in the de-convolved mass spectrum, in order of decreasing abundance. The number of fragment ions used in the reference code was determined by the minimum required to generate a unique identifier. The resultant list of retention indexed breath components was used to create a library to integrate the original sample data using Varian MS Workstation software (Varian, UK) for each compound's extracted ion chromatogram. The resultant data was consolidated into breath-matrix that contained the extracted peak areas for each breath VOC feature listed against the sample name.

RESULTS AND DISCUSSION

Determination of system variability using internal standards quality control chart

Peak areas of internal standards from every breath sample and background samples were deconvoluted and recorded throughout the 12 month storage period. The variation in the peak areas of these internal standards reflects the overall stability of the analytical and internal standard systems. Importantly, knowledge of the variability in the system's operational performance across the study was a pre-requisite in assessing potential changes in the VOC profiles observed after different storage times.

40 quality control data sets were generated from 25 breath samples, 10 background air samples and five internal standards blanks. The global %RSD across the complete

sample set for the 12.5 month duration was 22.1% for Toluene-d_8, 26.9% for Decane-d_{22} and 24.2% Hexadecane-d_{34}. The peak areas of the internal standards were derived from the integrated peak areas of individual quantitation ions. For toluene-d_8, these were m/z 98, 100, 99, 70 and 66. For decane-d_{22} these were m/z 66, 46, 50, 82 and 80, and for hexadecane-d_{34} these were m/z 66, 82, 50, 46 and 64. Figure 2 shows the z-scores of the internal standard peak areas across the whole experiment, and defines the analytical variability within the study. Further, a combined internal standard score S_{IS} was defined according to Equation 1, where A_{IS_n} (n = 1 to 3) is the individual peak area for each of the three internal standards and $\overline{A_{IS_n}}$ is the mean of the 40 values determined for each of the three internal standards. Figure 3 also shows the S_{IS} trend over the 12.5 month of the study and what is immediately evident is the general reduction in variability as the study progressed. This may be attributed in part to improvements in the operation and hence stability of the test atmosphere generator across the 12.5 month study.

$$S_{IS} = \sqrt{\sum_{n=1}^{n=3} {A_{IS_n}}^2} \qquad \text{Equation 1}$$

The systematic variability of other instrumentation factors was further examined through unsupervised principal component analysis (PCA) of the three deuterated standards' peak intensities across the study. Three distinct groupings of responses are discernible. The largest group contains data from the 0 month, 10.5 month and 12.5 month study. No systemic differences in the analytical and quality systems were discernible in these data. The responses from 1.5 month show variability with an overall increase in the observed responses for internal standards, while at 6 month there is a reduction in variability and an overall decrease in the observed responses for the internal standards. No systemic trend was present within these responses, which reflected the levels of variability associated with trace analysis [16].

Relationship between breath components and storage duration

The breath matrix contained 161 VOCs that were observed exclusively in breath samples (These were designated as the endogenous VOC). 423 other VOCs isolated from the breath samples were also observed in the environment or the purified-air samples. These

latter 423 VOCs were classified as exogenous VOC, while acknowledging that a proportion of this group had endogenous attributes.

Exhaled breath is saturated with water and the largest mass of material trapped from breath within the adsorbent trap is water. Trapped water interacts with the stationary phase of the GC column, creating cyclic siloxane compounds; additional reactivity is seen with other silicon treated surfaces throughout the analytical path. The level of these siloxane compounds increases with the level of degradation level of the GC stationary phase. Consequently, all cyclic siloxane compounds were removed from the data set. Further, compounds that were present in less than 30% of the breath samples were also excluded from data modelling. Multivariate analysis was performed on the remaining data using SIMCA-P+ software (Version 12.0.1.0, Umetrics, UK) to determine if there were molecular discriminators of storage in the VOC profile. All variables were assigned to single block weight of 1(1/SQRT) and Pareto base scaling (Par). Pareto base scaling was selected due to its ability to magnify low to medium range signals in analysis without inflating the background noise. This scaling subtracts each variable from the mean of the data set and divides the difference by the square root of the standard deviation. Unsupervised principal components analysis (PCA) was performed to establish if the presence of storage related changes in the breath VOC profiles was discernible, and Figure 4 shows the PCA score plots for the endogenous VOCs and it was not possible to distinguish between the endogenous VOC profiles obtained from samples stored for 1.5 month and those analysed immediately after sampling; 0 month, the reference samples. Thereafter however it was apparent that the longer the samples were stored the greater the observed differences between them and the reference samples became. The dendrogram from hierarchical cluster analysis in Figure 5 illustrates how the difference between the sets of samples becomes stronger with increasing storage time. The effect of storage on the exogenous profile was also evident, although not as pronounced as the endogenous profile, see Figure 5. Of note in Figure 4 and Figure 5 is the isolation of endogenous compounds in the PCA-plot and dendrogram.

The trend in the data was modelled using partial least squared regression (PLS) to explore the relationship between peak intensities (predicted variable X) and storage duration (response variable Y). The model generated three principal components with a R^2 (fraction of sum of squares explained by PC1) of 90.8% and Q^2 (fraction of total variation of Y predicted by PC1) of 91.2% for principal component 1 (PC1) indicating a

strong relationship between X and Y variables. The relationship between X and Y variables was modelled by plotting PC1 scores against storage duration, see Figure 6 and Equation 2 which indicate sample stability up to a storage duration of 1.5 month. By 6 month the deterioration in the sample integrity is well established increasing with time through the 10.5 month datum and up to the 12.5 month final point.

$$Y = 0.8821X^2 + 12.564X - 130.41$$ Equation 2

A paired two-tail t-test performed on all the endogenous VOC peak intensities revealed the extent of statistically significant changes with storage duration. After 1.5 month storage storage compounds were identified to be present at significantly different levels (p = 0.05) suggesting that 94% of the VOC profile was stable over this storage time. By 6 month 44 compounds were observed to have change significantly in their intensity (p=0.05); 73 % of the endogenous profile was stable. By 10.5 the majority of breath components were unstable, with 41% of the profile showing no significant change. Of particular interest were 33 compounds that were either completely lost from the VOC profile on storage or were absent in the reference samples at time = 0 month and then went on to develop during storage; 25 compounds were eliminated after 6 month, and these compounds were notable for their low peak intensities compared to many of the endogenous VOCs recovered from breath samples; the mean estimated on-column mass was ca. 55 pg (referenced to internal standards).

Examination of the deconvolved mass spectra of these compounds and data base matching (NIST MS library) resulted in six tentative identities being tested against retention index library matches and those found to be within ± 50 IU of retention index value are included in Table 2. Some of these low intensity compounds were close to the limit of detection of the instrument and it is reasonable to conclude that compounds were still present but below the detection thresholds of the instruments and methodologies. Six compounds that show increased presence were also identified using the same methodology and are also included in Table 2, and the selected ion chromatograms of these 12 compounds are presented in Figure 7.

It is helpful to note that while the disappearance or emergence of compounds from stored samples is arguably the most significant of changes observed the low intensity of these feature means they were not the primary discriminators of storage identified from

multivariate analysis. The most significant VOC breath storage discriminators with the highest correlation score from a PCA S-plot were elucidated and are listed in Table 3.

SUMMARY

A 12.5 month storage study was carried out to assess the stability of exhaled breath compounds trapped on a dual-bed adsorbent trap at -80°C. Previously a storage study focused on the stability of target compounds sub-sampled from a bag sample, and reported that samples appeared stabled for up to 2 weeks following sampling, transport and storage at temperatures from 4°C to 7°C [17], the effect of sample handling on the VOC profile was not described. The current study was the first to characterise changes associated with longer term storage of up to 1 year.

Collecting 25 breath samples from a single participant required care for it was important to ensure that normal and relaxed breathing patterns were sustained throughout. The sampling campaign needed to be undertaken over as short a time period as possible, with changes in breath profiles due to digestion and fasting minimised (acetone from fasting for example), and that possible artefacts from psychological challenges were suppressed [18]. Potential confounding factors resulting from such stresses were further minimised by randomisation of allocation of the samples across the storage durations. The reproducibility of the sampling approach has been evaluated previously where the within-subject variability was found to be significantly lower than between-subject variability (p = 6.23 ×10^{-23}) [19].

Instrument and method reproducibility at picogramme levels were another confounding factor in this study. Different instrument and method operational states were identified across the 12.5 month of this study, however no overall trends were discerned and the overall variability was within the ranges associated with TD-GC-MS at the concentrations encountered. The changes observed in the VOC profiles recovered after different storage durations could not be attributed to variations in the instrumentation's performance.

Examination of the data with different statistical approaches revealed that the longer the adsorbent tubes were stored the greater the differences in the recovered VOC profiles were. Care needs to be exercised to avoid over fitting MVA models to limited sample sizes, such as this, which is why it is helpful to emphasise the results from the paired two-tailed t-tests. Overall it would appear that breath samples obtained with the method used may be stored under the conditions of this study for up to 1.5 month with

potentially 6 % of the trapped compounds affected. Increasing the storage time to longer duration appears to be accompanied by an increased prevalence of storage artefacts within the resultant analysis. A detailed characterisation of the storage sensitive components was beyond the scope of this work; however some observable patterns were noted. The fragmentation patterns of most the storage sensitive VOC contained m/z 57, 43, 71, 85, 41 or m/z 69, 83, 41, 55 indicative of hydrocarbon, ester, aldehyde, alcohol or fatty acid functionalities.

Storage factors are likely to include the type of adsorbents used and the structure of the adsorbent tubes and whether multi-sorbents designs are used. It would appear that migration of VOCs within dual beds may still occur at -80°C, albeit at lower rates than previously reported at higher temperatures. The relatively high levels of water may be associated with the increase of hydrolytic products such as acetone after storage for 6 month. A proportion of the exogenous components of this study were also apparently sensitive to storage conditions, and interactions between environmental VOC contamination and endogenous breath VOC are additional potential mechanisms for storage instability.

In addition to storage factors it is also helpful to note that this study did not, focus on potential disease biomarkers. In the first instance collecting 25 samples from a participant, or participants, with a confirmed diagnosis was not ethically justifiable. Further, the effect of physical stress and/or distress on the subsequent breath profile risked the introduction of experimental artefacts into the study that would not be representative of in-clinic operations. Finally to include a representative collection of potential disease biomarkers of interest would represent an undertaking beyond the resources available to this study. Nevertheless it is helpful to reflect that disease biomarkers range in reactivity and many of the compounds of current interest are reactive (S-, N-, and carbonyl) and as such caution in setting storage times and conditions with breath samples taken from cohorts with disease appears appropriate.

The rate of reaction of a trapped compound in storage will vary as the concentration of the volatile in the exhaled breath sample varies; even endogenous VOCs can have an order of magnitude concentration variation, both inter- as well as intra-subject. The rate of change in the stored volatile compounds observed in this study may not be universally applicable, and another subject could well exhibit a different panel of compounds that differentiated storage time. At the end of this study it seems that the longer an adsorbent

sample is stored the greater the potential for storage artefacts across a study cohort might be.

At the outset the motivation for this study was the validation of a proposed longer-term storage protocol to facilitate gains in operational efficiency through: reduced transport costs; stabilised instrumentation; and, the adoption of automated processing of consolidated sample batches. However, the hypothesis of this study was rejected: samples were found not to be stable over the 12.5 month duration of the study. Indeed at 1.5 month some instability was apparent. It would appear reasonable to adopt a cautious approach to the use of stored samples in breath analysis. Studies with targeted metabolites will enhance the reliability of their findings by the verification of the storage stability of their analytes. The elimination of storage-time and storage-conditions as discriminant variables in non-targeted metabolomics profiling may also be seen as a helpful quality assurance check. Further research and development in the storage and stabilisation of breath samples is a logical development from this study and the authors suggest that, until proven otherwise, it would be prudent to validate storage protocols prior to undertaking studies, and to seek to analyse adsorbent based breath samples rapidly, with a minimised storage time.

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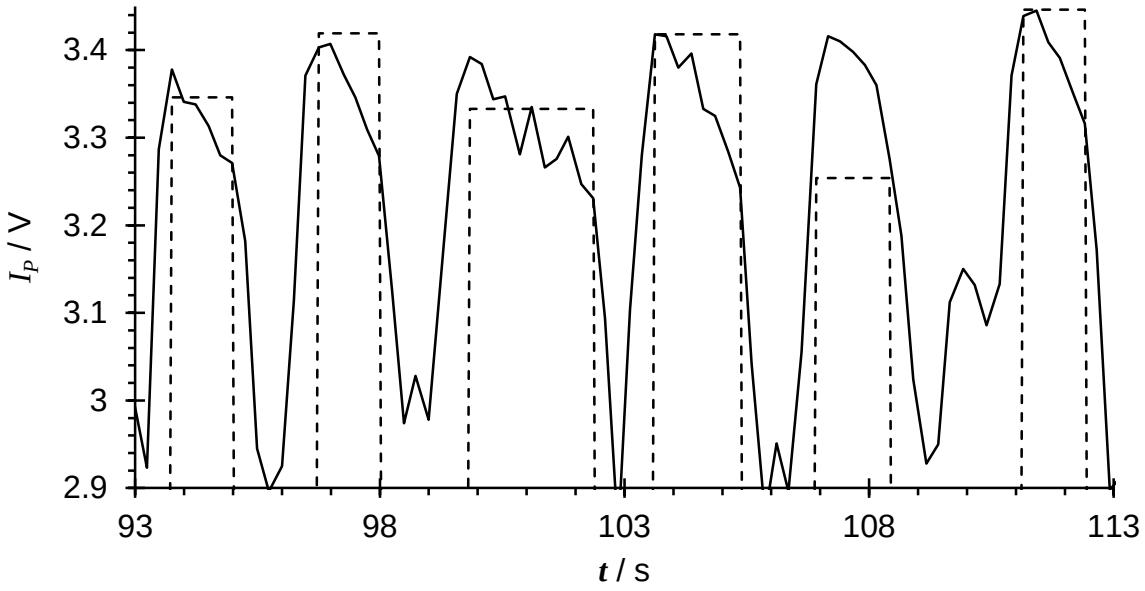

Figure 1 The solid line is the output from the pressure transducer ($\boldsymbol{I_P}$) used to monitor and control the adaptive sampler used to acquire the 25 breath samples used in this study. The dashed trace indicates the control signal used to actuate the sampling valves. This example of six breaths taken from one of the samples over a period of 20 s is typical of the variability of the participant's breathing pattern and indicates the portions of each breath that were sampled.

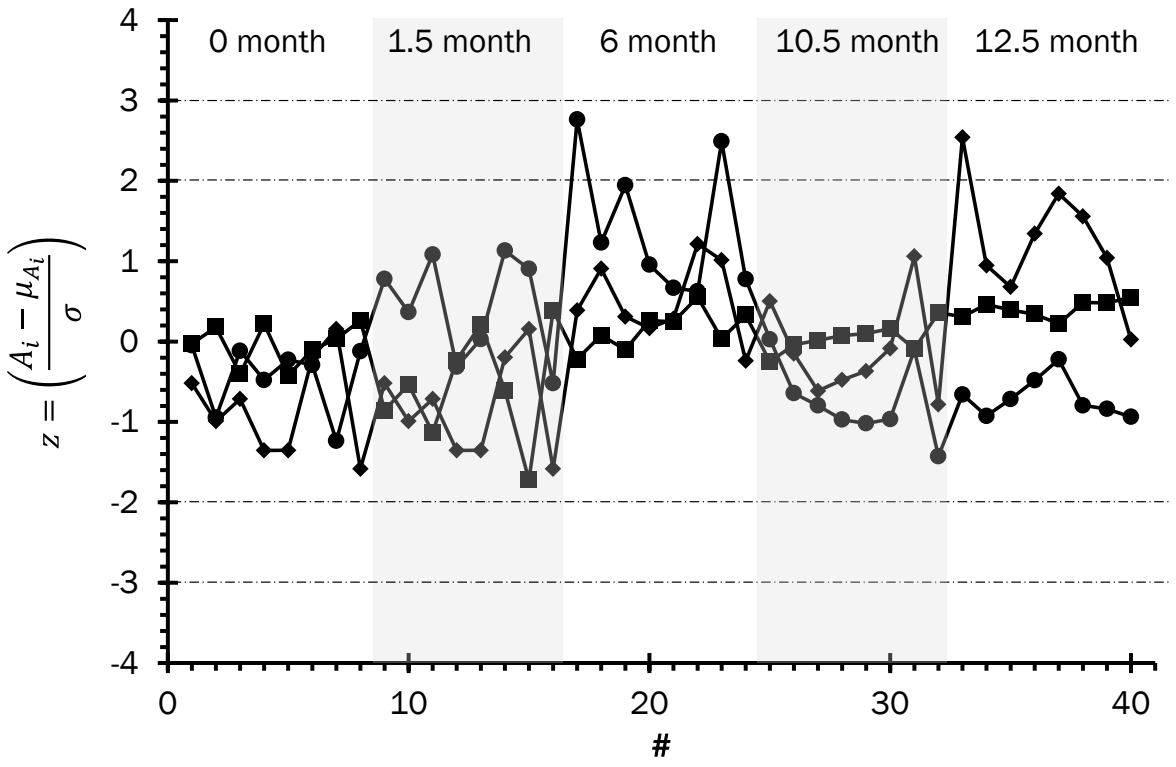

Figure 2 Z-scores for the peak areas of the internal standards for all the TD-GC-MS runs undertaken in the experiment. Although no statistically significant deviations were observed throughout the experiment, the variability in the 1.5 month and 6 month analytical campaigns noted in Figure 3 is also indicated here.

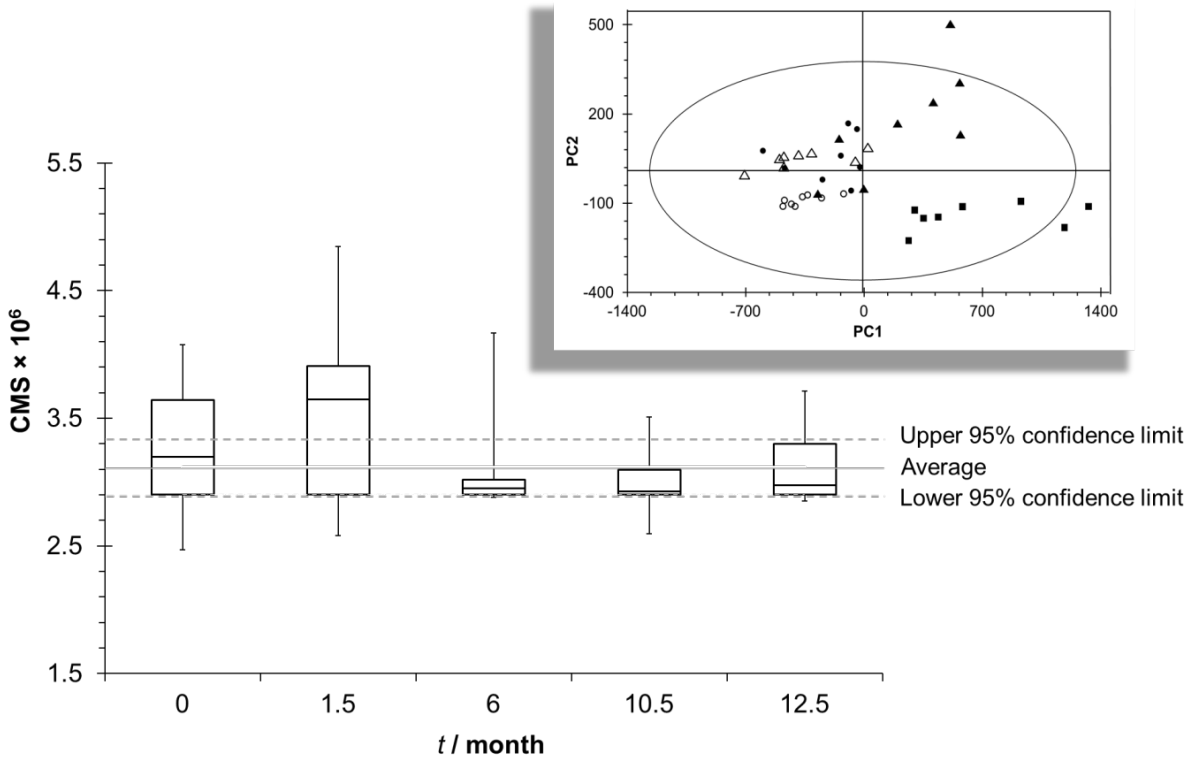

Figure 3 A box-whisker chart of the combined marker scores (CMS, Equation 1) observed for the five analytical campaigns run across the experiment. Insert top right. Unsupervised principal component analysis (PCA) of the 3 deuterated standards peak areas for the five analytical campaigns undertaken throughout the experiment. (5 observations respectively for each time points) 0 month (solid circles), 1.5 month (solid triangles), 6 month (squares), 10.5 month (open triangle) and 12.5 month (open circle).

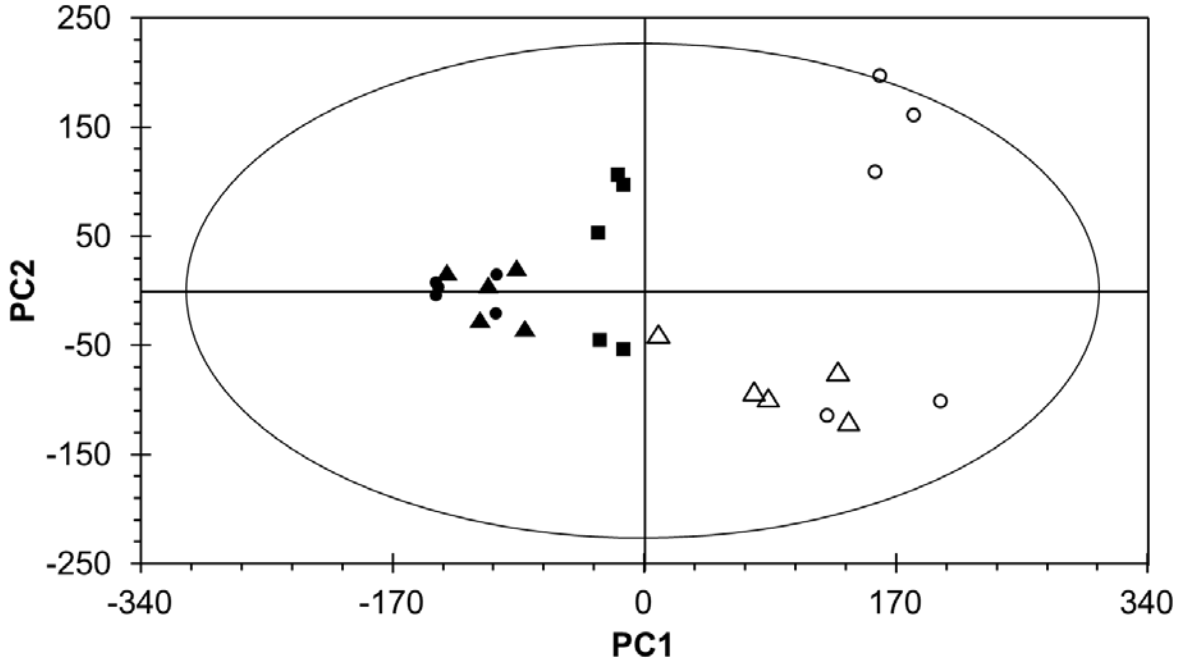

Figure 4 Unsupervised principal component analysis (PCA) generated from 151 endogenous breath components. 0 month (black dots), 1.5 month (triangle), 6 month (square), 10.5 month (open triangle) and 12.5 month (open circle) are shown. The separation of the analytical campaigns is distinctly different from the internal standard data and quality assurance z-scores, increasing with storage time.

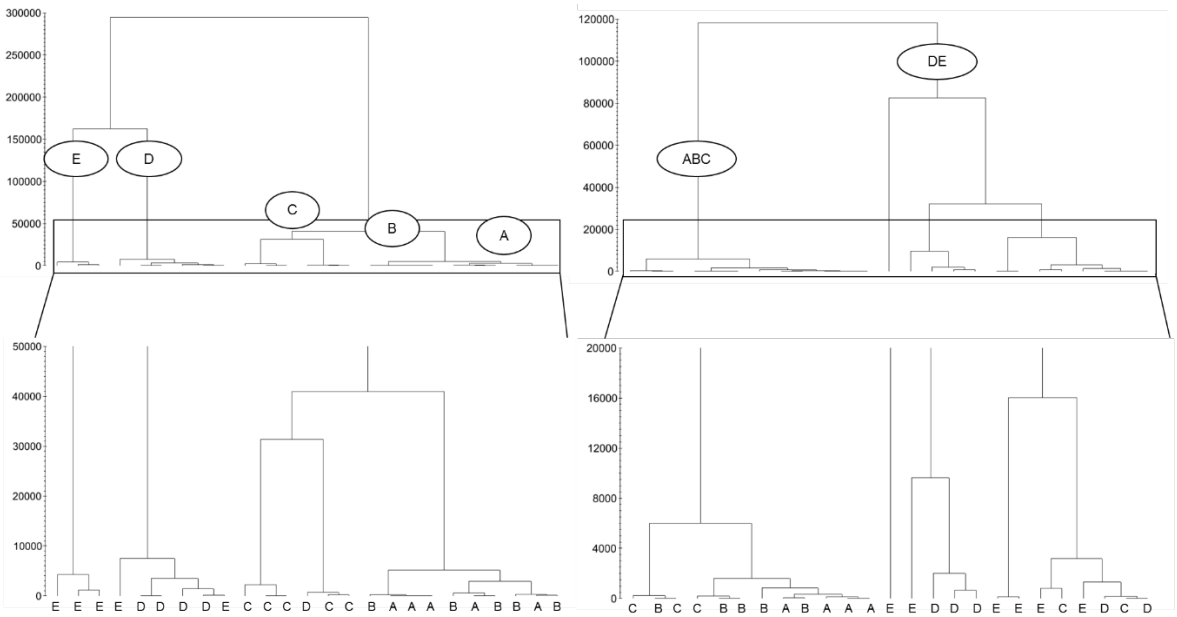

Figure 5 Dendrogram of endogenous (left) and exogenous (right) VOC generated from hierarchical cluster PCA analysis. The letters A, B, C, D and E stand for time points 0, 1.5, 6, 10.5 and 12.5 month respectively. Differences in apparent stability between endogenous and exogenous components are evident, although both datasets show distinct separation by 6 month. At 6 month the endogenous VOC formed a distinct grouping that was not observed in exogenous dataset.

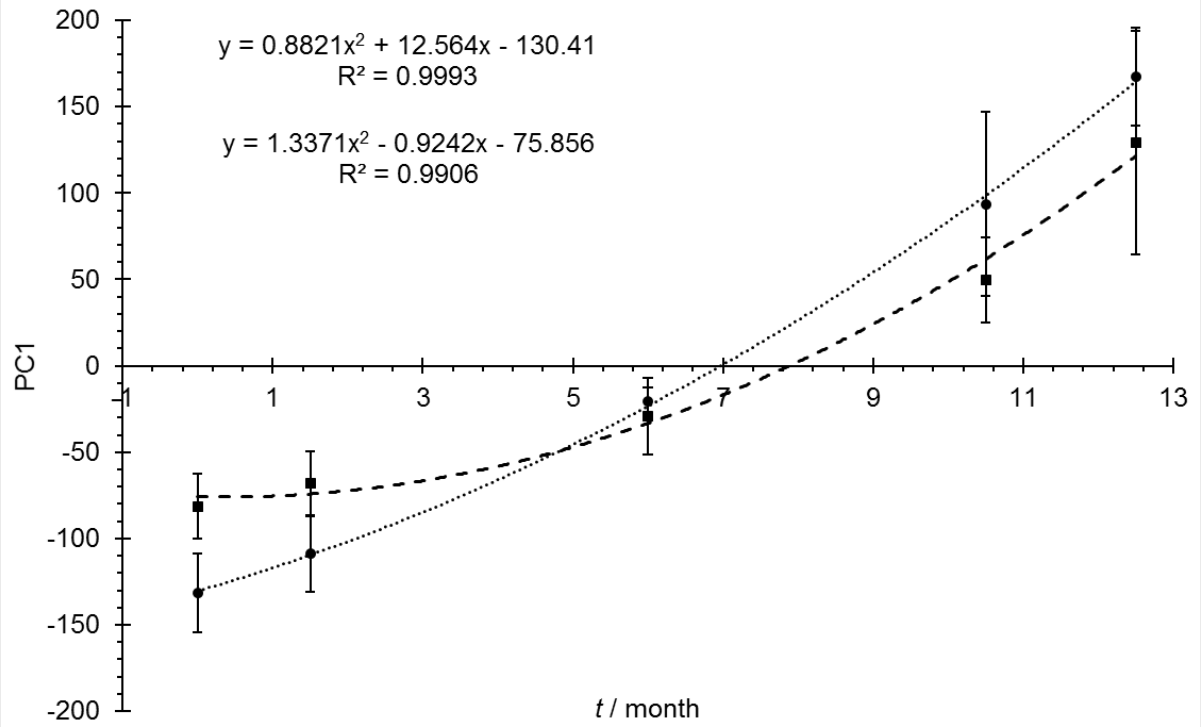

Figure 6 PLS regression of the PC1 scores generated from partial least squares regression (PLS) of endogenous breath components (black dot with dotted line) and exogenous breath components (square with dashed line) against storage duration indicates that the storage time may be predicted with some reliability from the recovered VOC profiles.

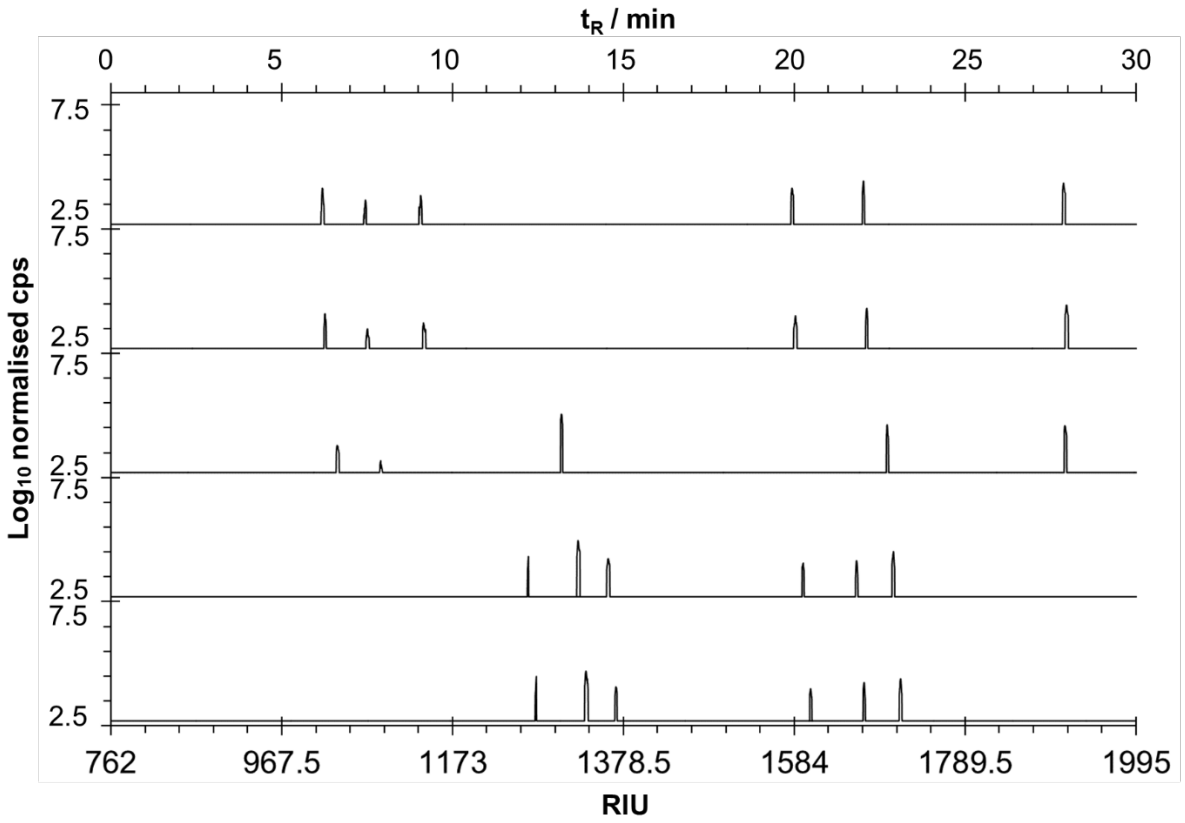

Figure 7. Extracted ion chromatograms of 12 endogenous compounds that were either lost or created during storage showing log_{10} intensity vs. retention index/retention time, See Table 2. Lower x-axis shows retention index units (RIU) and upper x-axis shows their equivalent retention time (t_R). From top to bottom: 0 month (reference); 1.5 month; 6 month; 10.5 month and 12.5 month.

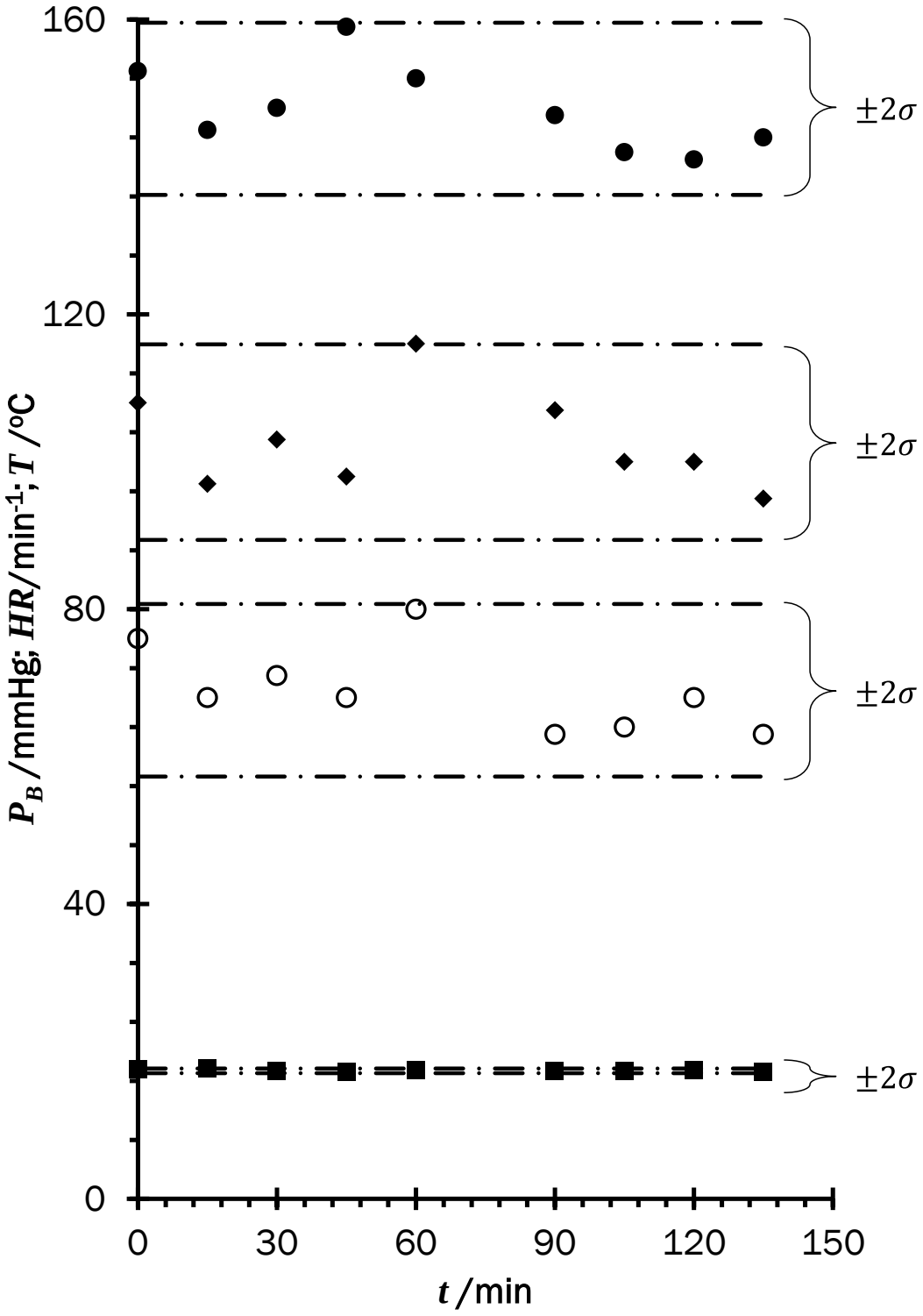

Figure S 1. Observed systolic blood pressure (Solid circles) diastolic blood pressure (Open circles) heart rate (solid diamonds) for the participant during the breath sampling procedure. Room temperature (Solid squares) was also recorded. Some indication of stress towards the end of the first 60 min was apparent followed by a relaxation back to starting levels. No significant (>2σ) deviations were recorded.