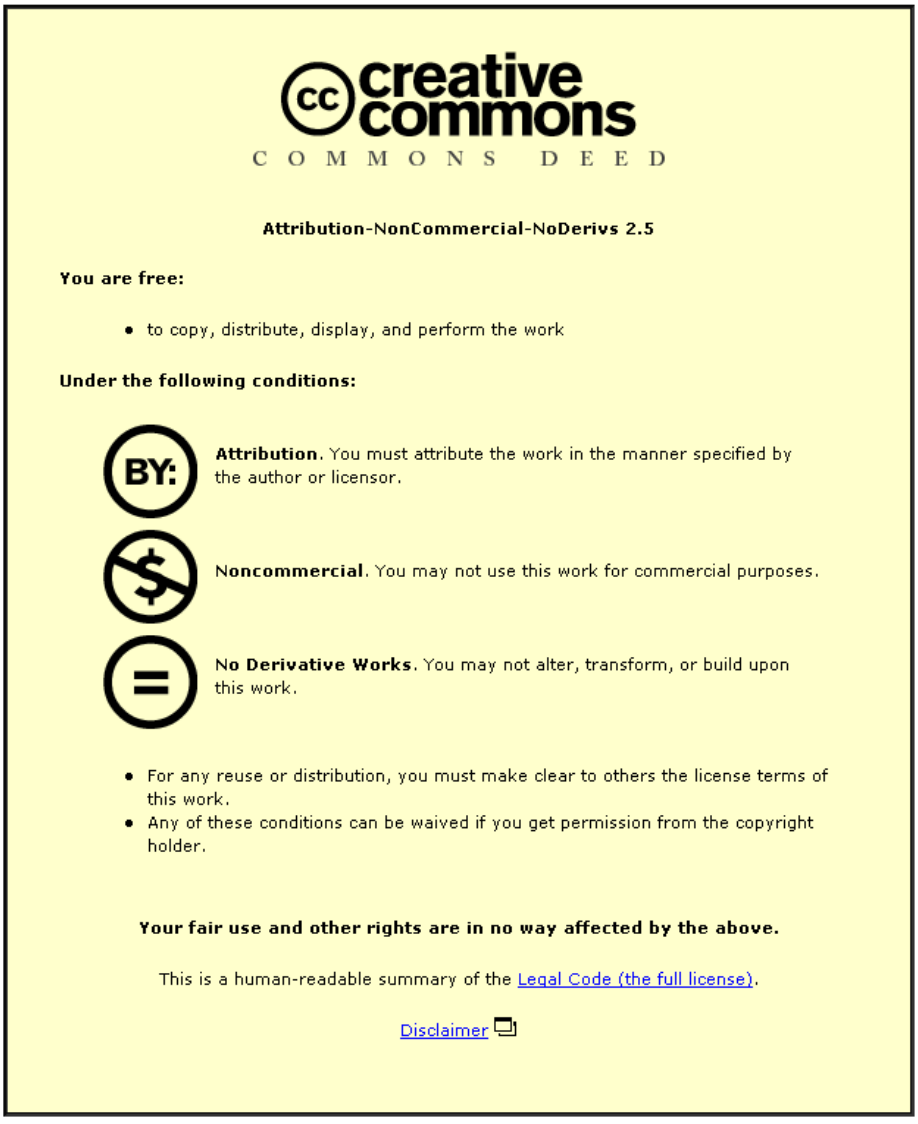

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THE EFFECT OF A PACED AUDITORY SERIAL ADDITION TEST (PASAT) INTERVENTION ON THE PROFILE OF VOLATILE ORGANIC COMPOUNDS IN HUMAN BREATH: A PILOT STUDY.

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ABSTRACT

This study sought to identify if detectable changes in human breath profiles may be observed following a psychological intervention designed to induce stress; a paced auditory serial addition test (PASAT).

Breath samples were collected from 22 participants (10 male and 12 female) following a double cross-over randomised design with 2 experimental interventions. One intervention required participants to listening to classical music chosen to be neutral. The other intervention required participants to undertake a PASAT that induced cardiovascular responses consistent with acute stress. Both interventions also involved two sequences of cognitive function tests.

Blood-pressure and heart-rate were recorded throughout each intervention and distal breath samples were collected onto Tenax® TA / Carbograph 1 thermal desorption tubes, using an adaptive breath sampler. Samples were collected before and after the PASAT. Breath samples were analysed by thermal desorption gas chromatography-mass spectrometry. Data registration using retention indexing and peak deconvolution followed by partial least-squares discriminant analysis identified 6 stress sensitive compounds. A principal components analysis model based on these components generated a model with that predicted post-PASAT vs post-Neutral intervention samples with a sensitivity of 83.3% and a selectivity of 91.6% for females, compared to 100% sensitivity and 90% selectivity for males.

Of the six compounds indole, 2-hydroxy-1-phenylethanone, benzaldehyde, and 2-ethylhexan-1-ol were identified on the basis of mass spectral, retention indexing and confirmation against pure standards. 2-methylpentadecane was tentatively identified from mass spectral and retention indexing, whilst one component has yet to be assigned, although the mass spectrum is indicative of a terpene. Indole and 2-methylpentadecane concentrations increased in response to the PASAT intervention, while the other compounds reduced in their abundance in human breath, possibly as a result of ventilation effects.

KEYWORDS

Breath, Psychological stress, VOC, Volatile organic compounds, adsorbent traps, GC-MS.

INTRODUCTION

Breath profiling is innately attractive for it offers a non-invasive, safe and straightforward observation of multiple biochemical processes occurring within the human body. A fast and affordable broad-spectrum health-screen run in-clinic is a common ambition held by many researchers in this area. Recently the non-invasive detection of biomarkers in breath associated with tuberculosis (TB) [1], chronic obstructive pulmonary disease (COPD) [2], asthma [3] and an array of cancers [4-6] have been reported. Currently it is possible to isolate volatile organic compounds (VOC) from a breath sample at concentrations ranging from mg m^{-3} (ppmv) down to ng m^{-3} (pptv). The composition and concentration of the profile of VOC changes as a result of many factors not necessarily associated with disease; diet [7], environment [8], and exercise [9] for example.

Psychological stress (stress) is experienced by everyone and it is a healthy response to a challenging or threatening environment, evoking what is known as the general adaptation syndrome (the fight or flight response). It is conceivable that participants providing breath samples for diagnostic research during a visit to a clinical facility might be experiencing differing degrees of stress. The UK Health and Safety Executive (HSE) reported over 400,000 cases of psychological stress related complaints in the UK from 2009 to 2010 [10]. Psychological stress disorders such as anxiety and depression have also been shown to have a higher prevalence in patients suffering from COPD [11] and lung cancer compared to healthy volunteers [12]. Stress may be a common concurrent factor in many breath samples.

Stress causes a cascade of hormonal releases. The most well-known is the secretion of cortisol, but this is only one aspect of the body's response. Gluconeogenesis, glucogenolysis and lipolysis are also stimulated and increased levels of renin and angiotensin II enzyme are produced causing elevation in blood-pressure (BP) and heart-rate (HR) [13]. Research into stress exposure prior to influenza vaccination has claimed increases in the level of antibody response in women [14]. Long-term exposure to psychological stress has been reported to cause headaches [15], back pain [16] and reduced cognitive function [17]. Such observations arouse curiosity as to whether stress may be identified in breath VOC profiles, and if so whether any such putative markers of stress might be potential confounding factors for diagnosis of disease by breath analysis. The observation and description of the effect of psychological stress on breath VOC, is currently unreported. This study, therefore, sought to engender a stress response in

human breath using a PASAT intervention [18 and 19]. The underlying hypothesis was that induced stress would not be associated with identifiable changes in exhaled VOCs across the participant cohort. An adjunct to this study would be a tentative examination of any exhaled VOC compounds that appeared to be associated with a stress response in the event of the hypothesis being disproven.

METHODS AND MATERIALS

Volunteer participants.

The study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. This research followed the protocols that were reviewed and granted a favourable opinion by the local area ethics advisory committee, Loughborough University; G08-P7 and G09-P5. All participants taking part in the study gave written informed consent.

23 male and/or female volunteers aged between 19 and 26 years participated in the study, and 22 complete sets of breath samples were obtained. The study used a randomised cross-over design with the participants attending two sampling sessions. (neutral and stress), each comprising an intervention. In the neutral session, they were asked to sit comfortably and listen to music (neutral intervention), chosen to be non-stressful. In the stress session they were asked to undertake a mental arithmetic test (PASAT intervention), chosen to elicit stress [19]. They were also asked to take a cognitive function assessment tests. (See Supplementary Table 1) before and after the intervention in both samplng sessions, See Figure 1).

The cognitive function assessments do not induce a stress response, and were run as a part of another study into the effect of stress on cognitive function. The cognitive function study was not germane to the current study and will not be discussed further in this paper.

The sampling/test sessions ran in the morning. Before the sampling/test sessions the participants had fasted for a minimum of 9 hr and had drunk only water during this time. Further, the participants had refrained from washing or using oral hygiene products on the morning of their visit. In each session, two VOC breath samples were collected. The first breath sample was taken prior to beginning the experimental interventions (neutral /PASAT intervention) and the second directly after completion of the intervention Figure 1.

Heart-rate and blood-pressure measurements

Heart-rate (HR/bpm) and blood-pressure (BP/mmHg) were recorded using a Datex Ohmeda S/5 patient monitoring system. A finger clip was attached to the index finger of the participant's left hand in order to monitor the HR. A Critikon 23 -33 cm Dura-Cuf® blood-pressure cuff was applied to the participant's upper left arm. Following a rest period baseline BP and HR readings were taken at 2-minute intervals whilst the participant sat quietly. A further 13 readings were taken over the course of each experimental session at 12, 16, 19, 23, 30, 42, 45, 48, 49, 57, 60, 65 and 71 minutes. HR, and BP (systolic, diastolic and the mean arterial pressure) were recorded at each time point, see Figure 1 .

2.2.3 Paced Auditory Serial Addition Task (PASAT)

The PASAT, originally developed to assess the effects of neurological injury on cognition [18], was used to induce mental stress in a reproducible manner in participants [19]. Whilst remaining seated and following a 30-second practice, participants completed a 10-minute PASAT. The PASAT required participants listen to a recording of a series of random single digit numbers (1 to 9) with intervals of 4.5-seconds between digit presentation in the first 2-minutes, decreasing by 0.5-seconds every subsequent 2-minute period [19].. Participants were instructed add the current number to the previously presented number and promptly call out their response. An incorrect response was followed by a loud auditory error-alert to indicate a wrong answer. At pre-defined points over the task (approximately every 10 trials) the error-alert sounded, regardless of the correctness of an answer. Participants were asked to watch a live view of themselves during the task, displayed on a monitor in front of them. The experimenter gave prompts during the task if participants deviated from task instructions. Participants were told their performance would be recorded and displayed in a performance table.

The neutral intervention required participants to listen to recordings of ten minutes of classical music comprising four instrumental pieces; Truman Sleeps composed by Burkhard Dallwitz, Guitar Flute & String composed by Moby, Gabriel's Oboe composed by Ennio Morricone and Suite Bergamasque: Clair De Lune performed by Dame Moura Lympany. This intervention was designed to be non-stressful.

Breath sample collection and analysis

Distal airway breath (4.0 dm3) was collected from participants at each sampling time in the test procedure (Indicated by Ⓑ in Figure 1). Participants donned a non-vented full face mask fitted with a silicone pillow (ResMed, Oxfordshire, UK), supplied with purified 'medair' while they breathed in a relaxed and natural manner during the sampling process. Every breath was recorded with pressure sensors, and the signal from the these sensors was processed using analogue to digital conversion, and fed to a virtual instrument that controlled the sampling micro-valves that ensured a high-precision sampling pump (sample-flow = 1 $dm^3 min^{-1}$) was maintained at a constant operating flow throughout. Digital signal processing was used to continuously analyse the breathing profile and control the switching signals to the micro-valves that ensured a portion of air from the distal airways was reproducibly sampled from each breath through an inert capillary onto a mixed bed sorbent trap (Tenax® TA/Carbograph 1 TD, Part No C2-AAXX-5032 Markes International, Llantrisant, UK).

Supplementary Figure 1 is a photograph of the breath sampling mask and Supplementary Figure 2 compares examples of the sampling breath profiles acquired from the same participant before and after the PASAT. A more complete description of the sampling system has been reported previously [20] and the confounding factors associated with the analysis of human breath by thermal desorption GC–MS especially those associated with the high water levels encountered in human breath samples have been reviewed [21]. Further a central composite design optimisation studied sample volumes and analyte breakthrough with multi-linear regression analysis. Breath sampling-flow and breath sample-volume over the ranges (0.5 to 1.4 $dm^3 min^{-1}$, and 2.5 to 10 dm^3 respectively) for propanone, isoprene, 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene, butylbenzene, 4-isopropenyl-1-methylcyclohexene, phenylmethanol and 1,3-benzothiazole were studied and a maximum sample volume of 6.2 dm^3 was achievable without breakthrough of the most volatile components [21]. The reproducibility of this approach has also been evaluated and the within-subject variability was observed to be significantly lower than between-subject variability ($p=6.23 \times 10^{-23}$) [22] and more recently this method has been used to non-invasively phenotype asthmatics [23].

Participants wore the breath sampling masks for 5 min to enable them to acclimatise to the procedure, and to develop a normal relaxed breathing pattern from which the breath

samples were collected. The mean respiration rate across the cohort was 15 ±4 breaths per minute for the pre-PASAT samples and 17±3 breaths per minute post PASAT. (The figures in brackets indicate the 95 % confidence limit).

The trapped volatiles were analysed with a Varian 3800 GC interfaced to a Varian 4000 ion-trap mass spectrometer controlled using the Saturn Workstation software package (Varian). Sample introduction was by two-stage thermal desorption (Markes International Unity Series 1 fitted with a general-purpose hydrophobic cold trap.) The instrumentation parameters are summarised in Table 1. The quality assurance of the analytical system was assessed by the analysis of a retention index standard mixture (RI STD) of 16 components of known concentration every six breath samples. Two blanks were run between analytical samples to ensure the absence of any VOC carry over. Collected breath samples were stored at 4^{o}C prior to analysis by TD-GC-MS.

RESULTS AND DISCUSSION

Blood pressure and heart rate

Individual HR, systolic BP and diastolic BP were collated and the average observations show the cardiovascular responses to the PASAT and neutral interventions. Figure 1 contrasts the changes in the observed in cardiovascular response during the PASAT (labelled P) and the neutral sessions .

PASAT Response Score.

The participants' individual cardiovascular responses to the PASAT and neutral interventions were scored on the basis of observed changes in their systolic blood pressure and heart rate. The average of the 3 initial baseline observations were used to give BP and HR start values (P_S^{start} and HR^{start}). The maximum observed value of the 4 readings taken during the intervention periods ($P_S^{\max}$ and $HR^{\max}$) were assigned the points of maximum "stress". Equation1 defines a PASAT response score (PSR) derived for the PASAT (PSR_S) and neutral interventions (PSR_N).

$$PSR = \frac{\Delta P_S}{P_S^{start}} \times \frac{\Delta HR}{HR^{start}} \times 1000$$

where

$$\Delta P_S = P_S^{\max} - P_S^{start} \quad (1)$$

and

$$\Delta HR = HR^{\max} - HR^{start}$$

The PSR sought to emphasise the relative change in systolic blood pressure that occurred during the intervention (ΔP_S) compared to baseline observations(P_S^{Start}). Heart rate was also included in the same manner providing a ratio between the overall change (ΔHR) and the baseline values. The PSR score in Equation 1 was framed to give higher score for those individuals showing the largest cardiovascular responses under the interventions. The PSR scores for the participants are summarised in Table 2 and the box-whisker plot in Figure 1 contrasts the responses for the male and female participants. One male participant did not exhibit a stress response (PSR_S = 0.9, PSR_N =5.3). The average responses for the remaining 21 participants was a PSR_S of 64.5 ± 24 compared to PSR_N score of 4.5 ± 2) The median PSR score during the neutral intervention was 3.5 compared to 428 for the PASAT intervention. Male and female responses were not statistically different with Female PSR scores showed a greater range of responses during the PASAT intervention (PSR = 12 to 233), while it was the male participants who had the greatest variability (PSR = 0.1 to 18.6) in the neutral state. Overall, the cardiovascular responses summarised in Figure 1 and by the PSR scores are consistent with the participants experiencing psychological stress from the PASAT intervention.

Chromatographic data

The gas chromatographic-mass spectrometric (GC-MS) data were processed following a work-flow that has been described previously [21]. Retention times were correlated to a retention index scale constructed from a primary retention ladder generated from a quality assurance standard for the analysis, and a secondary retention index scale based on the retention times of 5 ubiquitous siloxane components present in all breath samples as a result of hydrolysis of the active phases within the analytical system. Figure 2 illustrates a typical example of the total ion current (TIC) chromatograms with both

retention time and retention index values (RIU) obtained from the blanks and breath samples collected under the two experimental sessions.

The GC-MS data were deconvolved using AnalyzerPro (Spectral Works, UK), and a typical breath sample would yield between 300 and 400 individual components. A breath matrix was constructed that contained 154 breath VOC that were unique to breath and not found in any background sample. Each component in the breath matrix was assigned a unique reference number that began with the prefix BRI – followed by the retention index value for that component. The final 5 numbers in the reference were the 5 most abundant ions in the components de-convolved spectrum. So for example the compound indol has a retention index of 1286 and the five most abundant peaks in decreasing order are m/z 117, m/z 90, m/z 89, m/z 63 and m/z 73, so the reference for indole was BRI1286-117-90-89-63-73. Saturn MS workstation software (Varian inc, UK) was used to integrate peak areas for each breath component's extracted ion chromatograms (XIC). Compounds that were present in less than 30% of the breath samples collected were also excluded from the data modelling, and this left 50 breath components that were used in the resultant multi-variate analysis (MVA) [24,25].

Multi-variate analysis

The breath matrix consisted of the peak areas of the 50 VOC breath components (variables) against the participants (observations). MVA was conducted using SIMCA-P + software (Version 12, Umetrics, UK). Partial least squared discriminate analysis (PLS-DA) was initially performed on the post intervention samples from both PASAT and neutral experimental sessions. All variables were assigned to a single block and a weighting of $1/\sqrt{Block}$ was applied making the total variance of all variables equal to 1. In addition Pareto Variance (ParN) was selected for base scaling of the data set; this scaling method takes each variable, subtracts it from the mean of that variable set and divides it by the square root of the standard deviation for the variable set [26,27]. The S-plot generated from this statistical model (Figure 3) indicated six possible VOC variables (α1 to α6) that changed in response to interventions experienced by the participants. Principle component analysis (PCA) of these six VOC variables enabled the separation between the PASAT and neutral observations to be evaluated. The score plots generated from these data are given in Figure 4. In each plot responses from the PASAT and neutral interventions are seen to cluster and a distinction between breath profiles obtained under the two experimental sessions can be observed. The principle components

identified, exhibited 44.1% [PC1] and 15.7% [PC2] of the total explained variance of the data set for male participants yielding a 100% sensitivity (% of correctly assigned true positive observations) for the male PASAT observations and 90% selectivity (% of correctly assigned true negative observation) for the neutral observations. In contrast to this the female observations produced a weaker model and yielded a sensitivity of 83.3% and a selectivity of 91.6%. The total variance modelled by each component was 34.5% and 28.4% for [PC1] and [PC2] respectively. Further when the multivariate results were cross-correlated to the PSR scores the outliers were found to have anomalous PSR scores. Participants 4 and 18 had low PSR scored during the PASAT intervention and Participant 6 scored a higher PSR score in the neutral intervention than the PASAT intervention (Table 2).

Preliminary identification of PASAT sensitive breath components.

Elucidation of the VOC components α1 to α6 was based on three tests. The first was a "satisfactory" match of the de-convoluted spectrum generated from Analyzer pro to the respective entry in the NIST MS library (NIST05). "Satisfactory" defined in this instance as forward (direct matching of spectrum peaks to library reference spectrum) and reverse (matching by omitting spectrum peak not present in the library spectrum) match factor with a value greater than, or equal to, 650, see Table 3 [28].

The second test was a "satisfactory" match between the observed retention index value (IU) and the estimated retention index value obtained from NIST05 MS library. "Satisfactory" was defined as a difference not greater than ±50 IU. The recorded retention index value for α2, see Table 3, of 1063 IU for BRI1063-105-77-52-106-51 (indicated by mass spectrometric library matching to be 2-Hydroxy-1-phenylethanone) did not match the estimated NIST retention index value of 1272 IU. No record of an experimental retention index for this compound for a DB5 (5% phenyl 95 % methyl) stationary phase appears to be currently available.

The third and final test was based on a matching the preliminary assignments in Table 3 to standards analysed under the same conditions, Table 1. An adsorbent sampling tube was spiked with 0.1 µl of a 50 µg ml^{-1} methanol solution of: indole (α1), 2-hydroxy-1-phenylethanone (α2), 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl) (α4), benzaldehyde (α5), 2-ethylhexan-1-ol (α6); all reagents were purchased from Aldrich. The an on-column masses of each of the components were 500 pg. 2-methylpentadecane (α3) was not available commercially and was not included in this test.

These three tests confirmed the assignments of indole, 2-hydroxy-1-phenylethanone, benzaldehyde, and 2-ethylhexan-1-ol. α4 was not confirmed as 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl) however, the mass spectrum indicates that α4 was likely to be a terpene. 2-methylpentadecane (α3) remains a tentative assignment and Table 3 summarises the PASAT sensitive components.

The origins of these VOC in breath are not well defined and may be generated through a combination of independent mechanisms. Indeed it is perhaps to soon postulate biological origins and roles for these VOC's as part of a stress-sensitive response in breath. At this stage it is helpful to note that benzaldehyde is a common food component and occurs naturally in fruits like apples and honey [31,30] likewise terpenes occur naturally in foodstuffs, [29]. These components may be due to dietary intake and may not have an endogenous origin. Indole is associated with the production of the essential amino acid tryptophan, which in turn is part of the pathway that produces serotonin; previously reported to be involved in cardiovascular and psychological response to acute stress [32]. Indole may also be generated from tryptophan metabolism by indole positive bacteria in the gastro intestinal system. 2-Ethylhexan-1-ol has been associated with poly vinyl chloride (PVC) [33] and also as a potential VOC marker of lung cancer [34]. In addition 2-ethylhexan-1-ol has also been observed in the headspace of in-vitro cultures of lung carcinoma A549 cells [35]. Methylated hydrocarbons have been reported as the endogenous products of oxidative stress and numerous VOCS in this class have been identified as potential biomarkers of various cancers [6, 33].

Example responses from Participant (14, Table 2) for these six compounds are presented in Figure 5. Indole and 2-methylpentadecane exhibit an increased or up-regulated response during the PASAT intervention. Across the cohort, indole signals increased from +140% to +360% (compared to the Neutral intervention samples) in response to the PASAT intervention. 2-methylpentadecane increased to a lesser extent, between +30% to +80% increases compared to the Neutral intervention. The remaining four components show a reduced or down-regulated response compared to the Neutral Intervention.

The collective scores for the six VOC markers were combined by taking their euclidian distance, based on the peak intensities (I_R) to generate a marker score (CMS), see Equation 2 and the box whisker plots in Figure 6. The median CMS for the neutral intervention was 5.42×10^4 compared to 4.62×10^4 for the PASAT intervention. Female participants exhibited the greatest range and variability during the PASAT intervention. In

contrast male participants showed the widest range and variability during the neutral intervention. In comparison to the PSR (Figure 1) the CMS results show equivalent trends in the female participants with an increase in range and variability in breath samples collected under PASAT intervention when compared to the results from the neutral intervention. This correlation was not reflected in the male participants, one reason for this maybe due to the lower intensities observed for the down regulated breath components; indicating a higher rate of clearance.

$$CMS = \sqrt{\sum_{\alpha=1}^{6} I_R^2} \tag{2}$$

SUMMARY

The PASAT intervention was used to induce cardiovascular reactions indicative of acute psychological stress in the participants. Our starting hypothesis that no change in the VOC profile would occur was not proven and these preliminary findings are consistent with the possible existence of a stress-related response in the VOC profiles of humans. Four of the six most PASAT responsive VOCs have been identified through combined mass spectral, retention index and chemical standard matches. A combination of all six of these PASAT responsive components, enabled the population to be classified as PASAT responsive or Neutral with a sensitivity of 83.3% and a selectivity of 91.6% for females, compared to 100% sensitivity and 90% selectivity for males. Further the individuals who were not correctly classified though these six VOC were either observed to exhibit stress, during their neutral visits (induced we think by the unfamiliar surroundings of the test rooms and laboratory environments), or who had low PSR scores; indicative of not experiencing stress during the PASAT intervention.

It seems to reasonable to take as a starting preposition that stressed people breath faster, with increased pulse rates and elevated blood-pressure, and this is likely to change their VOC profile; indeed the four down-regulated VOCs may have been depleted through increased ventilation from elevated heart-rate, and respiratory rate during the PASAT. If stress induced enhanced ventilation causes a reduction in levels of markers, or perturbs patterns of markers then additional controls in breath sampling protocols may need to be developed. In contrast 2-methyl,pentadecane and indole, which were

observed to be up–regulated in this study need to be verified in larger studies before any indication could be derived that they were indicative of a metabolic/biochemical response to the PASAT intervention and by inference stress.

The retrospective quantitation of the six VOC was not possible, however the levels are low, approaching the limit of what is feasible to analyse in a clinical setting. If the sampling and transfer efficiencies were similar to those obtained during the verification with standards then the concentrations involved would lie across an estimated that would be unlikely to be greater than 20 ng m^{-3} for indole, and 4.8 µg m^{-3} for benzaldehyde, for example. Targeted studies with labelled standards with a larger participant cohort would be a logical follow up to this study.

Demonstrations of the utility of breath analysis in providing next generation in-community diagnostic capability through monitoring biochemical changes in the human body continue to be reported [1]. This study indicates that the human VOC profile may also be sensitive to non-physical stimuli. Physical disease may not be the only contributing factor in breath profiling and the acquisition of samples and data from people undergoing traumatic and emotionally challenging diagnoses associated with serious diseases may need to consider this factor.

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REFERENCES

[1] Phillips M et al. 2012 Point-of-care breath test for biomarkers of active pulmonary tuberculosis Tuberculosis 92 314 - 320

[2] Van Berkel JJBN, Dallinga JW, Möller GM, Godschalk RW, Moonen EJ, Wouters EF, Van Schooten FJ, *2010* A profile of volatile organic compounds in breath discriminates COPD patients from controls *Res. Med.* **104** 557-563

[3] Dallinga JW, Robroeks CM, van Berkel JJ, Moonen EJ, Godschalk RW, Jöbsis Q, Dompeling E, Wouters EF, van Schooten FJ. 2010 Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children

Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology **40** 68-76

[4] Andreas W, Schmid A, Mechtcheriakovc S, Ledochowskid M, Grabmere C, Gastlf G A, Amann A, 2007 Lung cancer detection by proton transfer reaction mass-spectrometric analysis of human breath gas *Int. J. Mass Spec.* **265** 49-59

[5] Phillips M, Cataneo RN, Saunders C, Hope P, Schmitt P, Wai J. 2010 Volatile biomarkers in the breath of women with breast cancer *J. Breath Res.* **4** 026003

[6] Peng G, Hakim M, Broza YY, Billan S, Abdah-Bortnyak R, Kuten A, Tisch U, Haick H 2010 Detection of lung breast colorectal and prostate cancers from exhaled breath using a single array of nanosensors *British J. Cancer* **103** 542-551

[7] Statheropoulos M, Agapiou A, Georgiadou A 2006 Analysis of expired air of fasting male monks at Mount Athos *J. Chrom. B* **832** 274-279

[8] Adgate JL, Church TR, Ryan AD, Ramachandran G, Fredrickson AL, Stock TH, Morandi MT, Sexton K 2004 Outdoor, Indoor, and Personal Exposure to VOCs in Children *Environ .Health Perspect.* **112** 1386-1392

[9] King J *et al* 2009 Isoprene and acetone concentration profiles during exercise on an ergometer *J. Breath Res.* **3** 027006

[10] Health and Safety Executive 2011 Stress and Psychological Disorders (UK Health and Safety Executive) www.hse.gov.uk/statistics/causdis/stress/stress.pdf.

[11] Hynninen KMJ, Breitve MH, Wiborg AB, Pallesen S, Nordhus IH, 2005 Psychological characteristics of patients with chronic obstructive pulmonary disease: a review *J.Psychosomatic Res.* **59** 429-443

[12] Heffner KL, Loving TJ, Robles TF, Kiecolt-Glaser JK 2003 Examining psychosocial factors related to cancer incidence and progression: in search of the silver lining *Brain behavior and immunity* **17** S109-S111

[13] Standfield CL 2011 Principles of human physiology Pearson Benjamin Cummings: London (4th edition)

[14] Edwards KM, Burns VE, Reynolds T, Carroll D, Drayson M, Ring C 2006 Acute stress exposure to influenza vaccination enhances antibody response in women *Brain Behav. Immun.* **20** 159-168

[15] Houle T, Nash JM 2008 Stress and headache chronification *Headache* **48** 40-44

[16] Dahl J, Wilson K, Nilsson A, 2004 Acceptance and commitment therapy and the treatment of persons at risk for long-term disability resulting from stress and pain symptoms: A preliminary randomized trial *Behavior Therapy* **35** 785-801

[17] Marin MF, Lord C, Andrews J, Juster RP, Sindi S, Arsenault-Lapierre G, Fiocco AJ, Lupien SJ. 2011 Chronic stress cognitive functioning and mental health *Neurobio. Learn.Mem.* **96** 583-95

[18] Gronwall DM 1977 A Paced auditory serial-addition task: a measure of recovery from concussion *Percept. Mot. Skills* **44** 67-373

[19] De Boer D, Ring C, Wood M, Ford C, Jessney N, Mcintyre D, Carroll D 2007 Time course and mechanisms of mental stress-induced changes and their recovery: Hematocrit, colloid osmotic pressure, whole blood viscosity, coagulation times, and hemodynamic activity *Psychophysiology*, **44** 639–649.

[20] Basanta M, Koimtzis T, Singh D, Wilson ID, Thomas CLP 2007 An adaptive breath sampler for use with human subjects with an impaired respiratory function *Analyst* **32** 153-163

[21] Guallar-Hoyas C, Turner MA, Blackburn GJ, Wilson ID, Thomas CLP, 2012 A work-flow for the metabolomic/metabonomic investigation of exhaled breath using thermal desorption gas chromatography mass spectrometry *Bioanalysis* **4**, 2227–2237

[22] Basanta M, Jarvis RM, Xu Y, Blackburn G, Tal-Singer R, Woodcock A, Singh D, Goodacre R, Thomas CL, Fowler SJ. 2010 Non-invasive metabolomic analysis of breath using differential mobility spectrometry in patients with chronic obstructive pulmonary disease and healthy smokers. Analyst. **135**, 315-320.

[23] Ibrahim B, Basanta M, Cadden P, Singh D, Douce D, Woodcock A, Fowler SJ. 2011 Non-invasive phenotyping using exhaled volatile organic compounds in asthma. Thorax. **66**, 804-809.

[24]URH Massart DL, Vandeginste BGM, Deming SN, Michotte Y, Kaufman L. "Chemometrics: a Textbook (Data Handling in Science and Technology volume 2)" Publ. Elsevier 2003, ISBN: 0-444-42660-4

[25] Umetrics AB, "User's Guide to SIMPCA-P, SIMPCA-P+" Version 11. Publ. Umetrics, Box 7960 Umea, Sweden (2005). URL:http://www.umetrics.com/Content/Document%20Library/Files/UserGuides-Tutorials/SIMCA-P_11_UG.pdf

[26] Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM, 2006 Targeted profiling: quantitative analysis of 1H NMR metabolomics data *Anal. Chem.* **78** 4430-4442

[27] Ramadan Z, Jacobs D, Grigorov M, Kochhar S, 2006 Metabolic profiling using principal component analysis discriminant partial least squares and genetic algorithms *Talanta* **68** 1683-1691

[28] The NIST mass spectrometry data centre NIST Standard Reference Database 1A: User guide release date: June 08

[29] Jerković I, Marijanović Z, Kezić J, Gugić M, 2009 Headspace volatile and semi-volatile organic compounds diversity and radical scavenging activity of ultrasonic solvent extracts from Amorpha fruticosa honey samples *Molecules* **14** 2717-2728

[30] Mattheis JP, Buchanan D A, Fellman JK, 1991 Change in apple fruit volatiles after storage in atmospheres inducing anaerobic metabolism *J. Agri. Food Chem.* **39** 1602-1605

[31] Vikram A, Lui LH, Hossain A, Kushalappa AC, 2006 Metabolic fingerprinting to discriminate diseases of stored carrots *Annals of App. Biol.* **148** 17-26

[32] Davies SJ, Hood SD, Argyropoulos SV, Morris K, Bell C, Witchel HJ, Jackson PR, Nutt DJ, Potokar JP, 2006 Depleting serotonin enhances both cardiovascular and psychological stress reactivity in recovered patients with anxiety disorders *J.Clinical Psychopharm*. **26** 414-418

[33] Tuomainen A, Stark H, Seuri M, Hirvonen M, Linnainmaa M, Sieppi A, Tukiainen H 2006 Experimental PVC Material Challenge in Subjects with Occupational PVC Exposure *Environ. Health Perspect.* **114** 1409-1413

[34] Haick H; Gang P; Tisch U; Zilberman Y; Pisula W; Feng X; Müllen K (2009) Sniffing out cancer in the breath: detection of non-polar volatile compounds through carrier scattering in random networks of carbon nanotubes *Proc. SPIE* **7418** 741809-741809-15 (**DOI: 10.1117/12.824444)**

[35] Filipiak W, Sponring A, Filipiak A, Ager C, Schubert J, Miekisch W, Amann A, Troppmair J. 2010 TD-GC-MS analysis of volatile metabolites of human lung cancer and normal cells in vitro Cancer Epidemiol. Biomarkers Prev. 19 182-195

THE EFFECT OF A PACED AUDIO SERIAL ADDITION TASK (PASAT) INTERVENTION ON THE VOLATILE ORGANIC COMPOUNDS IN HUMAN BREATH PROFILES: A PILOT STUDY.

FIGURES AND TABLES

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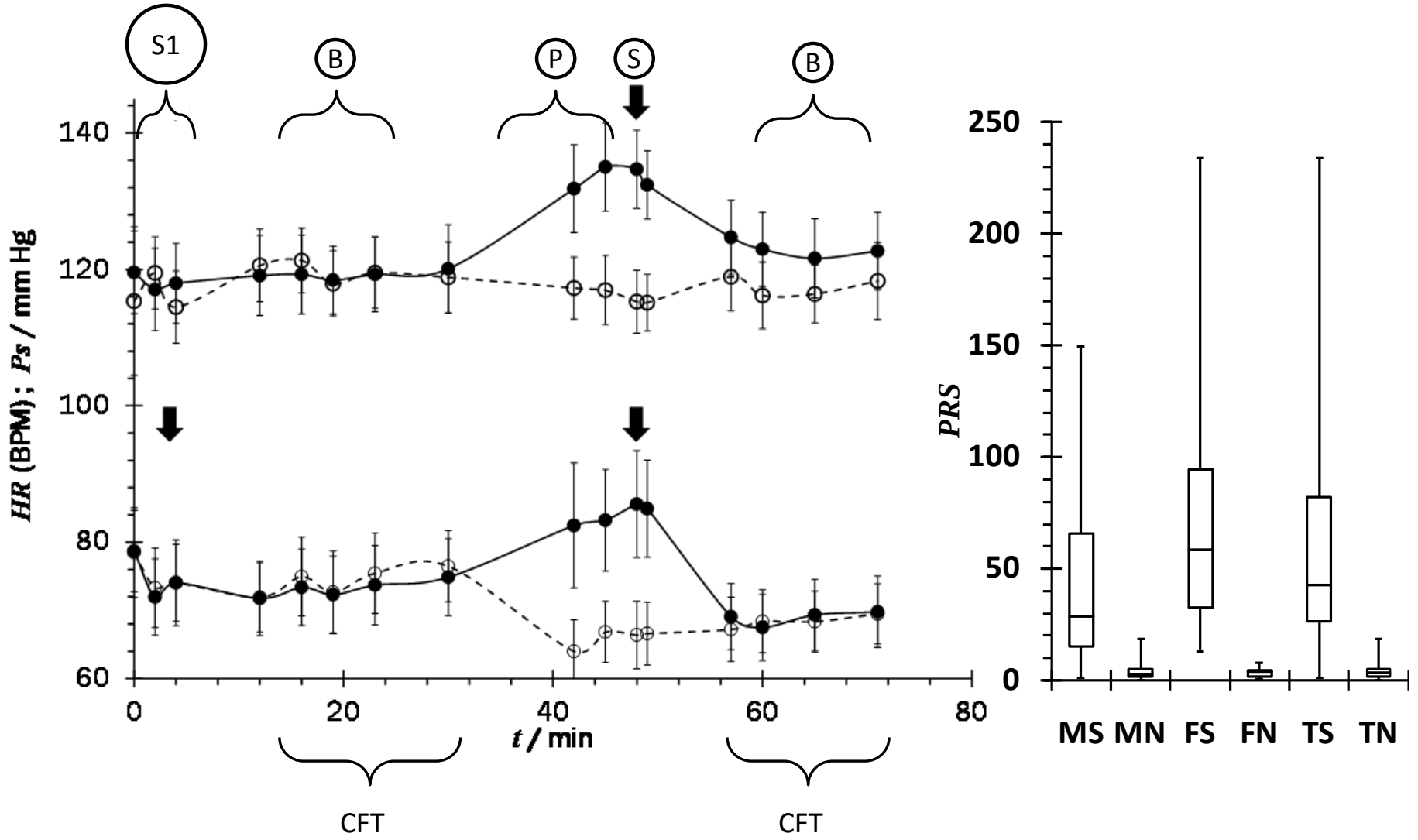


Figure 1. Left. Mean responses of 22 participants for systolic blood pressure (P_S, top), and heart rate (HR, bottom) over the 70 min stress (solid line) and neutral (dashed line) sessions. The error bars designate the 95 % confidence limit on the mean. Right box-whisker summaries of the physiological stress response (PSR, see Equation 1) scores classified by male stressed and neutral interventions (MS and MN), female stressed and neutral (FS and FN) and the total group stressed and neutral (TS and TN) results. The PRS scores the cardiovascular response observed at the baseline (5 min after the sampling mask was fitted) S1 and at the maximum S. The PASAT exercise is denoted by P and the breath samples were collected during the CFT sessions as indicated by the bracketed periods (B).

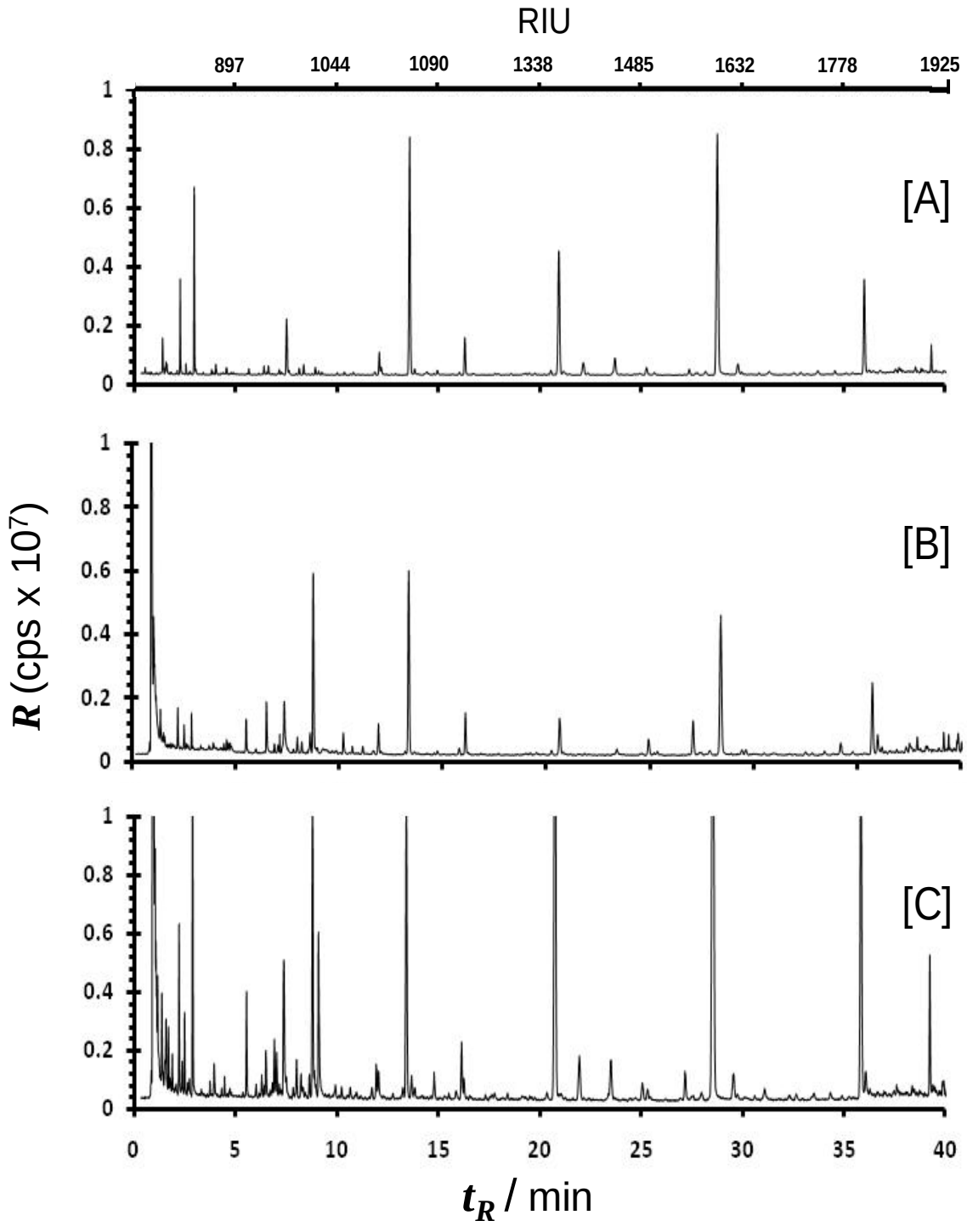


Figure 2 Examples of total ion current (TIC) chromatograms of [A] filtered air, [B] neutral session and [C] Stressed session breath samples. Lower x- axis shows retention time (t_R) and upper x-axis illustrates the equivalent time in retention index units (RIU).

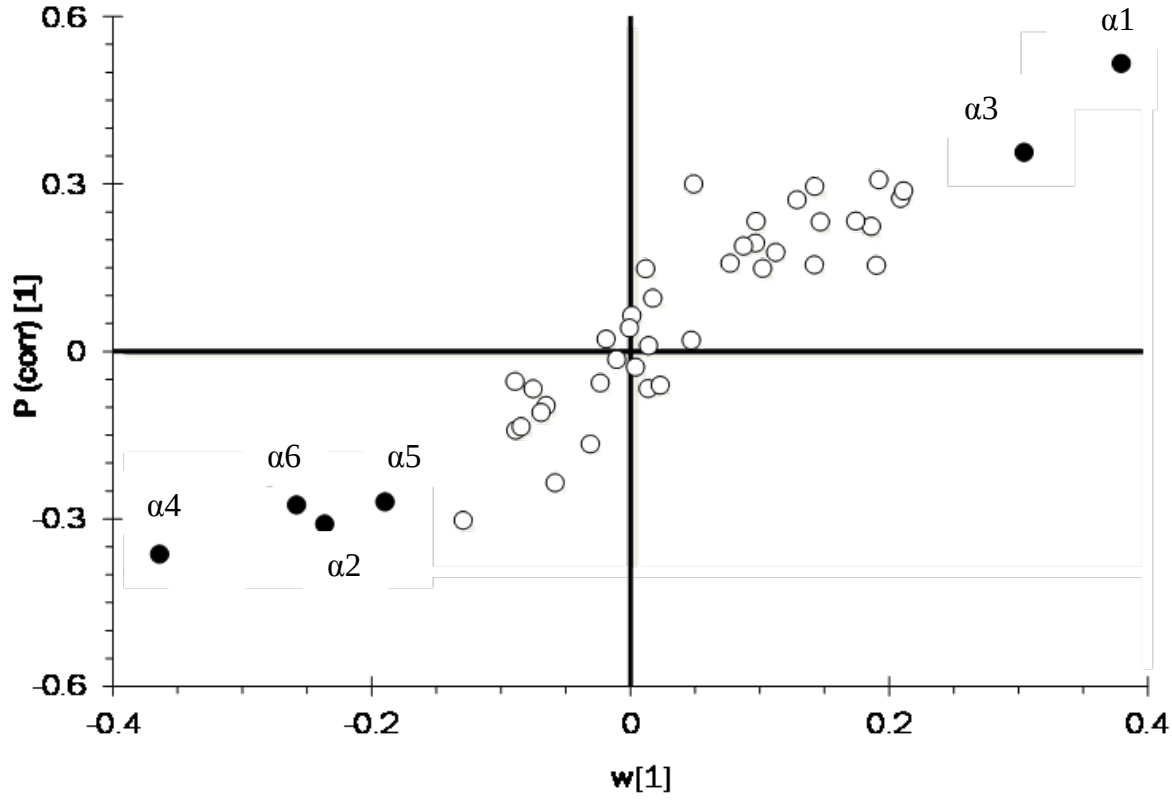


Figure 3. S-plot generated from the modelled covariance (P(corr)[1]) and modelled correlation (w[1)] of the partial least square discriminate analysis (PLS-DA) from the intensities (peak areas) of 50 breath components (variables) from 44 breath samples (observations). The six variables highlighted (black dots) were found to provide the highest magnitude (intensity) of the variables in the data set and greatest reliability (P(corr)[1] axis) of the variables in the data set; indicating potential putative markers sensitive to PASAT intervention.

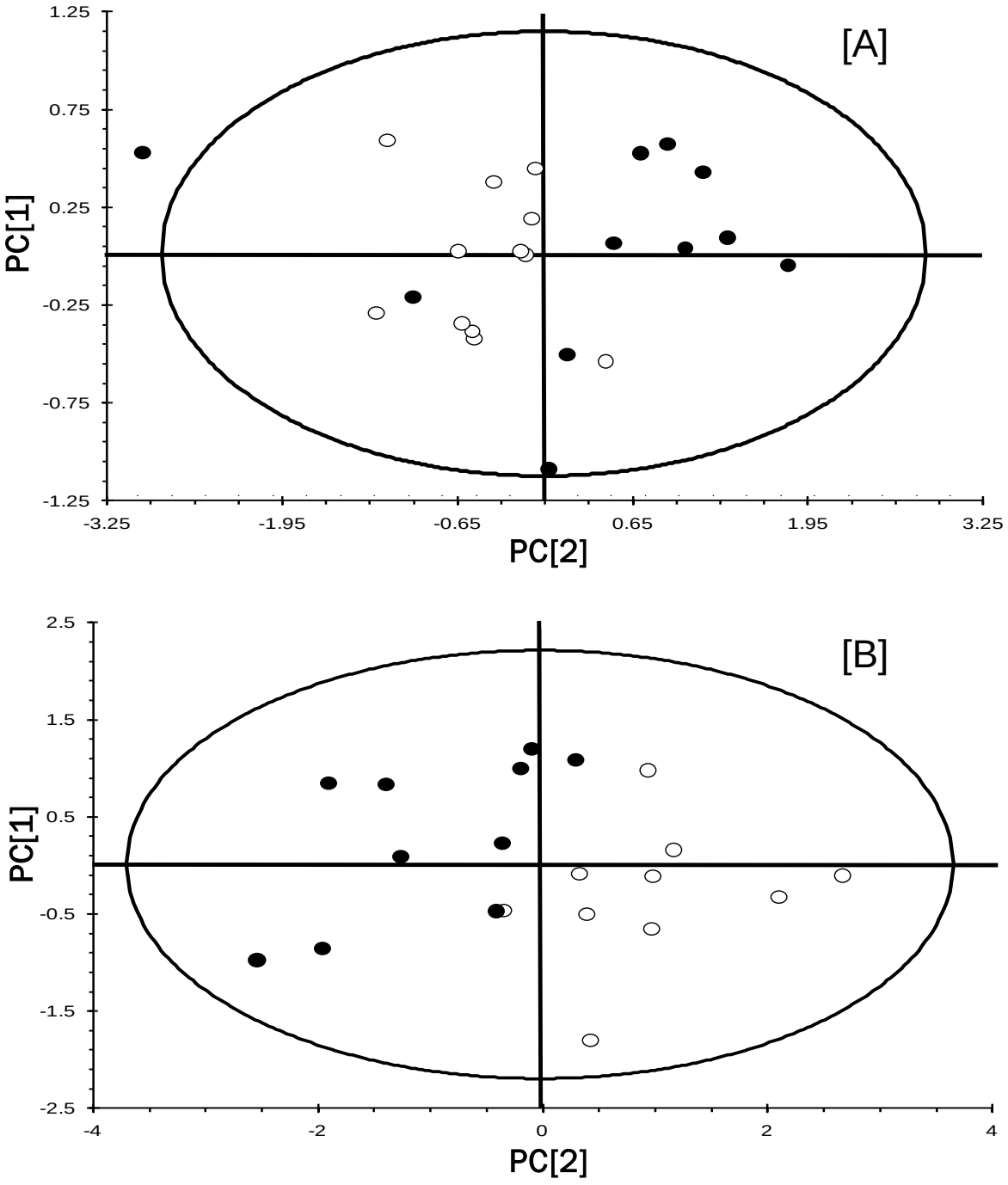


Figure 4. Unsupervised principle component analysis(PCA) of six stress sensitive breath components identified from PLS-DA (Figure 3) model on [A] 12 observations from the stressed (black dots) experimental session and 12 observations from neutral(open circles) experimental session from female participants. [B] 10 observations from the stressed experimental session and 10 observations from the neutral experimental session from male participants. 100 % sensitivity was obtained for the male stressed observations and 90% selectivity was obtained for the neutral observations. In contrast to this the female observations yielded a sensitivity of 83.3% and a selectivity of 91.6% was observed.

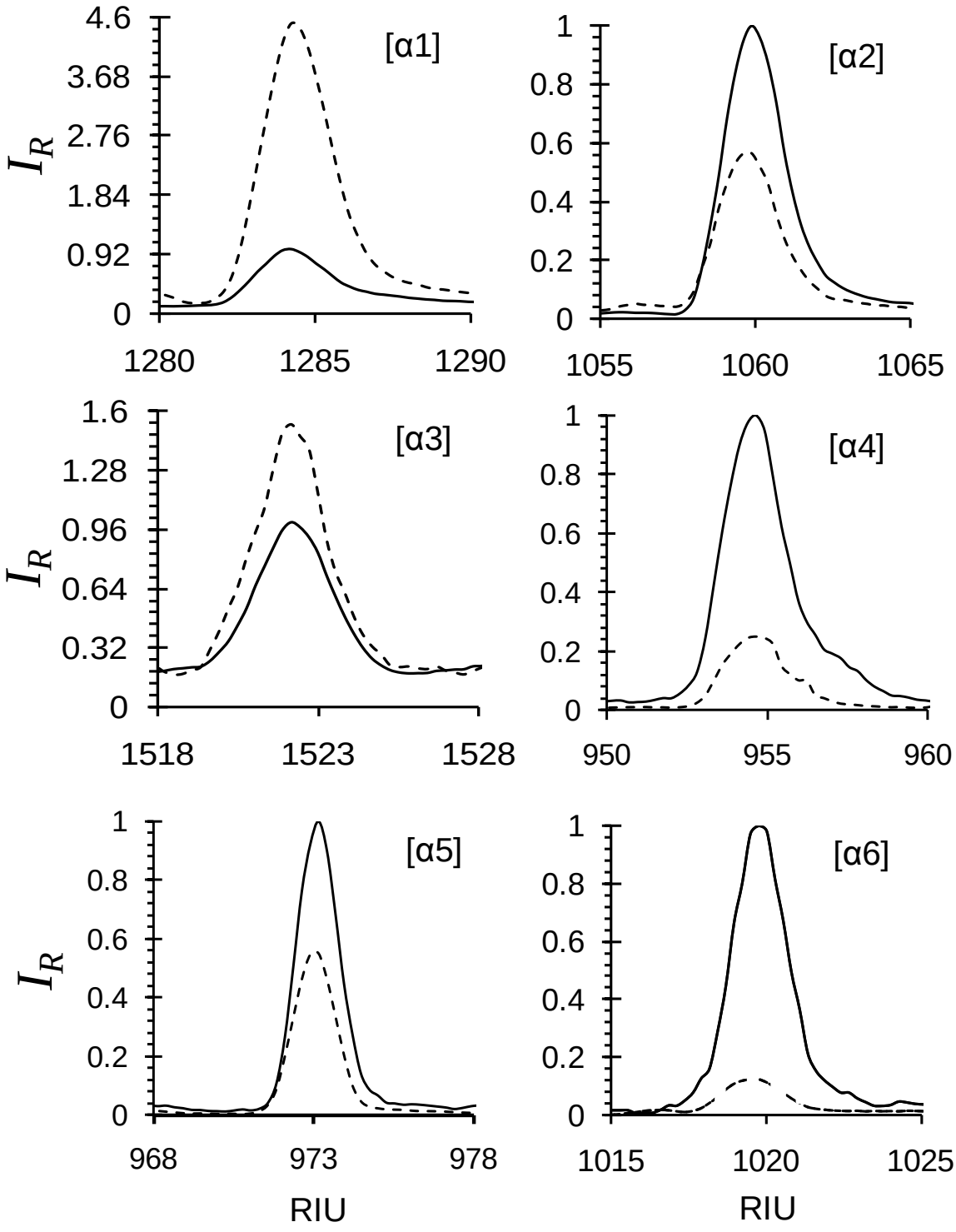


Figure 5. Overlaid extracted ion chromatograms (XIC) responses for stressed experimental session (dashed line) and neutral experimental session (solid line) for stress sensitive breath components, Indole (α1), 2-hydroxy-1-phenylethanone (α2), 2-methylpentadecane (α3), unkonwn terpene (α4), Benzaldehyde (α5) and 2-ethylhexan-1-ol (α6) from a single male participant. The intensities (I_R) displayed have been normalised with respect to the neutral experimental session to indicate those components that have been up-regulated and down-regulated as a result of undertaking the PASAT.

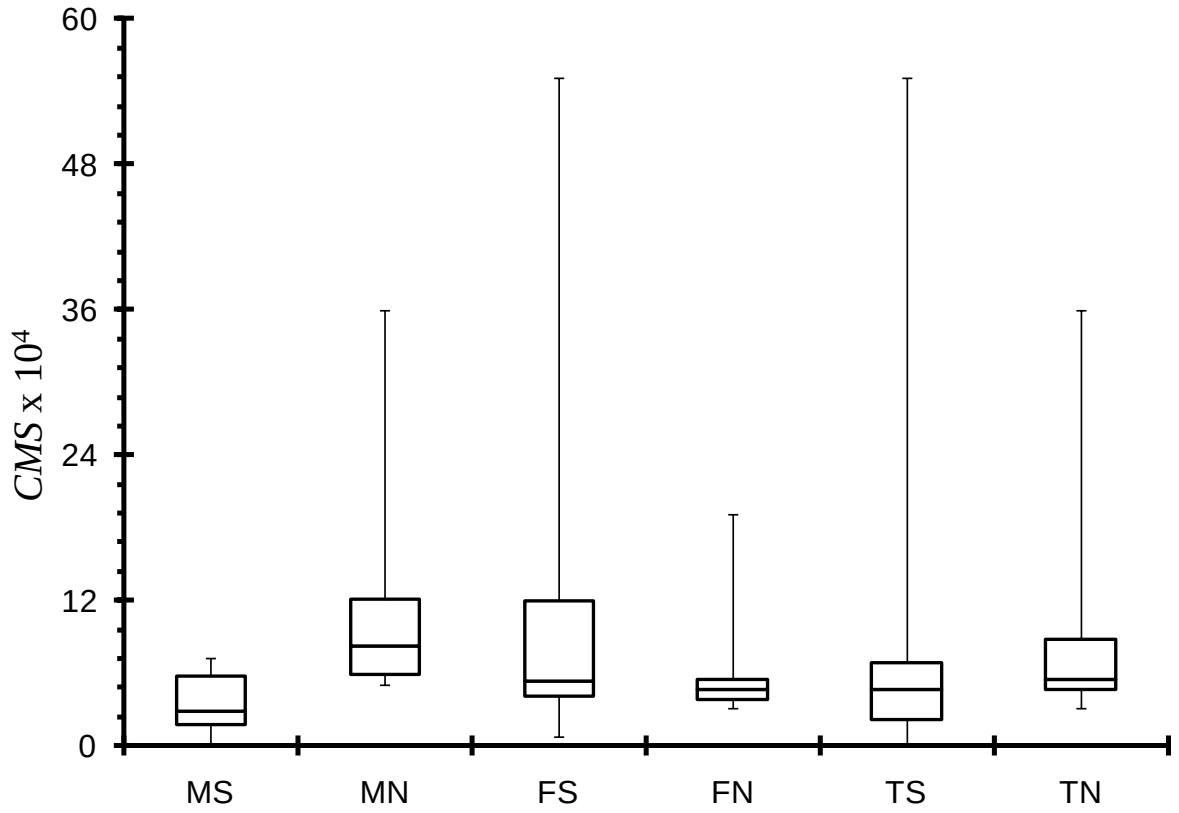


Figure 6. Box-whisker summaries of the combined marker scores (*CMS*) classified by male stressed and neutral interventions (MS and MN), female stressed and neutral (FS and FN) and the total group stressed and neutral (TS and TN) results.

Table 1 **Instrument parameters**

Parameter	Level	Units
Thermal Desorption		
Pre-purge Temperature	20	°C
Pre-purge time	1	Min
Pre-purge flow	18	$cm^3\ min^{-1}$
Tube desorption	300	°C
Primary desorption time	5	Min
Desorption flow	45	$cm^3\ min^{-1}$
Cold trap packing		general purpose hydrophobic
Trapping Temperature	-10	-°C
Trap desorption	300	°C
Secondary desorption time	5	Min
Trap desorption flow	2.0	$cm^3\ min^{-1}$
Split ratio	Splitless	
Flow path temperature	180	°C
Gas Chromatography		
Column	DB 5 MS 60 m long x 0.25 mm id x 0.25 µm film thickness	
Temperature programme		
Initial temperature	40	
Stage 1 $\frac{\delta T}{\delta t}$, ($T_{\text{Stage 1}}$), Hold time	3.3 (90) 0	°C min^{-1}(°C) min
Stage 2 $\frac{\delta T}{\delta t}$, ($T_{\text{Stage 1}}$), Hold time	2.5 (140) 0	°C min^{-1}(°C) min
Stage 3 $\frac{\delta T}{\delta t}$, ($T_{\text{Stage 1}}$), Hold time	10.0 (300) 8.9	°C min^{-1}(°C) min
Total analysis time	60	Min
Carrier gas	He	
Carrier gas flow	2.0	$cm^3\ min^{-1}$
Ion Trap Mass Spectrometer		
Trap temperature	150	°C
Manifold temperature	50	°C
Transfer line temperature	300	°C
EI scan mode	EI Auto	
EI scan frequency	2	Hz
EI scan range	(m/z)40 to (m/z) 450	

Note: Split ratio of 1:10 used for analysis of standard solution.

Table 2 Cardiovascular response scores of participants during the neutral (PSR_N) and PASAT intervention sessions (PSR_S).

Ref	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
M/F	M	F	M	M	M	F	F	M	F	M	M	F	F	F	M	M	F	F	F	F	F	M
	PASAT intervention																					
HR^{max}	90	119	70	92	80	77	105	91	90	90	96	100	82	134	95	84	146	66	97	95	105	85
HR^{start}	78	94	54	90	69	68	81	76	66	73	59	66	72	84	75	76	88	42	82	61	65	54
P_S^{start}	141	121	112	136	121	113	105	120	129	141	113	119	102	117	107	138	112	112	116	126	110	144
P_S^{max}	152	148	122	142	144	124	125	136	155	168	129	138	117	146	121	153	152	120	133	154	132	162
PSR_S	11.6	60.6	27.5	0.9	30.3	12.9	56.7	26.1	74.2	44.8	87.6	84.5	19.7	149	34	11.1	233	40.8	27.5	125	125	72.6
	Neutral intervention																					
HR^{max}	81	102	65	72	74	83	98	85	69	101	71	89	101	73	82	95	113	54	79	94	71	70
HR^{start}	75	90	58	65	68	79	85	82	61	81	60	72	84	56	73	78	81	49	60	79	68	56
P_S^{start}	126	120	125	118	120	107	102	125	120	135	113	113	111	116	107	133	118	108	120	119	103	147
P_S^{max}	127	121	125	124	122	122	101	118	123	138	122	111	109	123	114	131	117	107	122	116	105	148
PSR_N	1.0	0.8	0.1	5.3	1.6	7.4	1.9	2.0	3.2	4.7	15.5	5.0	4.2	18.6	7.7	3.9	2.7	1.2	4.5	4.5	1.0	1.4

HR^{start}: Baseline heart rate recorded at start(BPM); HR^{max}: maximum heart-rate (BPM); P_S^{start}: baseline systolic blood pressure recorded at start (mmHg): P_S^{max}: maximum systolic blood pressure (mmHg) and PSR Pasat Response Score, see Equation 1.

Table 3. Identification of PASAT sensitive breath components C1 to C6 with reverse and forward spectral match and de-convoluted spectrum lists.

Ref	Name and library entry	Deconconvolved mass spectra	MS F/R	RI O/E	CAS
α1	Indole BRI-1286-117-90-89-63-73	117 (999); 90 (411); 89 (310); 63 (151); 73 (130)	850/889	1286/1276	120-72-9
α2	2-hydroxy-1-phenylethanone BRI-1063-105-77-52-106-51	105 (999); 77 (311); 52 (172); 106 (73); 51 (66)	889/885	1063/1272	118-93-4
#α3	2-methylpentadecane BRI-1527-41-57-43-85	41 (999); 57 (786); 71 (776); 43 (619); 85 (412)	650/658	1527/1548	1560-93-6
*α4	unkonwn terpene compound BRI-951-93-91-92-79-121	93 (999); 91 (435); 92 (430); 79(264);121 (217)	-	951/ -	-
α5	Benzaldehyde BRI-976-105-77-52-106-95	105 (999); 77 (239); 52 (222); 106 (103); 95 (72)	813/813	976/982	100-52-7
α6	2-ethylhexan-1-ol BRI-1019-41-57-70-85-55	41 (999); 57 (756); 70 (620); 85 (543); 55 (389)	732/830	1019/995	106-76-7

Note: MS F/R Forward (F) and reverse (R) mass spectral match of deconvolced mass spectrum to NIST05 library search.

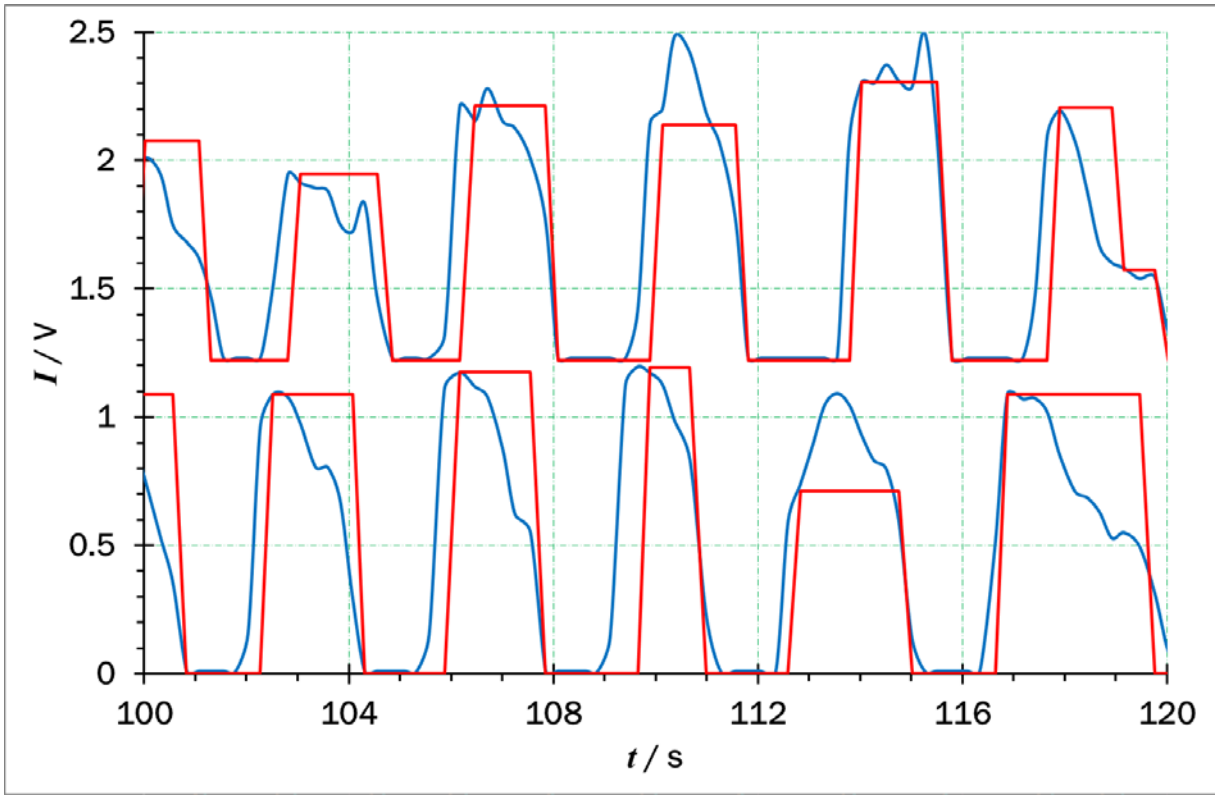
RI O/E Observed (O) and estimated (E) retention index value for deconvolved peak.

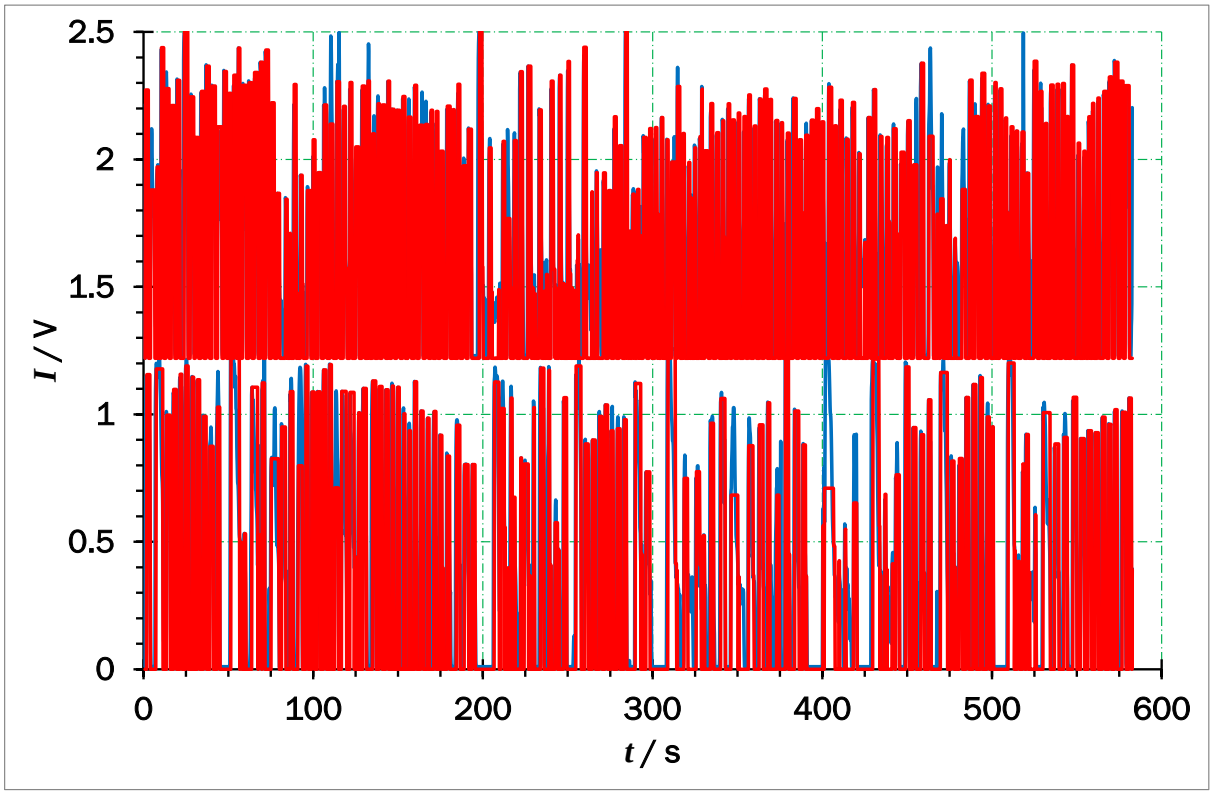
Confirmation by spectral match with NIST05 Library only as reference standard is not commercially available.

* BRI-1527-41-57-43-85 NIST match indicates 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl), this was not confirmed with a reference standard.

JBR 394750 PAP Supplementary Figure 1.

Showing the sampling system in use. The participant is supplied with purified air at a constant flow rate (Supplementary Table 2); not visible in this picture. A disposable rebreathing valve prevents background air from contaminating the experiment. The changes in upper airway pressure are monitored continuously and the silicone tube connects to a pressure transducer; not shown. The mixed bed adsorbent trap (shown in the process of being fitted) is connected to the participant's respiratory zone via a deactivated capillary (not visible) fitted into an adapted Swaglock/PTFE luerlock connector mounted on the full face respiratory mask. The signal from the pressure transducer is used to control the sampling valves and the designated part of each breath is sampled over many breaths. Every breath is continuously recorded along with the sample valve activation, see Supplementary Figure 2.





JBR 394750 PAP Supplementary Figure 2.

A close up of part of the breath sampling sequence (top), and the whole sampling sequence (bottom) taken from the same participant following a PASAT intervention top trace, and neutral session bottom trace. The changes in respiration profiles and variability are evident for this participant.

Blue trace is the pressure trace, Red is the sample valve control signal, high = sample.

JBR 394750 PAP Supplementary Table 1. Cognitive function tests

During each of the two visits the participants also under took a four cognitive function tests before and after the intervention period. The tests were a Stroop test, a Flanker test, a Sternberg paradigm, and a Rapid Visual Information Processing Test (RVIP). Each test lasted approximately 5 minutes and they were always given in the same order. In brief, the Stroop test, involved presenting the participants with words for a colour eg blue, red, green etc. The text colour for these words was different to the written colour presented. The aim was to discern the written colour and not the colour it was presented in. The Flanker task required the participant to view an image of three arrows in a line, each pointing randomly left or right. The aim was identify correctly the direction of the centre arrow while ignoring the flanking arrows. The Sternberg test is a memory test and a measure of working memory. Finally, the RVIP test presented the participants with a series of random numbers from 1 to 9 and required the participant to indicate when they observed three consecutive odd numbers. This battery of tests were prepared at the School of Sport, Exercise and Health Science at Loughborough University

JBR 394750 PAP Supplementary Table 2 Breath Sampling **parameters**

Parameter	Level	Units
Sampling tube, 3.5 "stainless steel standard thermal desorption tube.		
Mass of Tenax	150	mg
Mass of Carbograph 1TD	200	mg
Breath sample flow	1.0	$dm^3\ min^{-1}$
Breath sample volume	4.0	dm^3
Indicative numbers of separate breaths sampled $\pm$ 95% confidence limit	185 $\pm$ 57	
Filtered Air sample flow	2.0	$dm^3\ min^{-1}$
Filtered Air sample volume	4.0	dm^3