

VOLATILE ORGANIC COMPOUND MARKERS OF PSYCHOLOGICAL STRESS IN SKIN: A PILOT STUDY

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ABSTRACT

The forehead was studied as a possible sampling site for capturing changes in volatile organic compound (VOC) profiles associated with psychological-stress. Skin-VOCs were sampled with a polydimethylsilicone (PDMS)-coupon and the resulting VOCs were recovered and analysed with two-stage thermal desorption gas chromatography-mass spectrometry.

15 young adult volunteers (19 yr to 26 yr) participated in two interventions run in a randomised crossover design. One intervention, termed “Neutral”, required the participants to listen to peaceful music, the other, termed a “paced audio serial addition task”, required the participants to undertake a series of rapid mental arithmetic calculations in a challenging environment that induced a stress response. Skin-VOC samples were taken during each intervention. The resultant data were processed with dynamic background compensation, deconvolved, and registered to a common retention index scale.

The importance of freezing skin patch samplers to -80°C was determined during the method development phase of this study. The cumulative distribution function of the GC-MS data indicates the possibility that PDMS-coupons are selective towards the lower volatility VOC components in skin. The frequency distribution of the GC-MS data was observed to be approximately log-normal, and on the basis of this study, a further two-orders of magnitude reduction in sensitivity may be required before the complete skin-VOC profile may be characterised.

Multi-variate analysis involving Pareto-scaling prior to partial least squares discriminant analysis identified four VOCs with the highest probability of contributing to the variance between the two states, and the responses to these VOCs were modelled with principle components analysis (PCA). Two VOCs, benzoic acid and n-decanoic acid were upregulated (14- and 8-fold respectively) and appear to be PASAT sensitive, with areas under (AUC) their receiver operator characteristic (ROC) curves of 0.813 and 0.852 respectively. A xylene isomer and 3-carene were down regulated 75 % and 97% respectively, and found to be predictive of the neutral intervention (ROC AUC values of 0.898 and 0.929 respectively). VOC profiles in skin appear to change with stress either due to increased elimination, elevated bacterial activity, or perhaps increased oxidative pathways.

INTRODUCTION

In a previous study the effect of a “stress-test” on the profile of volatile organic compounds (VOCs) in exhaled breath was described [1], and six potential breath-markers of stress were identified. Levels of indole and 2-methylpentadecane were observed to increase, while benzaldehyde, 2-hydroxy-1-phenylethanone, 2-ethylhexan-1-ol and, and a terpene compound were shown to decrease in response to induced stress. The current study sought to test the proposition that the VOC-profile associated with skin will also change when an individual is subjected to stress.

Stress

The potential confounding effect of stress VOC-profiles on the detection of VOC markers of chronic diseases in exhaled breath, skin, urine and saliva has yet to be completely described. Stress may also be viewed as a co-morbidity prevalent in people with chronic conditions, and a rapid non-invasive diagnostic of stress may be viewed as helpful in the treatment and management of a broad range of diseases. For example, patients with chronic obstructive pulmonary disease (COPD) have been reported as also having comorbid psychological problems that increase their functional disability and reduce their quality of life. The researchers concluded that psychological characteristics were important variables that interacted with physical symptoms and that that psycho-social variables should be considered when treating patients with COPD [2].

‘Stress’, as it is understood in modern biology, was first discussed by Hans Selye who described it as the failure of an organism to respond appropriately to perceived threats, whether emotional or physical [3]. Selye proposed three stages of threat-response: alarm, adaptation, and finally, exhaustion. Alarm is the initial response that causes the hypothalamus to activate the pituitary gland which in turn activates the adrenal glands to release adrenaline, nor-adrenaline and cortisol. The autonomic nervous system is also activated which leads to elevated heart rate and blood pressure, along with faster and shallower breathing and sweating. Sugars, cholesterol and fatty acids are released from the liver to supply energy rapidly for the ‘fight or flight’ response, muscles tense for action and the blood’s clotting ability increases to prepare for potential injury [4].

Adaptation follows in which a resistance to the stressor is built-up, and the body remains mobilised to fight. Finally if the stress is prolonged, exhaustion occurs, at which stage the body’s resources are depleted, and the individual suffering from prolonged perceptions of threat becomes more vulnerable to stress-related conditions [3]. Previous research has focussed on calibrating physiological responses as indicators of stress [5].

The selection of the sampling site.

Skin is the largest organ with different *micro-flora* and *micro-fauna* colonies distributed across the various anatomic structures; increasing further the metabolomic complexity [6]. The selection of the sample site was an important element in the experimental design of this study.

VOCs recovered from skin originate from: VOCs permeating through the skin from the underlying tissues and bio-fluids (mostly blood); glandular secretions/sweating; microbial conversion of lipids into VOCs (notably volatile fatty acids); and, exogenous deposition, which also includes VOCs arising from skin reactions with ozone and UV. The main types of glands in human skin are: eccrine; apocrine; and, sebaceous. Eccrine glands are distributed across the body with the highest densities on the forehead, palms and feet. The main function of the eccrine glands is thermal regulation. However, eccrine glands on the palms of the hands and soles of the feet respond to emotional stimuli [7,8].

Apocrine sweat glands are also stimulated by emotional stress, and open into hair follicles mainly in axillae. Apocrine glands are also found at the perineum, ears, eyelids, nipples and the external genitalia [9]. Sebaceous glands are distributed across the body apart from the hands and feet with the highest densities found across the forehead, as well as the upper torso and arms.

Emotional sweating has been evaluated in several studies as a measure of mental strain; measurements are usually based on electro-dermal activity, using skin conductivity, also known as the galvanic skin response. One such study measured the electrodermal activity across the palm during three activities, arithmetic calculation, a hand-grip exercise to induce physical strain and a deep breathing exercise to induce relaxation [10]. All participants showed a marked increase in palmar sweating while the three tasks were explained to them, which returned to baseline before they started the exercises. The participants also showed an increase in electrodermal activity at the beginning of each exercise, no matter what the stimulus was. (This observation highlights the importance of allowing time for equilibration with an intervention to enable such an introductory response to subside.) Palmar sweating returned to baseline rapidly once the deep inspiration intervention commenced, in contrast to the continued elevation above baseline that was observed with the physical stimulus, while the mental strain intervention resulted in an unstable high response, indicating the applicability of electrodermal activity as an assessment of mental strain [7]. It is also helpful to note that this study indicated that sweating responses to the mental strain intervention reduced with repetition, indicating learned behaviour, and the reduced efficacy of this stress-intervention if used more than once with the same cohort.

Observations of sweating from the palm and forehead during a set of stress interventions indicated that the rate of secretion of sweat was dependent on an individual's stress threshold [11]. In another study, emotional sweating from the foreheads of medical students during a practical examination was investigated [5]. The majority of the participants showed elevated sweating episodes during the particularly difficult sections of the task. Of interest, was the observation that participants who failed the first attempt showed significantly elevated sweating during the second attempt. This study controlled ambient temperature and humidity during the experiment leading to the conclusion that increases in forehead sweating were related to the emotional stress experienced by the participants.

Previous studies have established that emotional stress engenders a sweating response in the palms, axillae and forehead. As the palms and axillae were inaccessible during the PASAT used in the current study, the forehead was chosen as the sampling site for skin VOCs, a site that has been selected for observing emotionally induced sweat since the 1990's [12].

Sampling VOC-Profiles in skin

On-line and archival sampling techniques have been reported for the analysis of VOCs from skin. On-line approaches with ambient-ionisation mass spectrometry have obtained in-vivo mass spectral "fingerprints" with desorption electrospray ionisation (DESI) [13], secondary electrospray ionisation (SESI) [14,15] and extractive electrospray ionisation (EESI) [16]. Sealing a participant's limb into a ventilated Tedlar bag and analysing the exhaust by selected ion flow tube mass spectrometry (SIFT-MS) provided targeted on-line acetone [17] and ammonia levels [18]. A proton transfer reaction mass spectrometry (PTR-MS) technique demonstrated how to sample synthetic-air directed over the skin's surface [19]. The benefits of these on-line approaches to the researcher are speed, selectivity and sensitivity, and for time-resolved targeted analysis they represent the

current state-of-the-art. Nevertheless, such approaches require significant capital outlay, along with environmentally regulated rooms staffed with skilled-specialists to operate them. Further, compliance with infection-control and patient-safety protocols may be problematic for they are difficult to sterilise, and some ambient ionisation approaches use high-voltages at, or close to, the sample site. The burden on the participant also needs consideration for they are required to present part of their body to the instrument for the duration of the sampling process. Smaller units with lower operational overheads are needed, such as sensor arrays (enose) [20] or gas chromatography-ion mobility spectrometry [21]. Such approaches are limited currently by the size of their spectral/response libraries, and deconvolution of complex signals remains difficult. While undoubtedly useful for targeted hypothesis testing they require parallel GC-MS analysis [20] if they are to be used in a non-targeted metabolomic design.

Archival techniques have adopted: whole-sweat collection; headspace analysis; and adsorption/absorption from direct surface contact. Whole sweat sampling often uses cotton or gauze pads subsequently resampled by solvent extraction or headspace analysis with adsorbents, including solid phase micro extraction (SPME) [22,23,24]. Other approaches describe encapsulation of a limb, or the entire body, to collect sweat that is subsequently resampled for analysis [25,26,27]. Headspace sampling approaches have also used bags as well as, petri-dishes, vials, or jars to create a sealed headspace around limbs, appendages or areas of skin from which sampled headspace was directed onto a 'cold trapping' device [28] or adsorbent sampler, including SPME, [29,30,31,32,33,34]. In a successful attempt to reduce the sampling burden on the participant, a headspace comprised of a polydimethylsilicone (PDMS) coupon sandwiched between two layers of thin, flexible stainless steel mesh has been developed [35]. One mesh holds the PDMS sampler away from the skin creating the sampled headspace, while the other fixes the unit in place. Preliminary results are promising with reproducibility demonstrated for a panel of test compounds, along with the potential for quantitative methods once the inherent reactivity of stainless steel has been addressed.

Direct contact sampling methods trap VOCs from skin with a sampling medium that absorbs or adsorbs the VOCs from the skin's lipid layer with recovery of trapped VOCs by thermally desorption. SPME fibres used to "stroke" the sampled area of skin gave results comparable to headspace SPME sampling [36]. Glass beads have been used, instead of SPME fibres, to roll over the skin [35,37,38,39]. This approach has been refined by incorporating a higher affinity PDMS adsorptive phase coated onto a stir-bar (developed for flavour analysis) and fitted into holder that enabled the stir-bar to be rolled across the sampled skin surface. Impregnating the stir bar with internal standards prior to sampling gave semi-quantitative results [41,40]. This skin-sampling method has shown significant promise as a simple, non-invasive sampler. Some further development to improve the stir-bar handling post sampling and to ensure reproducible pressure, velocity and sample area will enhance this approach still further [40].

The principle approach used for direct contact sampling from human skin with PDMS has been a thin film that conforms to the contours of the body giving a sampling approach with a negligible sample burden [41,40,42,41,42]. Such direct contact samplers are compatible with thermal desorption analytical systems. PDMS based headspace and direct contact samples have generally been analysed by a two-stage thermal desorption process, with cold trapping to provide a second pre-concentration stage before GC analysis, providing significant enhancements in sensitivity.

An important consideration in this study was a reduction in the sampling-burden on the participants to enable them to undertake the PASAT and Neutral interventions without hindrance; hence a direct PDMS sampling approach was used.

EXPERIMENTAL

Ethics

The study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. This research followed 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.

Participant intervention

The PASAT was first developed by Gronwall in 1977 to monitor the recovery of patients who had sustained mild head injuries [43]. The task involves single digits presented to the participant via an audio feed, the participant is required to verbally add each newly presented digit to the one immediately preceding it. Subsequently the PASAT has been found to be an effective psychological stressor, and it was used in this study concurrently with the breath sampling campaign noted above [1,44]. 10 male and 10 female volunteers across the age range 19 yr to 26 yr were recruited from Loughborough University staff, students and social networks into a randomised cross-over design with two sampling sessions. In one they were asked to sit comfortably and listen to classical music, this was denoted the 'neutral' intervention. In the other, participants were asked to undertake a PASAT, the 'stress' intervention. Each of the interventions lasted 11 min during which the participants' blood pressure and heart rate were recorded at 4 time points. The adoption of a narrow age range for the participants reduced the potential for skin-ageing related factors confounding the study.

Heart-rate (HR) and blood-pressure (BP) measurements were recorded using a Datex Ohmeda S/5 patient monitoring system. Heart rate was monitored via a finger clip attached to the index finger of the participants' left hand, a Critikon 22 cm – 33 cm Dura-Cuf blood pressure cuff was applied to the upper left arm. At each time-point heart rate, systolic blood-pressure, diastolic blood-pressure and mean arterial-pressure were recorded. Upon arrival 3 replicate measurements of the participants' blood pressure and heart rate were taken at 2 minute intervals to provide a baseline level, a further 12 readings were taken over the course of each experimental session see Figure 1.

Skin Sampling and analysis.

[45,46]. VOC-profiles were sampled with a polydimethylsiloxane (PDMS) coupon (5mm x 15mm x 0.45mm) that conformed to the contours of the participants' foreheads as it was held in place by a conditioned cotton-wool pad. The methodology used has been described previously [43] where the analytical figures of merit were summarised with limits of detection in the 170 pg.cm⁻¹ to 200 pg.cm⁻¹ range accompanied by relative standard deviations in the range 15 % to 32 %. This approach has been used for in-vivo applications such as: profiling chronic wounds; bacterial contamination of meat; and, the phenotypically classification of individuals with a specific (SNP) 538 G→A mutation in the human ABCC11 gene [44].

Before use the PDMS coupon was prepared and conditioned by ultrasonic cleaning with 5% aqueous Decon 90 solution followed by ultrasonic cleaning in high purity methanol, that was repeated three times using clean methanol for each repeat. Following the

washing steps the PDMS coupons were conditioned for 15 hr under vacuum at 180°C. This cleaning and conditioning protocol reduced the levels of non-siloxane VOCs in PDMS blanks to below detectable levels; less than 10 pg per Skin Patch. The conditioned PDMS coupons were transferred rapidly to an empty, inert coated stainless steel 3.5" x ¼" thermal desorption tube which was sealed immediately. The cotton-wool pads used to hold the Skin Patch against the skin surface were conditioning under vacuum at 70°C for 4 hr before being sealed, individually, in aluminium foil. The importance of rigorous cleaning and conditioning is exemplified in Figure S1 that contrasts the GC-MS total ion chromatogram obtained from a PDMS coupon used to sample the VOC from a cotton-wool pad before and after conditioning. It is important to note that every precaution to prevent contamination of the PDMS coupon must be taken at every stage of the workflow.

Participants were asked to fast from the previous evening and drink only water until they had completed their study visit. The PASAT, and associated samples were taken during the morning and the whole process lasted approximately 1.5 hrs. Skin-sampling started with removing the PDMS coupon from its thermal desorption tube, where it had been stored since conditioning, and placing it centrally on the participant's forehead where it was held in place with a conditioned cotton pad for a sampling time of 30 min while the participant undertook the PASAT, see Figure 1. The Skin Patch was removed using tweezers and immediately resealed into its thermal desorption tube and placed into -80°C storage until analysis. Note that the adsorbent tube seals were retightened 30 min after being placed in the freezer to ensure that the seals remained airtight as the tube contracted during cooling. The Skin Patch was analysed as soon as was possible after sampling by thermal desorption gas chromatography mass spectrometry (TD-GC-MS). The maximum permissible storage time was 21 days, after which the sample was discarded.

Analysis of the PDMS coupons entailed removing the adsorption tubes from the -80°C storage and allowing them to warm to ambient temperature, and for any condensate to evaporate. Before analysis a mixture of homologous n-alkane retention index (**RI**) standards was analysed (C8-C18) to: enable assessment of instrument performance, and calibrate the **RI**. In between each analysis a blank run was undertaken to ensure that no residual VOC were present within the instrument. A regime of regular quality-control retention-index standards (at the start of every shift and every six runs thereafter) and system blanks verified the performance of the instrumentation throughout the study.

Sample stability

As part of this study the storage of the PDMS coupons prior to analysis was investigated. The storage study investigated sample storage at -20 °C and -80 °C over 24 hr, 96 hr, 264 hr, 432 hr and 504 hr hours with five-replicate samples taken for each storage temperature/time combination. The simultaneous collection of these replicates required a large area of homogenous skin and so six sampling-locations were defined on the participants' upper-backs. The samples taken from each site were randomised to ensure that no bias occurred and each storage time contained samples from the randomised sampling locations.

Instrumentation

Recovery of trapped VOCs from the PDMS coupons was achieved by two-stage thermal desorption, (Markes International Unity 2, fitted with a Tenax TA/ Carbograph 1TD cold trap for refocusing). The PDMS coupon desorption temperature was 180 °C, and was selected to balance efficient desorption against maximum acceptable bleed from the PDMS phase with a desorption time of 10 min, see Table 1. The recovery efficiency under

these conditions was evaluated during a re-desorption study and was found to be greater than 99%, see Figure S2 for an example comparison of the desorption and re-desorption total ion chromatograms obtained.

The recovered VOC were separated using an HP 5890 gas chromatograph fitted with a 60m long DB-5 MS column with an internal diameter of 0.25mm and a 0.25 μ m film thickness operated in a constant pressure mode. The mass spectrometer was a Fisons Trio 1000 quadrupole mass spectrometer, operating in scanning mode, with an electron ionisation source, see Table 2.

Data Processing.

The data processing workflow was based on a breath analysis protocol that has been reported previously [47]. Each chromatogram was visually inspected using data handling software (Waters MassLynx (V4.1 SCN 779)) to verify the absence of instrument artefacts and chromatographic interferences. Inspection of the column bleed ions (e.g. m/z 207 from polysiloxane compounds) provided a reliable indication of: instrument state; the presence of unacceptable levels of water-loading; and ion-suppression artefacts from overloading (sometimes encountered with most volatile components recovered). This simple preliminary procedure identified and assessed confounding factors rapidly before progressing to the semi-automated deconvolution and integration techniques employed further down the workflow.

Once a chromatogram data-file had passed visual inspection it was converted to a *.cdf file format using the data-bridge capability of MassLynx, and imported into TargetView (ALMSCO International, Llantrisant, UK) for dynamic background compensation (DBC) and mass spectral deconvolution. The DBC algorithms distinguish between ions that contribute to chromatographic peaks and those that can be attributed to noise, column bleed, oil residue, and air/water ingress. The resultant mass spectra have enhanced fidelity with improved signal-to-noise ratios. The combination of the differences in chromatographic peak-widths, and the three-to-four orders-of-magnitude of ion intensities associated with the VOC mixture recovered from human skin made fully-automated deconvolution of the data problematic, so a deconvolution optimisation was performed. A series of test VOC, previously reported in literature to be present in human skin, within the GC-MS data were deconvolved under different peak-width settings, and the results were then compared against the NIST-database forward spectral match. The outcome of this optimisation was that peaks exhibiting a Gaussian distribution were deconvolved with a peak-width setting of 12s. For the remainder of the peaks that exhibited non-Gaussian peak-shapes a peak-width setting of 30s was used. In essence this meant that volatile fatty acids and primary amines were deconvolved with the 30s peak-width setting. Although manual intervention was required this approach resulted in the creation of reliably deconvolved spectral lists for inclusion into the study data matrix.

The penultimate step in the data processing workflow was incorporation of the compound information into a matrix for multi-variate analysis (MVA). The variables for the matrix were the resolved peaks and each was given a unique identify based on it's **RI** and the m/z values of the 5 most intense peaks present in the deconvolved mass spectrum in descending order of intensity. (Taking the form <**RI**_ m/z > <value base peak_ m/z > <value fragment ion 2_ m/z > <value fragment ion 3_ m/z >..., and so on.) Each separate sample was an observation thus creating the "skin data-matrix". **RI** registration of the resolved peaks was accomplished by constructing a secondary retention index ladder from the correlation of the retention times of a ubiquitous series of siloxane compounds, stripped from the PDMS sampling coupons during thermal desorption, to a primary index of the n-alkanes. Between run variations in the observed

RI for the same compound were assessed through the mass spectra, and the average **RI** was adopted. The final compound to elute from skin-samples was the skin-lipid squalene, which provided the final retention time marker for **RI** registration.

Exogenous compounds present in the sampling room environment, were identified from their presence in sampler blanks, taken at the same time as the skin samples. These were removed from the skin data-matrix, Between 200 and 300 VOC were isolated from a typical skin sample after deconvolution and exogenous compound removal.

The final step in the data processing workflow was the integration of the deconvolved peaks and this was based on the area under the curve for the extracted ion chromatogram of the designated quantitation ion for each compound (variable); in the majority of cases this was the base peak but where this did not offer sufficient selectivity a different ion was chosen for quantitation. Integration was performed on the raw data using MassLynx (Waters, Manchester, UK). If a compound was not isolated from the chromatogram a value of zero was entered into the skin data-matrix.

The skin data-matrix was imported to SIMCA-P v.12 (Umetrics, Umea, Sweden) for MVA. To give equivalent weighting to all the components in the matrix Pareto-scaling was applied. Pareto-scaling divides the peak area of each individual component in the skin data-matrix by the square root of the standard deviation of the peak areas for that component in the variable set, and compensates for the large range in responses between variables and assign equal significance to every variable, independent of relative magnitude [48, 49].

Supervised and unsupervised statistical models were applied iteratively starting with partial least squares discriminant analysis to generate an S-plot (a graphical representation all of the variables in the matrix distributed through the S-plot co-ordinates according to the probability that they contribute to the separation between the PASAT and neutral interventions). The variables (VOC) identified within the S-plot with the highest probability of contributing to the variance between the two states were extracted to a new matrix for unsupervised analysis by principle components (PCA). If separation between the classes was observed in PCA the variables were listed as candidate markers and a tentative assignment made. An important feature of the SIMCA-P v.12 modelling package was/is the integral boot-strapping routines that test and validate the PCA model using a 'leave-one-out cross-validation' algorithm.

Suggested assignments were based on two criteria: a satisfactory match between the deconvolved mass spectrum and the respective entry in the NIST MS library (NIST 11) and, a satisfactory match with the NIST estimated Kováts **RI**-value. A spectral match was deemed satisfactory if forward and reverse match factors gave values greater than or equal to 750. A **RI** match was deemed satisfactory if the observed **RI** value was within 50 retention index units (**RIU**) of the published estimate.

RESULTS

Sample stability was evaluated by selecting targeted VOCs at random from within defined 10 min periods of the chromatography to provide a representative volatility range. Analysis of variance (ANOVA) performed on integrated peaks areas tested the null hypothesis that storage conditions had no observable effect on the VOC-profile. After 4 days storage at -20°C one compound caused the null hypothesis to be rejected (6-methyl-5-hept-2-one, previously been noted to increase up to 300% on storage at 4°C [43]). After 11 days storage at -20°C other VOCs also exhibited increased responses causing the null hypothesis to be rejected, and at this point the -20°C storage study was

halted. Skin samples were not observed to be stable at this temperature. In contrast, after 21 days storage at -80°C none of the monitored compounds showed any statistically significant change and this was considered an adequate storage condition for skin VOC samples in these pilot metabolomic studies, with the proviso that analysis occurred within 21 days of storage. See Figure S3.

The Skin Patch was well-received by all participants and no adverse events were reported. It was observed to be unobtrusive, simple to use, and replicate samples could be taken. Replicates were not taken in this study because in this case the enlarged gauze pad would have interfered with the operation of the breath sampler that was being used at the same time. Further the data from the sample stability study indicated that the inter-subject variability was significantly greater than inter-subject variability between replicate skin samples. The intra-subject reproducibility may be estimated by looking at the variability of a targeted compound selected at random. 29 of the 30 z-scores for the individual peak areas for 6-methyl-5-hepten-2-one across the storage campaign fell within +/- 3 standard deviations of the mean observed for the 5 samples analysed at 0 hr storage. (One sample returned a z-score of 4.1.) The relative standard deviation across the whole stability data set was 32%; substantially less than the observed inter-participant variability.

At the end of the study a total of 5 samples had been discarded due to a combination of protocol-failures in instrumentation, or sample-storage. The nature of the study meant that the data from the other part of the cross-over design was excluded resulting in a final data-set that contained the data from 15 participants.

The physiological responses observed (Figure 1) were consistent with those described by Seyle as the 'fight-or-flight' stress response [3,4], and such responses were absent in the neutral session, indicating that the PASAT was an effective stressor. Figure 2 is an example pair of chromatograms contrasting the PASAT and neutral responses observed for one of the female participants. Differences in the chromatograms of the participant's skin VOC-profiles obtained from the two interventions are discernible, however the distinct and readily observable changes in these skin VOC-profiles appeared to have a high inter-participant variability. Consequently, MVA was used to establish if common candidate VOC discriminators of stress were present within the data.

VOCs were excluded from the MVA if they were found to be present in the field-blanks, or were present in less than 30% of observations [50]. The resultant skin VOC data-matrix contained 30 observations (15 participants) and 40 variables (VOCs). Partial least squares discriminate analysis (PLS-DA, Umetrics, Sweden) applied to the Pareto-scaled data-matrix generated the S-plot in Figure 3 that indicated 4 candidate compounds with a significant probability of contribution to differences between the profiles from the two interventions. Two of the candidate VOCs were up-regulated and the other two were down regulated, see Table 3. The PCA and associated dendrogram, based on the four compounds, is shown in Figure 4. These data support the proposition that stress is accompanied by specific and detectable changes in the VOC profile recovered from the forehead. Two neutral intervention observations were misclassified as stressed, although cardiovascular data for the two individuals was inconclusive, the implication was that these participants were not relaxed during the neutral intervention. It is possible that as this was their second visit they were anticipating their previous experience of a stress exercises in an unusual and unfamiliar environment that did not subsequently materialise.

Table 3 summarises the proposed assignments for the four PASAT sensitive skin-VOC and these are proposed, tentatively, as candidates for the classification of stressed and

neutral states: benzoic acid, n-decanoic acid, a xylene isomer and 3-carene. Figure 5 presents example chromatograms for these four compounds observed in the profiles obtained from a female participant under stressed and neutral interventions. Benzoic acid, n-decanoic acid and the xylene isomer were isolated from all the samples, 3-carene was isolated from 28 of the 30 samples analysed. The box-whisker plots summarise the relative responses and indicate a median 14.3 fold increase in the observed recovery for benzoic acid and a median 8.1 fold increase in n-decanoic acid recovery. In contrast the median recoveries for the xylene isomer fell by 75.3 % and 3-carene by 97.4 %.

Receiver-operator characteristic (ROC) curves were constructed for the four candidate stress sensitive VOC to estimate the quality of the prediction of the PASAT intervention based on their responses. Area under the curve (AUC) values for benzoic acid and n-decanoic acid were 0.813 and 0.852 respectively indicative of a potential stress diagnostic. On the other hand the AUC values for the xylene isomer and 3-carene were 0.102 and 0.071 respectively indicating that these compounds may not be used to provide a reliable indication of a stressed state. However, for the prediction of the neutral state these compounds' AUC were 0.898 and 0.929 respectively.

DISCUSSION

Efficacy of skin VOC profiling for non-targeted metabolomic approaches.

This investigation was a companion study to a breath-based investigation [1] in that a non-targeted global profiling approach was adopted to prospect for putative stress markers. Conceptually the prospect of injury-free, non-invasive breath sampling for metabolic investigation is attractive. However, isolation of the low-concentration (less than $1 \mu\text{g m}^{-3}$) endogenous VOC in exhaled air requires representative sampling of the alveolar, or end-tidal, portion of exhaled breath, requiring specialised equipment and training to ensure reproducible and representative breath-samples. Further, breath sampling is intrusive and necessarily impedes and interferes with normal behaviour while the sample is being taken. Despite many advantages over blood-, and urine-based approaches, breath sampling does not currently completely fulfil the criteria for the ideal non-invasive sampler. In comparison the skin patch does not require a trained scientist to use and is self-administrable, the method used here requires no external power, or other utilities, and is easily transported and stored allowing for batch sampling in the field, clinic or community setting for off-line analysis; demonstrated recently with a study where samples were obtained in the Philippines and analysed in the UK [46]. In many regards the Skin Patch fulfils the criteria for the non-invasive diagnostic sampling. Nevertheless questions remain about the quality and fidelity of VOC-profiles currently obtained from PDMS-based skin sampling; an evaluation of sample stability and selectivity is helpful at this juncture.

Data quality and selectivity

The cumulative frequency distribution, against **RI**, observed for a typical skin VOC-profile is shown in Figure 6. Most of the chemical information (80%) was contained in the last half of the chromatogram over the RI range 1600 to 3000. This is indicative of either a preponderance of lower volatility compounds present at the skin's surface, or an inherent selectivity in the skin patch methodology towards lower volatility compounds, or a combination of both of these factors. The location of the four PASAT sensitive compounds is indicated and tellingly these fall in the higher volatility, first half, of the chromatogram.

The frequency distribution of chromatographic features shown in Figure 6 is similar to that reported previously with thermal desorption techniques [46,51]. The observed distribution of intensities was approximately log normal, and extrapolation of this distribution suggests that the limit of detection of the methodology will need to be reduced by approximately two to three orders of magnitude before the complete VOC-profile for skin will be characterised.

Sample size is a factor in pilot studies and caution is always needed in the interpretation of the resultant data with regard to the possibility of false discovery and over-fitting of data. This challenge was addressed in part by the use of a cross-over design, which was appropriate in this instance for the effects of the PASAT intervention are short lived, see Figure 1, and carry-over between the neutral and PASAT interventions was limited. (Note that two participants indicated a stress response during the neutral intervention see above). Adoption of a cross-over approach enabled an efficient experimental design requiring fewer participants than would be the case for a parallel design, with each individual acting as their own control. The observed changes in the levels of benzoic acid and n-decanoic acid were statistically significant within the design, $p = 2 \times 10^{-7}$ and 0.0004 respectively; below a Bonferroni corrected level for p of $p = 0.003$, $n = 15$.

Metabolic origin

The origins of the two candidate upregulated PASAT sensitive VOCs observed in this study are not well defined and they may result from a combination of endogenous and exogenous origins.

Benzoic acid has been reported as a metabolite of both benzaldehyde and toluene [52], and the oxidation of benzyl alcohol to benzoic acid in skin by alcohol dehydrogenase with benzaldehyde as the presumed intermediate has been reported [53]. Benzoic acid is cited as being present within the cytoplasm, endoplasmic reticulum and as well in extracellular fluid. It has been isolated from blood, faeces, saliva and urine and has been found in samples from the bladder, kidney, liver, skin stratum corneum, testes and in fibroblasts. Of interest to this study was the observation that benzaldehyde was down regulated in breath following a PASAT intervention [1], raising the question of a possible oxidative pathway link between breath and skin. Occurring commonly in food stuffs benzaldehyde is readily absorbed through the skin and lungs and, along with its metabolites, distributes to all well-perfused organs and doesn't accumulate in any one tissue type. Its primary metabolite is benzoic acid which conjugates with glycine or glucuronic acid and is excreted in urine. Oxidation of benzyl alcohol and benzaldehyde appears to be the most commonly documented source of benzoic acid in urine, blood and on skin [53-54]. Benzoic acid has also been reported as a biomarker for asthma and lung cancer in exhaled breath [55,56].

N-decanoic acid is a medium chain volatile fatty acid (VFA) also known as capric acid (Lat: Goat) and has been reported in apocrine sweat as a source of malodour. Medium chain fatty acids are thought to be carried to the skin surface as L-glutamic conjugates and released by bacterial action [57]. An important route to VFA in axillary secretions is the partial catabolism of methyl branched longer chain fatty acids by the *Corynebacterium* bacteria; resident in the axilla and on the forehead [58,59] Methyl-branched fatty acids are reported to originate from the triacylglycerol component of sebum, and from apocrine sweat, and are released by bacterial lipases. Up-regulation of medium chain fatty acids such as n-decanoic acid reported in this study may be the result of: an increase in sebum secretion; elevated bacterial action with increased skin temperature; or, as the end product of a longer metabolic change increasing the secretion of methyl branched fatty acids precursors to the skin surface [30,29]. N-

decanoic acid may also be generated from decanal, associated with increased lipid peroxidation and oxidative stress. N-decanoic acid is found in cellular membranes and extracellular fluid, it has been isolated from blood, breast-milk, saliva and urine and it is associated with the stratum corneum.

The xylene isomer, one of the two candidate down regulated PASAT sensitive VOC, is likely to be attributable to exogenous exposure via household cleaning products, vehicle exhausts and cigarette smoke. Xylene is lipophilic, absorbed readily through the skin and lungs and promptly distributes throughout the whole body [60]. The reduction of the xylene isomer in response to the PASAT intervention may be due to enhanced elimination from elevated respiration during the PASAT intervention.

3-Carene the other down-regulated PASAT sensitive VOC had a good spectral match to the previously unidentified terpene reported in the previous breath study [1]. While a targeted study will be needed to confirm if this monoterpene is down-regulated in response to PASAT interventions in skin and breath, this observation also indicates relationships between breath and skin VOC profiles. The origin of terpenes in breath and skin profiles has yet to be defined, and it may be that terpenes are not endogenous in origin for they occur in foodstuffs [61]. However, mono-terpenes are associated with the mevalonate pathway and further exploration of monoterpene relationships and the derangement of the mevalonate pathway may prove to be a useful focus of future study.

SUMMARY

A stress-state study was chosen for two reasons: to compliment the breath study on stress and search for evidence of changes in skin-VOC profiles in response to stress; and, to explore the potential for skin-VOC profiles to be used to obtain non-invasive diagnostic markers, and in this instance a stress response to a PASAT intervention was used as a reproducible disease surrogate. This was necessarily a pilot study involving 20 participants and a 75 % compliance with the study protocol was achieved resulting in 15 sets of data being used in the MVA. Failures in compliance with sample storage and instrument performance were the reasons for excluding the samples from the MVA.

During method development, the importance of rapid freezing of the PDMS coupons to -80°C was indicated, and this observation is emphasised. No data were produced to support the storage of samples for longer than 21 days. No evidence was found that indicated sample deterioration during the maximum storage time of 21 days and it seems reasonable to conclude that samples may be stored for longer periods at -80°C.

The cumulative distribution function of the GC-MS data provides evidence of possible selectivity within the sampling method towards lower volatility VOC, while the frequency distribution of the same data indicates an approximate log-normal distribution with an estimated two to three orders of magnitude reduction in the limit-of-detection required to capture the complete profile. Another important aspect in the continued development of skin sampling techniques is the satisfactory trapping and analysis of reactive, pH sensitive, and highly volatile compounds such as formaldehyde and biogenic amines. Techniques for the characterisation of skin VOC profiles may still be considered to be in their early stages of development. Better-defined standards for sampling and data evaluation will facilitate data sharing and expedite progress in this area.

Two up-regulated PASAT sensitive VOC candidates have been isolated that may be potentially predictive of induced-stress in the cohort studied, Table 3. Median benzoic acid levels in the samples taken from stressed participants were observed to be up-regulated by a median factor of approximately 14-fold, with an AUC for its ROC curve of

0.813. N-decanoic acid was upregulated approximately 8-fold with an ROC AUC value of 0.852. The up-regulation of skin surface volatile fatty acids indicate a link between psychological stress and a potential increase in oxidative metabolic pathways. Continued studies into this observation are a logical continuation of the current study. Additionally, both breath and skin studies suggest that a terpene or terpenes (3-carene is proposed) are involved with the metabolic processes associated with the body's response to psychological stress.

This work indicates that skin-VOC profiles are complementary to breath-VOC profiles, and that there may be utility in combining these two measurement vectors to produce richer and more reliable panels of VOC diagnostic markers.

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VOLATILE ORGANIC COMPOUND MARKERS OF PSYCHOLOGICAL STRESS IN HUMAN SKIN: TABLES

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Table 1. A summary of the thermal desorption parameters.

Parameter	
Prepurge	
Flow	30 cm ³ min ⁻¹
Time	1 min
Primary Desorption	
Flow	50 cm ³ min ⁻¹
Temperature	180 °C
Time	10 min
Split Flow	0 (Splitless) cm ³ min ⁻¹
Cold Trap	
Low temperature	-10 °C
Secondary Desorption	
Flow	2 cm ³ min ⁻¹
Temperature	300 °C
Time	5 min
Split Flow	0 (Splitless) cm ³ min ⁻¹
Heating Rate	~100 (max) °C s ⁻¹
Flow path temperature	180 °C

Table 2. Summary of the gas chromatography-mass spectrometry parameters.

Parameter	Level
Gas Chromatography Conditions	
Initial Column Temperature	40 °C
Initial Hold Time	0 min
Initial Heating Rate	3.3 °C min ⁻¹
2 nd Column Temp	90 °C
2 nd Hold Time	0 min
2 nd Heating Rate	2.5 °C min ⁻¹
3 rd Column Temp	140 °C
3 rd Hold Time	0 min
3 rd Heating Rate	10 °C min ⁻¹
Final Column Temp	300 °C
Final Hold Time	8 min
Mass Spectrometer Conditions	
Scan Range	40 - 445 m/z
Scan Time	0.45 s
Interscan Delay	0.05 s
Electron Energy	70 eV
Source Temperature	200 °C
Interface Temperature	250 °C

Table 3. Proposed identities of the four candidate PASAT sensitive VOC isolated from skin samples.

Ref	Name / Library Entry	Deconvolved Mass Spectra	NIST (F/R)	RI (O/E)
$\sigma 1$	Benzoic Acid / Sk-1262-105-122-77-51	105(999), 122(857), 77(700), 51 (357)	866/924	1262/1271
$\sigma 2$	n Decanoic Acid / Sk-1380-60-73-41-43	60(999), 73(920), 41(508), 43(480)	764/782	1380/1371
$\sigma 3$	Xylene isomer/ Sk-862-91-106-105-77	91(999), 106(600), 105(260), 77(125)	914/914	862/872
$\sigma 4$	3 - Carene / Sk-995-93-91-77-79	93(999), 91(560), 77(320), 79(298)	872/879	995/1010

Note. The library entry may be interpreted as: <Sk (isolated skin volatile)> - <retention index-base peak> - <m/z of the next most intense fragment ion> - <m/z of the next most intense fragment ion> -... etc. up to five fragments, or the number needed to unambiguously identify the compound. NIST (F/R) the forward and reversed mass of the deconvoluted mass spectrum against the NIST data base. RI (O/E) the observed (O) retention index verses the (E) NIST estimated retention index values.

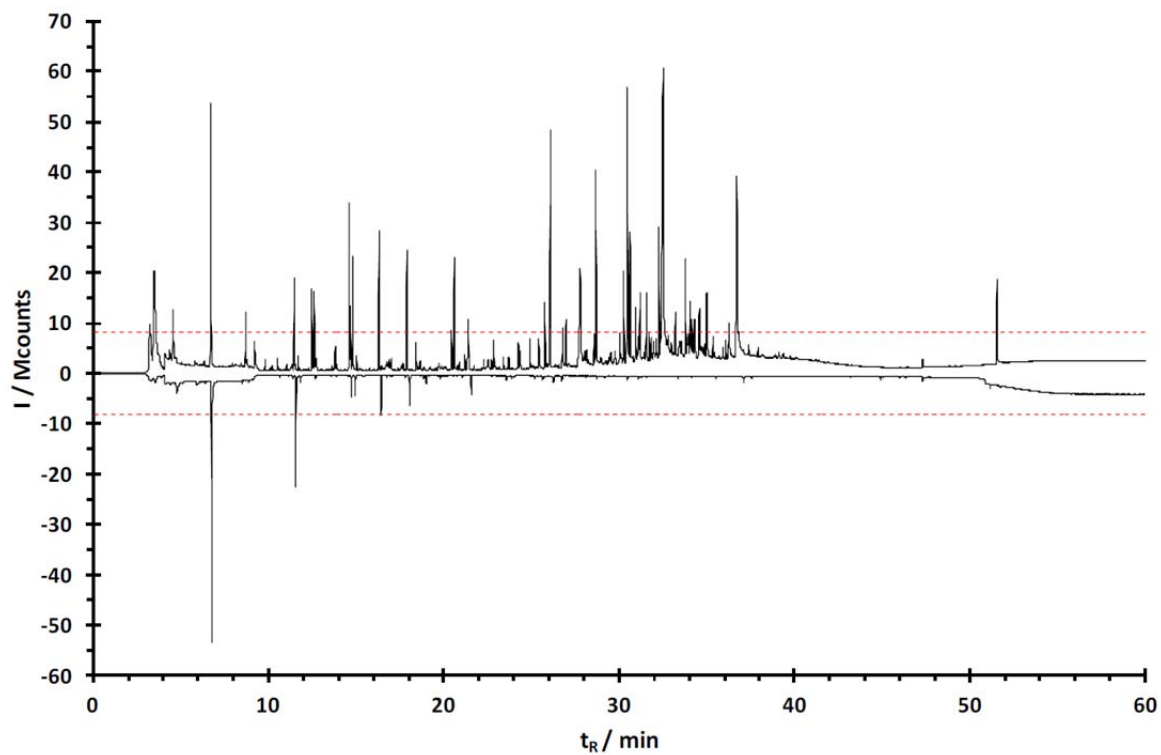


Figure S1 Example VOC profiles obtained from a cotton Skin Patch retaining pad pre (top) and post (inverted) vacuum conditioning. The red dashed line marks the response in M counts to 1ng in decane equivalents.

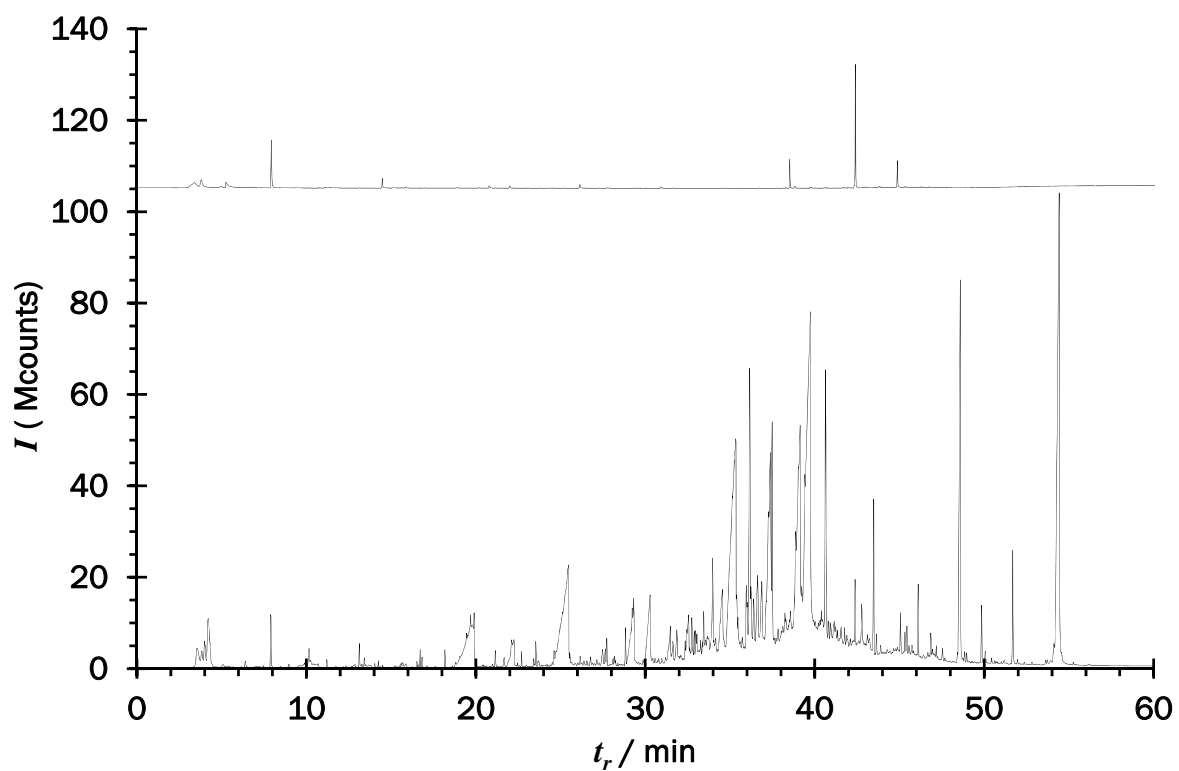


Figure S2 Example skin VOC profile (bottom) and subsequent re-desorption of the same Skin Patch (offset at 105 Mcounts). Less than 1% carryover is exhibited for any compound of interest, the major peaks observed in the blank are siloxane compounds from the PDMS coupon.

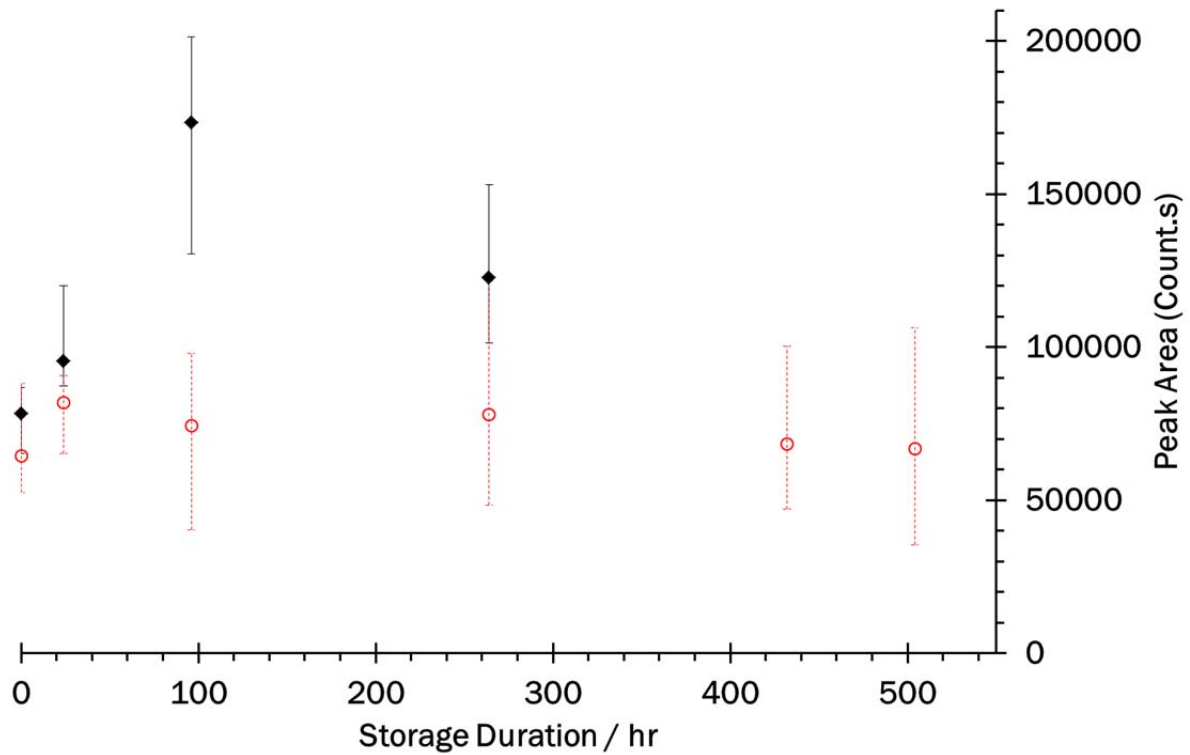


Figure S 3. An example of the effect of storage . Each data point represents the average peak area of 6-methyl-5-hept-2-one with the maximum and minimum range observed for the five replicates. The black trace shows data for -20°C storage and the red -80°C. The increase in 6-methyl-5-hept-2-one levels after 96 hours (4 days) is evident when stored at -20°C while at -80°C there is no statistically significant change over 504 hours storage.

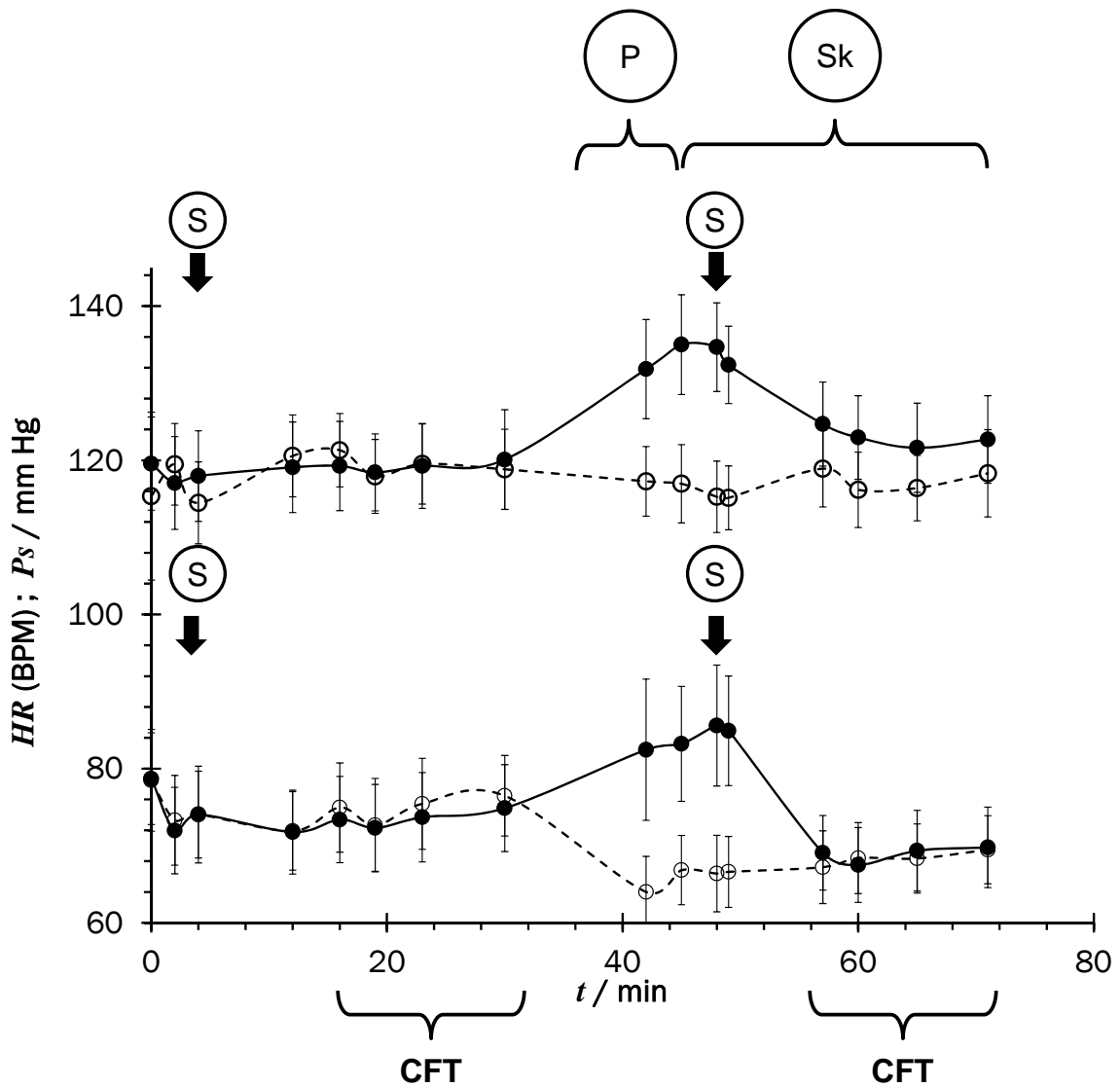


Figure 1 Mean responses of 22 participants for systolic blood pressure (PS, top), and heart rate (HR, bottom) over the 70 min stress (solid line) and neutral (dashed line) sessions. The error bars designate the 95% confidence limit on the mean. S designates when baseline and stress response physiological measurements were taken. CFT denotes the periods over which cognitive function tests were run. The PASAT exercise is denoted by P and the skin sample was collected during the interval bracketed by Sk. Figure adapted from [1].

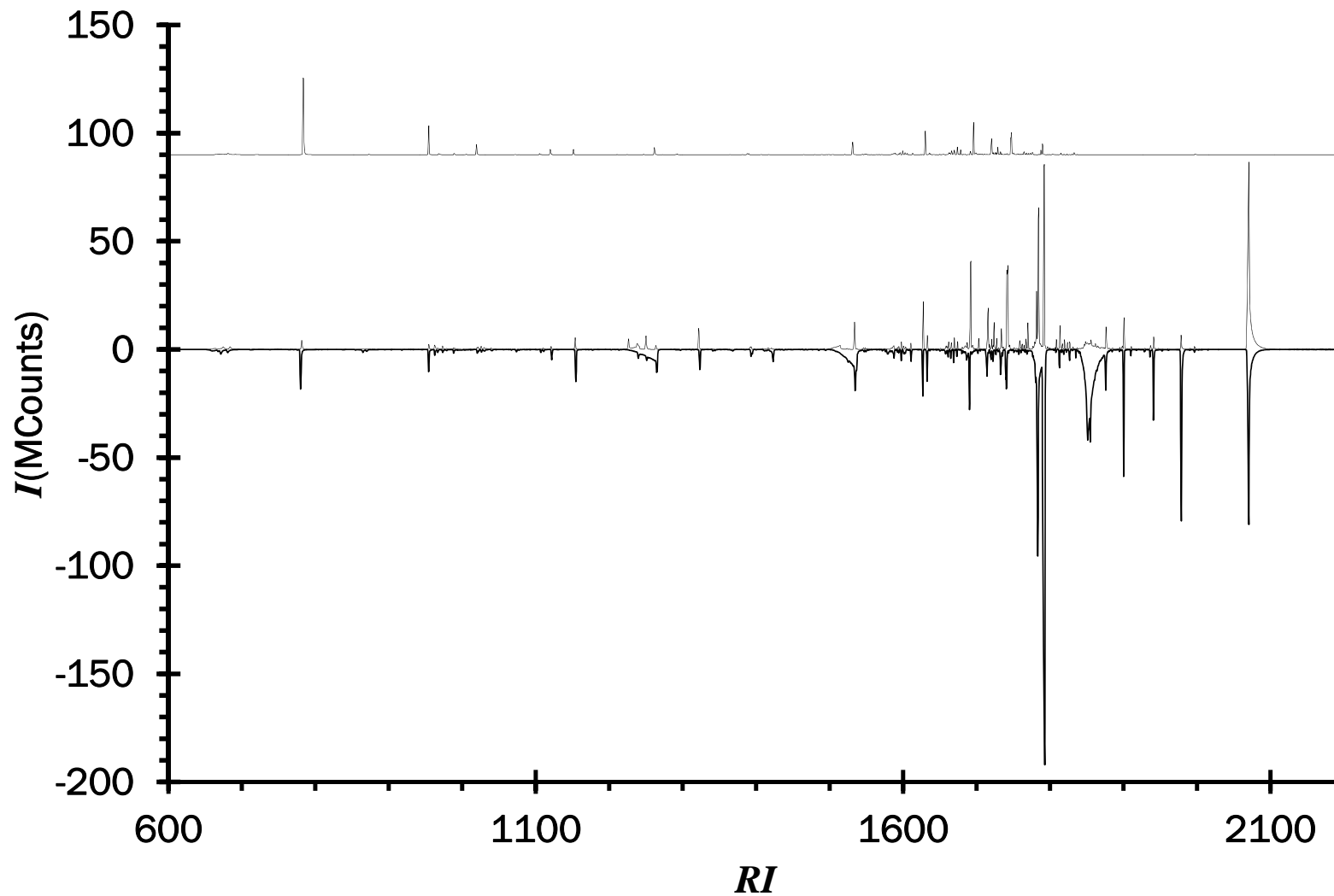


Figure 2 An example of dynamic background compensated, retention index aligned skin VOC chromatograms obtained from a female participant after the neutral intervention (top) and the PASAT (inverted), with a field blank (offset).

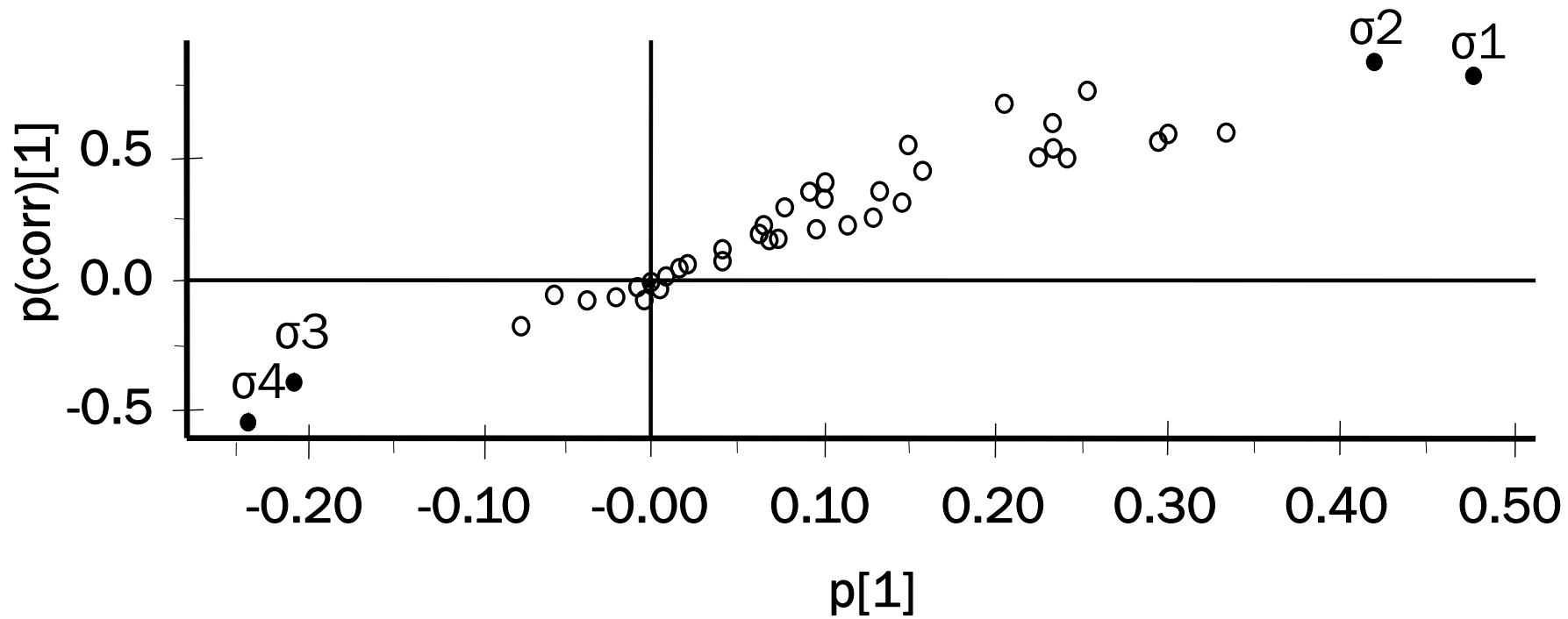


Figure 3 S-plot generated from the modelled covariance ($P(\text{corr})[1]$) and modelled correlation ($p[1]$) of the partial least square discriminate analysis (PLS-DA) from the peak areas of 40 skin components from 30 skin VOC samples. The four variables highlighted (solid dots) at the extremes of the S-plot indicate potential markers sensitive to the PASAT intervention and these variables were subsequently modelled using PCA.

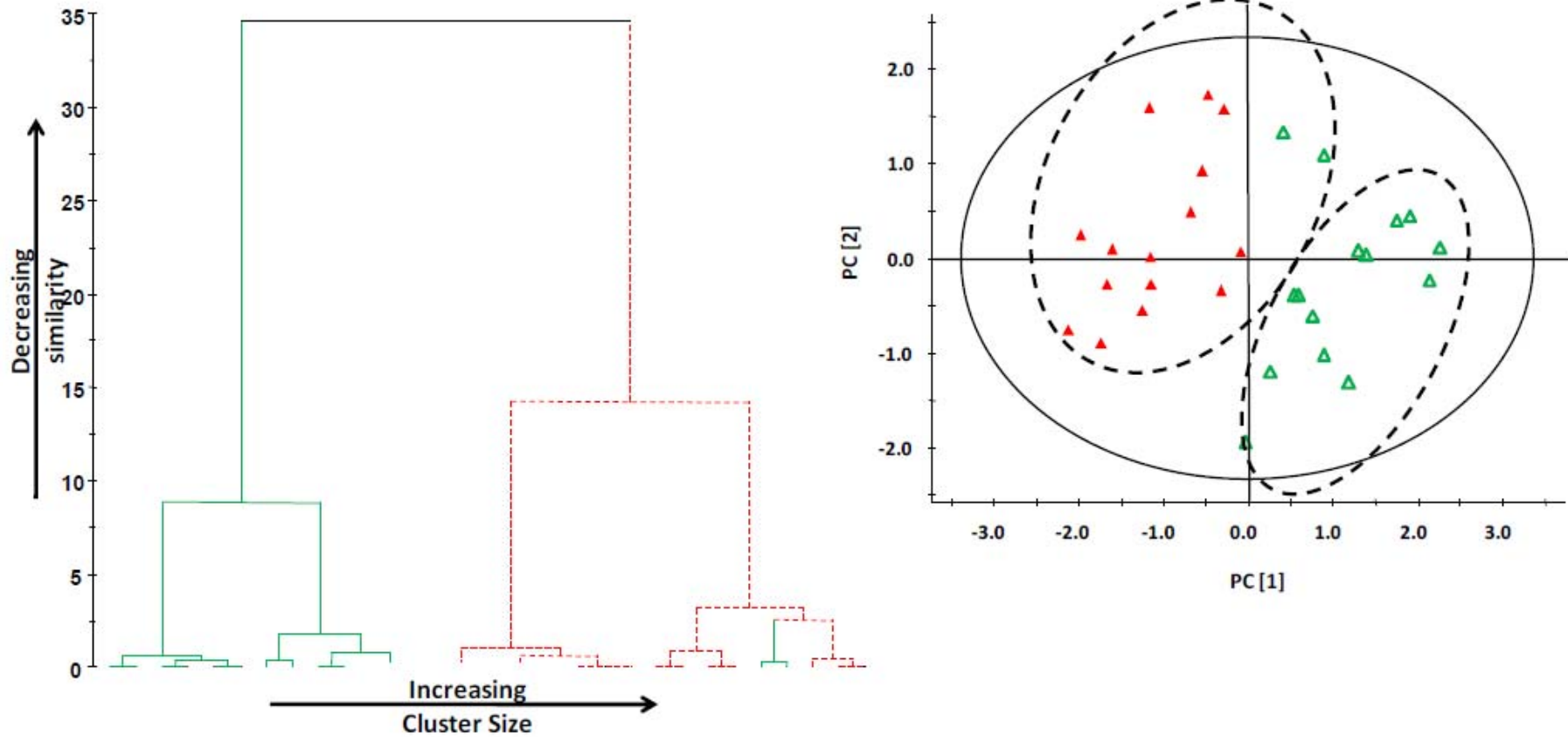


Figure 4 Left: Dendrogram constructed from scores and loading from unsupervised PCA, based on the four candidates identified by PLSDA, showing the nature of clustering between the neutral and stressed interventions. The solid green bars indicate neutral observations and the dashed red indicate the stressed; the length of the vertical bars indicates similarity with longer bars denoting a reduction in similarity. Two relaxed observations exhibit more similarity with the stressed than the other relaxed states and can be found to the right of the dendrogram. Right: the unsupervised PCA showing the nature of the clusters.

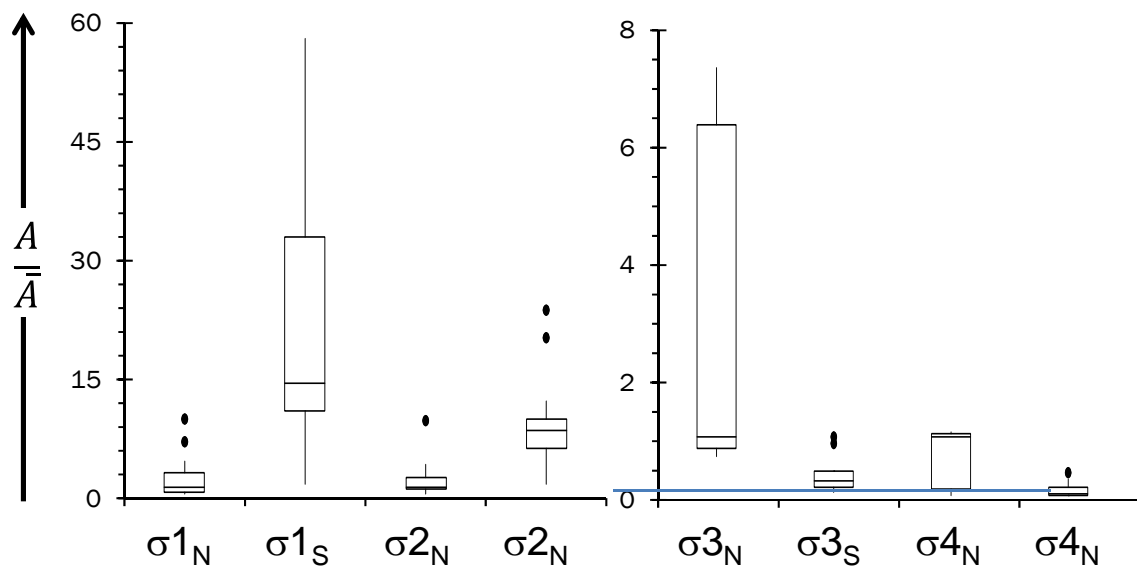
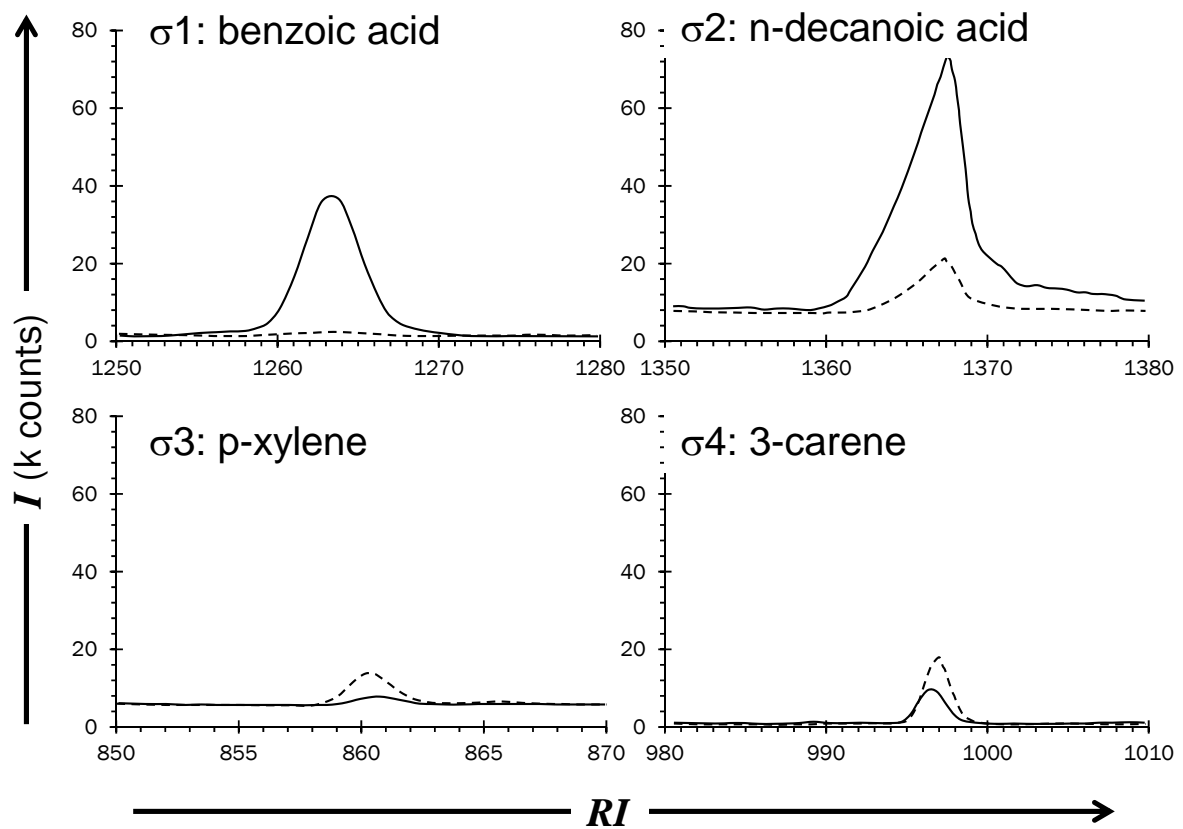


Figure 5 Top. Overlaid extracted ion chromatograms from a female participant for the four compounds found to differentiate stressed from neutral interventions (stressed: solid-line; neutral: dashed-line).
 Bottom. Box and whisker plots of relative peak areas for the four compounds showing the cohort's responses for the Stressed (s) and neutral (n) interventions. The peak areas have been normalised to the median of the neutral intervention, outliers are represented by the solid circles.

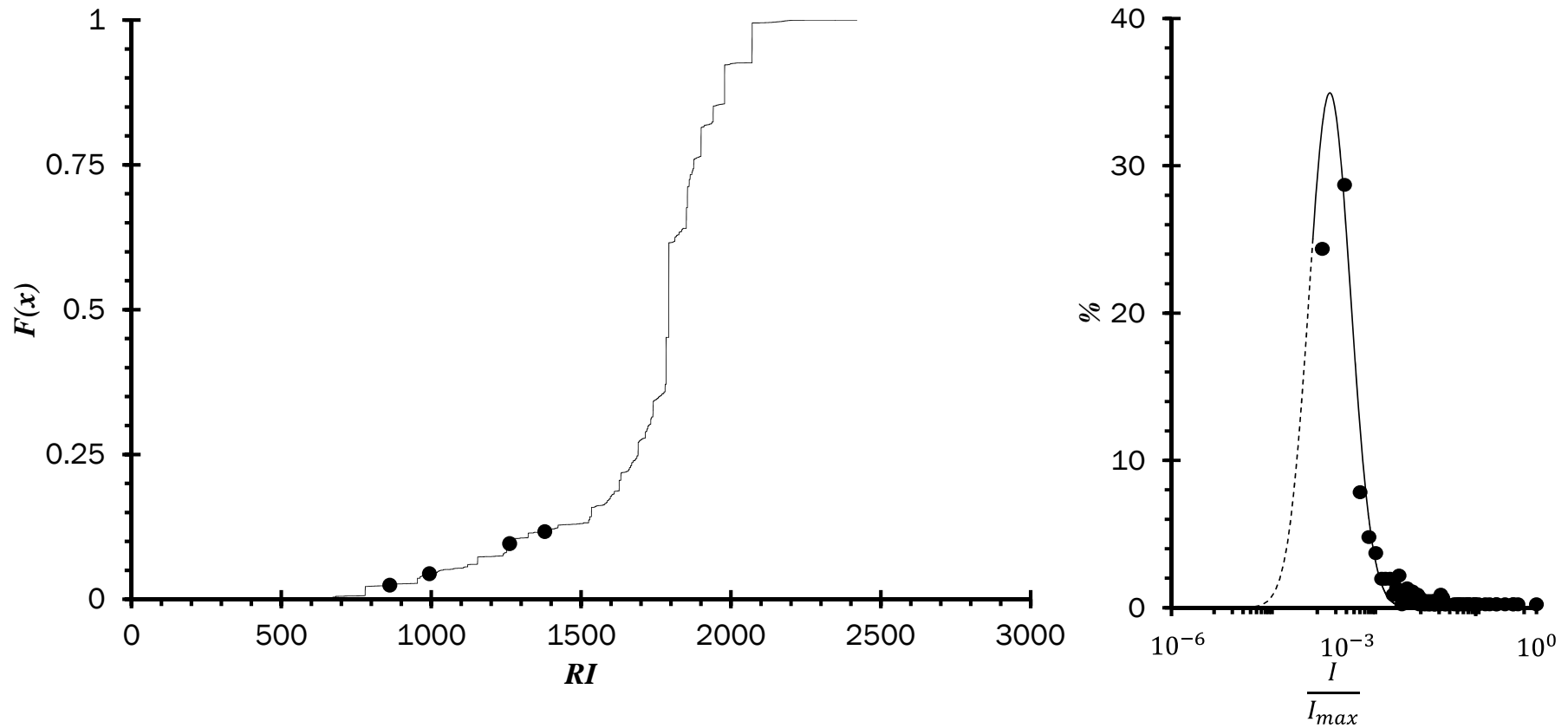


Figure 6. Left. Cumulative distribution frequency against RI showing that ca. 80 % of the cumulative response was associated with the last half of the chromatogram, indicative of possible selectivity towards lower volatility VOC. Right. The frequency distribution of the data showing the proportion (%) signals observed across the normalised range of intensities from the same chromatogram. A log-normal curve has been fitted to the data and extrapolated (dashed-line) to indicate the possible future nature of this distribution with enhanced sensitivity, and /or reduced sample selectivity. The extrapolated function indicates that the limit of detection will need to be reduced a further two to three orders of magnitude before the whole VOC profile will be observable.