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Published in:
Journal of Breath Research

DOI:
10.1088/1752-7163/ad7977

Publication date:
2024

Document Version
Publisher's PDF, also known as Version of record

Link to publication in ResearchOnline

Citation for published version (Harvard):
Ahmed, W, Wilkinson, M & Fowler, SJ 2024, 'Generating pooled quality control samples of volatile organic compounds', *Journal of Breath Research*, vol. 18, no. 4, 041004. https://doi.org/10.1088/1752-7163/ad7977

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Journal of Breath Research

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To cite this article: Waqar Ahmed *et al* 2024 *J. Breath Res.* **18** 041004

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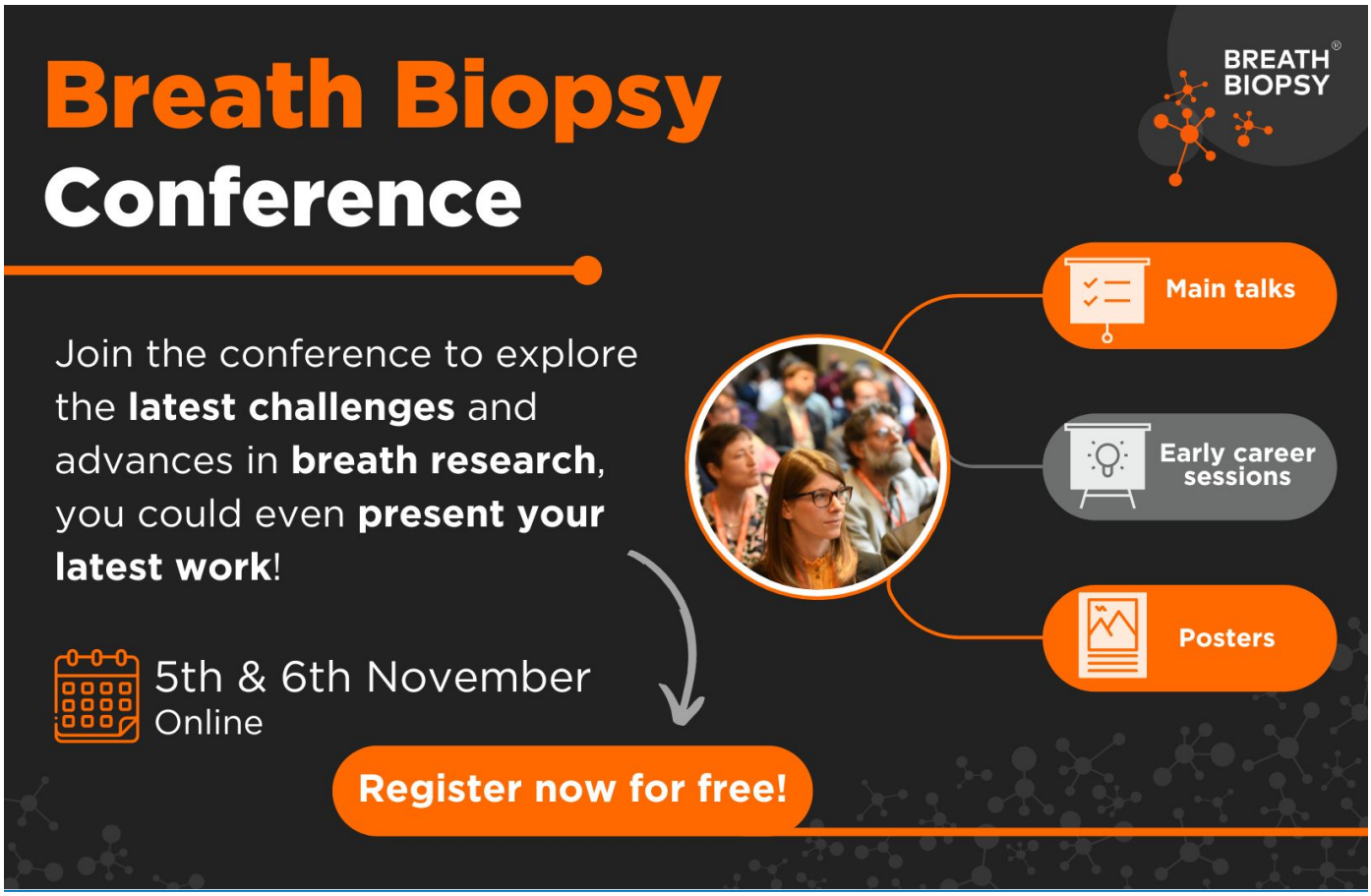

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NOTE

Generating pooled quality control samples of volatile organic compounds

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Keywords: metabolomics, standardisation, thermal desorption, breath analysis, GC-MS, quality control, pooled QC

Supplementary material for this article is available online

OPEN ACCESS

RECEIVED
2 July 2024

REVISED
19 August 2024

ACCEPTED FOR PUBLICATION
11 September 2024

PUBLISHED
19 September 2024

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Abstract

Untargeted analysis of volatile organic compounds (VOCs) from exhaled breath and culture headspace are influenced by several confounding factors not represented in reference standards. In this study, we propose a method of generating pooled quality control (QC) samples for untargeted VOC studies using a split-recollection workflow with thermal desorption tubes. Sample tubes were desorbed and split from each sample and recollected onto a single tube, generating a pooled QC sample. This QC sample was then repeatedly desorbed and recollected with a sequentially lower split ratio allowing injection of multiple QC samples. We found pooled QC samples to be representative of complex mixtures using principal component analysis and may be useful in future longitudinal, multi-centre, and validation studies to assess data quality and adjust for batch effects.

1. Introduction

Although exhaled breath volatile organic compounds (VOCs) have potential as non-invasive diagnostic biomarkers [1, 2], they are heavily influenced by confounding factors such as diet, medication, environment, and time of day [3, 4]. Therefore, experimental quality assurance and controls are essential in biomarker discovery [5].

Pooled quality control (QC) samples are comprised of a mixture of components representative of the target matrix, and are used to assess system suitability and to correct systematic analytical bias [6]. In contrast to biofluids such as blood and urine, exhaled breath cannot be readily mixed to form pooled QC samples, especially for large multi-centre studies [7]. Current strategies include analysing synthetic chemical standards alongside real samples or pooling breath samples using gas sampling bags which are limited to single centre studies and short-term storage [8–10].

Gas chromatography-mass spectrometry remains the gold standard for untargeted VOC analysis, and sorbent tubes are the most commonly used tool for sampling and pre-concentrating VOCs [11]. By splitting and recollecting samples after thermal desorption, a novel method of generating pooled QCs at the point of analysis is proposed here. To demonstrate this technique, pooled QC samples were generated using two sample matrices—exhaled breath after ingestion of a peppermint capsule or coffee, and culture headspace of three bacterial species.

2. Materials and methods

2.1. Sample collection

For the culture headspace study, *E. coli* ATCC 25 922, *S. aureus* ATCC 29 213, and *P. aeruginosa* PAO1 were cultured ($n = 5$ replicates each) in Luria–Bertani broth. 2 ml of overnight culture were standardised to OD_{600nm} of 0.1 in fresh media and aliquoted in

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20 ml crimp sealed headspace vials. A mixed bacterial sample was also created where 667 μl of the standardised culture of each bacterial species was added to one vial ($n = 5$ replicates). Vials were then incubated for 24 h at 37 °C under shaking conditions to collect VOCs at the stationary growth phase for all bacterial species. After 24 h, headspace was sampled from each vial onto sorbent tubes packed with Tenax GR at a flow rate of 100 ml min^{-1} using a low flow pump (Acti-VOC, Markes International, Bridgend, UK). To ensure all VOCs in the headspace were sampled, the headspace was purged with filtered air until a total volume of 200 ml was collected as described previously [12, 13].

Breath samples were collected under local ethical approval from the Manchester Allergy, Respiratory & Thoracic Surgery (ManARTS) Biobank and was conducted in accordance with the principles embodied in the Declaration of Helsinki and in accordance with local statutory requirements. Three groups of breath samples were collected: (1) initial baseline breath samples; (2) peppermint samples obtained 1 h after the consumption of a peppermint oil capsule (Boots, London, UK), and (3) coffee samples obtained after 1 h after drinking a shot of espresso. Initial baseline and peppermint samples were collected on the same day, whereas coffee samples were collected on the day after. The ReCIVA breath sampling device (Owlstone Medical, Cambridge, UK) was used. All four sampling ports on the ReCIVA were used concurrently, and three individual breath samples were collected, generating 12 tubes per participant ($n = 3$) per experimental group.

Breath samples were collected using a previously described method [14]. Briefly, a flow of 40 l min^{-1} of VOC-free air was generated by a CASPER pump (Owlstone Medical, Cambridge, UK). Participants were asked to breathe normally, and 500 ml of end tidal breath was drawn onto Tenax GR tubes at a flow rate of 200 ml min^{-1}.

2.2. Thermal desorption and QC generation

Using a thermal desorption unit (TD-100, Markes International, Bridgend, UK) sorbent tubes were pre-purged with 50 ml min^{-1} for 2 min before being loaded with 100 μl of a gaseous calibration standard (1 ppmv, 4-bromofluorobenzene in nitrogen, Thames Restek, UK).

Each tube was then desorbed at 280 °C in a flow of 50 ml min^{-1} He for 5 min onto a general-purpose hydrophobic trap held at 0 °C. Secondary desorption of the trap was carried out at 280 °C with a column flow of 1.3 ml min^{-1} and a split flow of 2.6 ml min^{-1} resulting in 1/3 of the eluent injected into the GC-MS column. Gas from the split flow was then recollected onto a new 'recipient' sorbent tube allowing portions of each sample to be pooled together. After all samples were pooled, the recipient tube underwent a repeated desorption and recollection cycle allowing for multiple QCs to be generated. With each cycle, the ratio of eluent passed to the GC-MS versus recollection was altered to ensure that each QC contained the same proportion (10%) of total stacked sample. This workflow is illustrated in figure 1 and specific split flows are shown in table S1.

2.3. GC-MS analysis

VOCs were carried into a GC column (DB-5 ms Ultra Inert, length 30 m × internal diameter 0.25 mm, film thickness 25 μm, (5%-Phenyl)-methylpolysiloxane, Agilent Technologies, SantaClara, USA) housed within a GC oven (7890B GC, Agilent Technologies, SantaClara, USA) using the following temperature ramp: 40 °C (no hold), ramp 6 °C min^{-1}–170 °C, ramp 15 °C min^{-1}–190 °C (total GC cycle time of 23 min) with an He carrier gas flow of 1.3 ml min^{-1}. A triple-quadrupole mass spectrometer (7010, Agilent Technologies, SantaClara, USA) was used in full scan mode where VOCs were ionised (EI^{+} 70 eV) and mass spectra acquired between 40–500 Da at 4 Hz.

2.4. Data processing and statistical analysis

All files were converted to mzXML format prior to pre-processing. Chromatograms were screened for inclusion in the final dataset by manual appraisal and all samples deconvolved and aligned using eRah [15]. Data pre-processing and statistical analysis was performed in R (v.4.2). Principal component analysis (PCA) was used to visualise how representative QCs were compared to distinct breath and culture headspace samples.

3. Result

Headspace VOCs (239 features) of axenic and mixed bacterial cultures were profiled and showed separation across PC1 (figure 2(a)). Pooled QCs were clustered and centred on PC2 and PC3 (figure 2(b)). The pooled QC formed a tighter cluster than the biological QC (i.e. mixed culture headspace) in PCA projection, the latter which may have been influenced by microbial growth and interactions in mixed culture.

Similarly, with exhaled breath VOC data (193 features), pooled QCs were tightly clustered and centred on PC2, with all samples separated from the pooled QC across PC1 (figure 3(a)). No separation was apparent for breath samples taken before and after ingesting a peppermint capsule was shown on this PCA, however samples after drinking coffee were separated across PC2. Separation was apparent on PC1 when comparing between baseline and post-peppermint ingestion breath samples only (figure S1). As with bacterial headspace samples, pooled QC samples were tightly centred around PC2 and PC3, between breath samples (figure 3(b)).

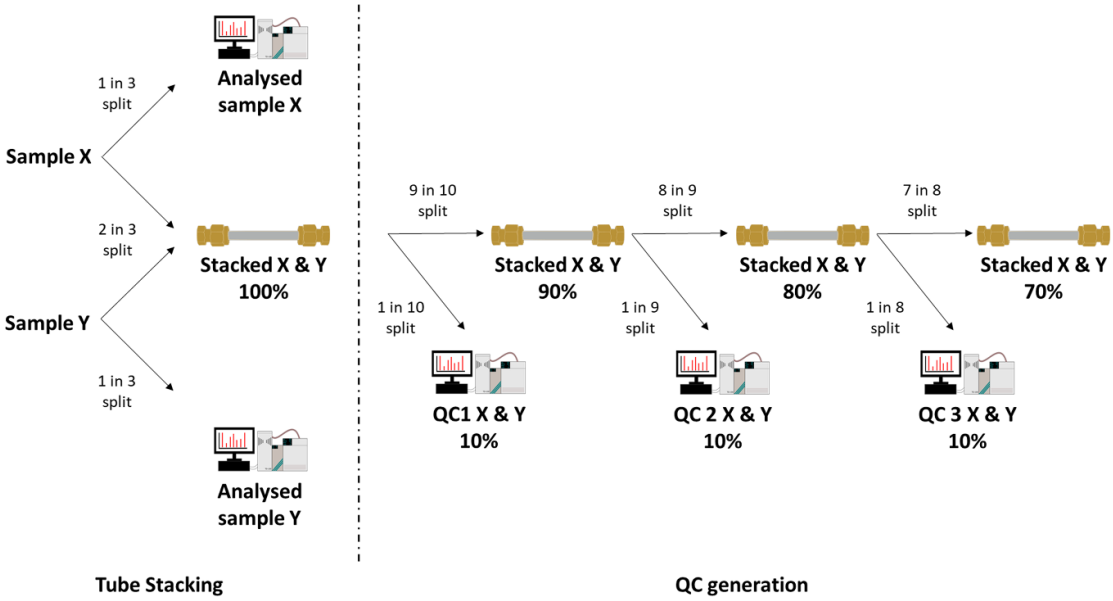

Figure 1. Workflow of VOC samples through the multiple stages of desorption and recollection within the thermal desorption unit. During the stacking phase each sample is desorbed onto the trap as usual then, during the trap desorption, 66% of the eluent is recollected onto a new recipient sorbent tube in a process known as stacking. Once all the samples in a batch are available, QC generation can be performed whereby the stacked tube is repeatedly desorbed to allow multiple QCs to be created. Each QC sample generated will have a consistent level of the VOCs present in the stacked tube due to changing split ratios with sequential cycles.

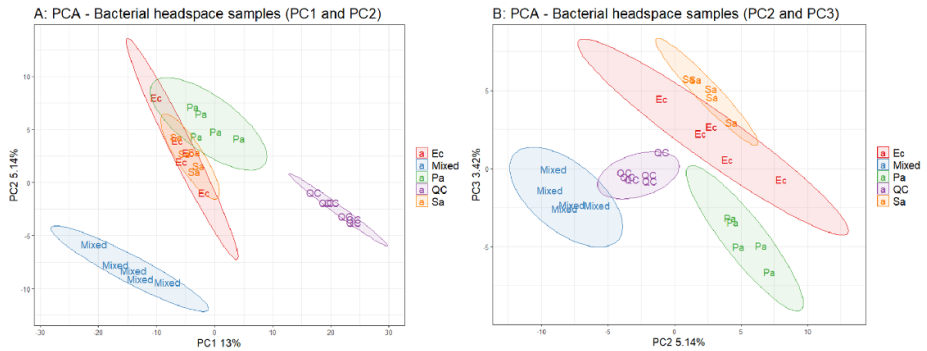

Figure 2. A: PCA scores plot of PC1 and PC2, and B: PCA scores plot showing bacterial culture headspace samples (Ec = *E. coli*; Pa = *P. aeruginosa*; Sa = *S. aureus*) where QC sample data are centred between mixed cultures and single cultures.

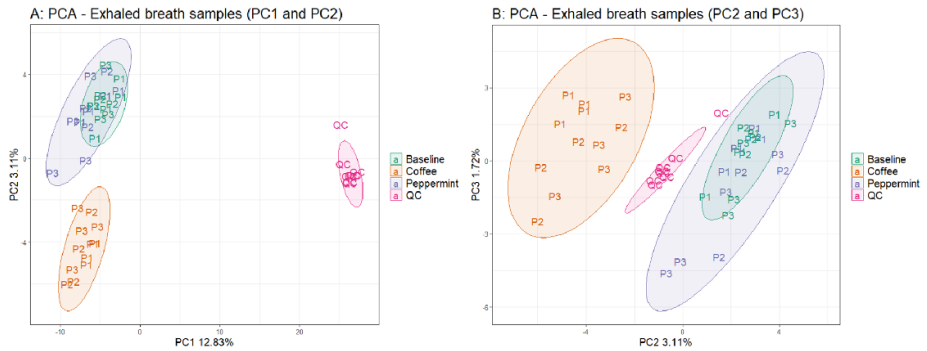

Figure 3. A: PCA scores plot of PC1 and PC2, and B: PCA scores plot of PC2 and PC3, showing breath samples separated based on coffee intake with QCs tightly centred between the two major sample groups. P1-3 indicate the individual participants.

4. Discussion

In this study, we showed proof-of-principle for generating pooled QC samples of VOCs, with applications in culture headspace and exhaled breath sample data interpretation, in similarity to untargeted metabolomic studies using pooled biofluid QC samples.

Pooled QC samples were representative of complex VOC matrices of distinct sample groups consisting of either different breath samples or microbial cultures. In both sample types tested, we were able to differentiate groups by PCA with the QC tightly clustered and centred compared to individual sample groups.

There were some limitations with using pooled QCs as in shown in this study: (1) concentrations of analytes in pooled QCs were not comparable due to the split ratio required to recollect multiple samples using the thermal desorption system used and (2) as pooled QCs were subject to multiple desorption cycles there is potential for trap artefacts to be introduced and accumulate with each cycle. These limitations likely contributed to variation as shown across the first principal components for both exhaled breath and culture headspace analyses. A potential solution is to develop and validate a method of splitting and recollecting VOCs independent of a thermal desorption unit as a GC-MS inlet, as demonstrated in other studies [16]. Furthermore, to make full use of pooled QCs for longitudinal clinical analysis, a thorough assessment of long-term sample storage for batched analysis will likely be required.

Breath VOC biomarker discovery studies would greatly benefit from pooled QCs in assessing sample, analytical, and data quality, as well as potentially adjusting for batch effects [5, 17, 18] as commonly seen in multi-site studies or longitudinal studies where analysis is likely to include systematic errors (e.g. instrument maintenance) or when samples are analysed in batches and require long-term storage.

Data availability statement

The data cannot be made publicly available upon publication because they are not available in a format that is sufficiently accessible or reusable by other researchers. The data that support the findings of this study are available upon reasonable request from the authors.

Conflict of interest

The authors declare no conflicts of interest.

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