A Methodological Framework to Profile Volatile Organic Compounds from Extracellular Vesicles using Gas Chromatography-Mass Spectrometry

Quynh-Mai Nguyen Le*, Anrui Ge*, Nayab Nadeem Abdul Kader*, Sanooj Soni* and Ilaria Belluomo*

*Department of Surgery and Cancer Imperial College London, London, UK

Abstract

Volatile organic compounds (VOCs) reflect cellular and microbial metabolism, but their transport within the body remains poorly understood. Extracellular vesicles (EVs), may contribute to VOC transport. Reliable EV-associated VOC profiling therefore requires effective vesicle disruption with minimal background contamination. This study evaluated water-mediated hypotonic disruption of EVs isolated from healthy human blood, followed by headspace thermal desorption gas chromatography–mass spectrometry measurement of VOCs. PCA showed substantial overlap between groups, while OPLS-DA and VIP analysis identified features associated with the EV + water condition, in chemical groups such as ether, alkene and ester. These preliminary findings support the feasibility of EV-associated VOC analysis but require confirmation with additional biological replicates, improved compound identification, and validated multivariate models. Further development may clarify VOC origin and transport and improve the interpretation and specificity of volatile biomarkers.

Introduction

Volatile organic compounds (VOCs) are metabolic by- or end-products of cellular and microbial processes that can enter the circulation and be excreted through the lungs (1,2,3). Consequently, VOCs can be indicative of endogenous processes occurring within the body. Altered VOC profiles have been reported in several pathophysiological conditions, supporting their potential use as non-invasive biomarkers (4,5). However, the mechanisms by which VOCs are transported within the body remain poorly understood. Extracellular vesicles (EVs), which mediate intercellular communication and carry diverse biomolecules, may represent a potential transport mechanism for VOC transportation as well as a promising source of biomarkers (6). Establishing whether VOCs are associated with EVs could therefore provide insight into VOC transport while identifying a novel diagnostic target. This study investigated whether VOCs can be detected in association with EVs using healthy human plasma donors. Disruption efficiency was assessed using flow cytometry while potentially released VOCs were analysed using headspace thermal desorption gas chromatography–mass spectrometry (HS-TD-GC-MS). Previous chemical disruption methods achieved substantial EV disruption but introduced VOC contamination, while mechanical approaches produced less background but insufficient membrane disruption. Therefore, the present study evaluated water-mediated hypotonic disruption combined with sonication as a low-background alternative (7). This methodological work will establish the foundational approach for EV disruption and subsequent VOC analysis. Reliable profiling of VOCs in patient-derived EVs could ultimately enable volatile signatures to be linked to the cellular or microbial origins of EVs, providing new insight into the biological sources, transport pathways, and mechanisms underlying VOC production. Such knowledge could improve the biological interpretation of volatile biomarkers and enhance their diagnostic specificity.

Methods and materials

Human Blood Stimulation for EV Production and Isolation Using LPS

Whole blood (WB) was collected from healthy human donors, after written informed consent were obtained. All procedures involving the collection of human samples were performed in accordance with the Human Tissue Act 2004. WB were induced in LPS followed by incubation for 4 h at 37°C under continuous rotation to promote EV release. Following stimulation, differential centrifugations were done then the resulting EV pellets were resuspended in liquid chromatography ultra-pure water (Water) or in phosphate-buffered saline (PBS) in sealed vials fitted with magnetic caps. This was followed by 1 h of incubation at 4 °C. In addition, EV suspensions were subjected to ultrasonic water bath treatment for 30 min to further promote EV disruption. Prior to osmotic disruption, plasma-derived EVs were counted using flow cytometry.

Headspace and Gas Chromatography-Mass Spectrometry

Sealed vials containing the samples were incubated at 63 °C for 1 h while agitated to promote partitioning of VOCs from the liquid phase into the headspace (HS). Then HS vapours were collected and concentrated onto thermal desorption (TD) tubes. Before use, the TD tubes were conditioned in continuous nitrogen flow. Following VOC capture, the TD tubes were introduced into GC time-of-flight mass spectrometry (GC–TOFMS) platform for untargeted VOC profiling. Analysis was performed using a TD100-XR automated thermal desorption system coupled to an Agilent 8890 gas chromatograph and a BenchTOF2 GC–TOFMS instrument. Chromatographic separation was achieved on an Rxi-624Sil capillary column, with helium used as the carrier gas at a constant flow rate. Mass spectral data were collected using electron ionisation. Controls included PBS-only, Water-only, PBS + EV, Water + EV controls.

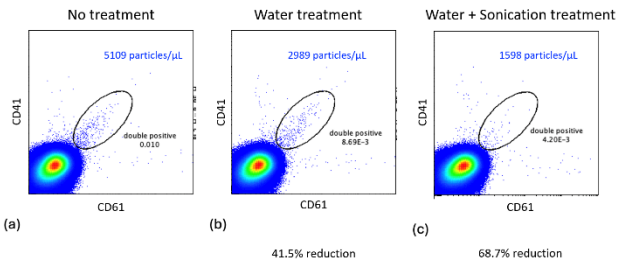
Data Processing and Statistical Analysis

Untargeted VOC data were processed in ChromSpace using an in-house Hanna VOC Lab pipeline, that enables the identification of 400 detectable analytes. Multivariate analyses, including principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), and variable importance in projection (VIP) analysis, were performed in R. PCA 95% confidence ellipses were used for visualization only. VIP scores were obtained from the OPLS-DA model, with VIP > 1 considered indicative of an important contribution to VOC profile differentiation. Analysis was helped by Anrui Ge (MSc).

Results and Discussion

Osmotic treatment combined with sonication efficiently reduces detectable EVs

The present study showcased that different disruption strategies produced distinct changes in the number of detectable EVs. The greatest reduction was observed when hypotonic treatment was combined with sonication. These findings indicate that Water treatment followed by sonication had the strongest effect on detectable EV integrity among the conditions examined and therefore represents the most effective disruption approach tested in this study. However, future studies should incorporate complementary EV characterisation techniques to distinguish true membrane disruption



from changes in surface-marker detectability. Transmission electron microscopy (TEM) could provide direct morphological evidence of vesicle structure and membrane integrity, while nanoparticle tracking analysis (NTA) could assess particle concentration and size distribution independently of antibody-based surface-marker detection (8).

Figure 1. Effect of osmotic treatment and sonication on PEVs. Representative flow-cytometry plots showing double-positive EVs following (a) no treatment, (b) Water treatment, and (c) Water treatment followed by sonication for 1 h. Detectable EV concentrations decreased 41.5% and 68.7% in the untreated sample and water + sonication respectively.

Overall variation in compound profiles across experimental groups

After untargeted analysis of the GC-MS data, PCA was used to assess overall variation in compound profiles across the PBS, Water, EV + PBS, and EV + Water groups. The PCA showed substantial overlap among the experimental groups, suggesting that treatment-related differences were not the dominant source of total variation in the dataset. This is particularly evident for the Water and EV + Water groups, which occupied overlapping regions of PCA space. The broad spread of the EV + Water samples also suggests appreciable within-group variability, which may reflect biological variation, technical variation, or both. The limited separation observed in PCA is important because PCA is an unsupervised method and therefore provides an indication of the dominant structure in the dataset without using group labels (9,10). The absence of clearly distinct clustering suggests that the overall compound profiles of the groups were relatively similar, even though differences may still have existed in individual features.

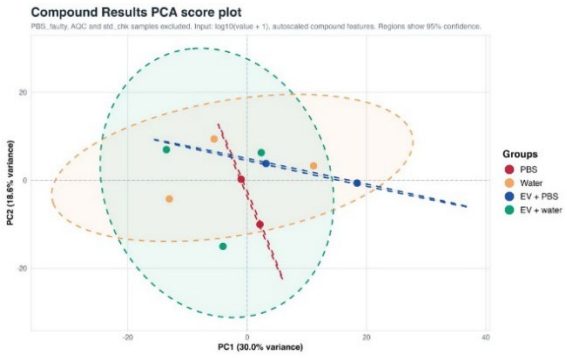
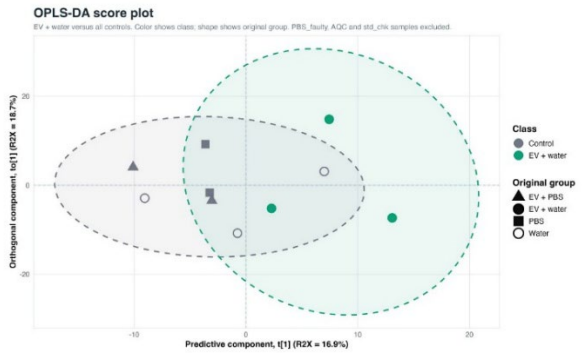


Figure 2. PCA of compound profiles showing PBS, Water, EV + PBS, and EV + water samples. PC1 and PC2 explained 30.0% and 18.6% of the total variance, respectively. Dashed ellipses indicate 95% confidence regions for the experimental groups.

OPLS-DA reveals partial separation of EV + water from controls

In contrast to PCA, OPLS-DA identified a treatment-associated multivariate pattern, with EV + Water samples tending to shift toward positive values on the predictive component. This indicates that a subset of compound features contributed to discrimination between EV + Water and the pooled control samples. However, the separation was incomplete, as shown by the overlap of the confidence regions and the position of one water sample within the EV + Water side of the predictive space. This suggests that the VOC profile associated with EV + Water was not entirely distinct from the control conditions. Because OPLS-DA is a supervised method, it can emphasize differences that are not dominant in unsupervised analyses such as PCA. Therefore, the OPLS-DA findings should be



first orthogonal component, to[1], explained 18.7%.

interpreted cautiously, particularly given the limited number of replicates. Validation using a larger dataset, cross-validation, permutation testing, and independent samples would be important to determine whether the observed class separation is robust (11).

Figure 3. OPLS-DA score plot comparing EV + water samples with pooled control samples. The predictive component, t[1], explained 16.9% of the X-variable variance, while the

VIP analysis identifies discriminating compound features

A previous study investigating VOCs associated with three human cell lines (EPI, Hep-G2, and HEK-293) reported eight compounds, with aldehydes, phenolic compounds, and terpenoid compounds being the most frequently represented chemical classes (12). In the present study, a different set of VOC features was associated with the EV + Water condition. VIP analysis identified a chemical compound in the ether group as the highest-ranking EV + water-associated feature, together with alkene, ester and several unidentified features. The absence of clear overlap between the compounds reported previously and those highlighted in the present study might be because previous work was based on defined human cell lines, whereas the present study examined plasma-derived EVs, which represent a more heterogeneous biological population and are exposed to a substantially more complex chemical background. These differences may influence both the VOCs associated with EVs and the compounds that can be detected analytically.

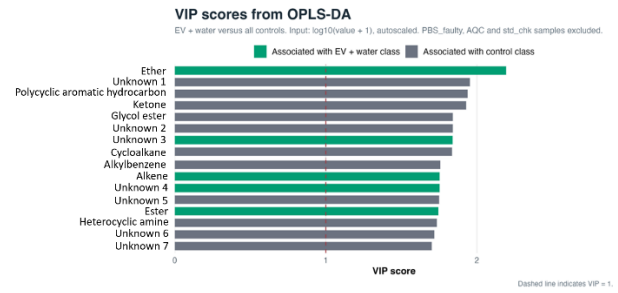


Figure 4. VIP scores from the OPLS-DA model comparing EV + water with pooled control samples. Compound features were ranked according to their VIP scores following analysis of log10(value + 1)-transformed and autoscaled data. The dashed vertical line indicates the conventional VIP threshold of 1.

Conclusion

In conclusion, water-mediated hypotonic disruption combined with sonication appeared to provide a useful compromise between EV disruption and minimisation of background VOC contamination. The detection of multiple VOC features following EV disruption supports the feasibility of investigating EV-associated volatile compounds. At the same time, increasing the number of biological replicates would improve statistical power and help determine whether the observed compound signals are consistently associated with EV disruption. Improved compound identification would also strengthen confidence in the chemical assignments.

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