Commentary

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Bioanalysis

Volatile metabolome: problems and prospects

"To fully implement biomarker discovery and realize the implied potential of volatile metabolomics, perhaps the methodology and practice of biomarker discovery must be re-examined."

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The prospect of exploiting body odor variation for disease diagnosis generates much excitement. However, the notion of diagnostic volatiles is not a recent one. Early diagnosticians relied significantly on body odor to diagnose conditions such as gangrene, smallpox, typhoid and others [1]. Numerous studies over the past two decades have provided overwhelming evidence that dogs and rodents can be taught (via associative learning) to discriminate among many health-related conditions on the basis of olfaction. The number of diseases (chronic or infectious) and injuries studied over the past two decades is impressive; as are the demonstrated abilities of the trained animals to detect these conditions via olfaction [2]. Yet, there is still debate. Do observed alterations in bodily odors represent a noninvasive portal into human (and animal) health? Or, are these observations distracting our pursuit of effective biomarkers?

Animals learn about volatile odors via the nose–brain axis that magnificently detects complex odor mixtures, processes these data and responds according to the training template. The pursuit of replicating this natural system with instrumental and statistical analyses relies on a sequential process:

- Sample analysis (sample prep and introduction, separation and detection);
- Data processing (noise reduction, peak alignment, peak integration);
- Metabolomic discovery (classification models, pattern recognition);
- Biomarker identification (structural elucidation and quantitation);
- Biomarker interpretation (determining physiological or biochemical pathway).

To fully implement biomarker discovery and realize the implied potential of volatile metabolomics, perhaps the methodology and practice of biomarker discovery must be re-examined.

Technical concerns

The first step in any chemical analysis scheme is to identify the sample. Published studies have examined every sort of excretion or emanation, including (but not limited to) urine, feces, sweat, serum, breath and sputum. The important decision regarding the sample can be made on the basis of economy, prior art or an understanding of the biological system being tested. In many studies, an assumption exists regarding urine as a 'window into individual health.' In fact, studies have demonstrated that the volatile information (as determined by trained animals) contained in serum is similarly available in urine [3]. However, comprehensive comparisons among sample types are not common in metabolomic studies of diseases (chronic or infectious) or injury.

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Headspace analysis

After making the difficult decision of what to sample (e.g., breath, urine, feces or sweat), nearly all studies of the volatile metabolome have employed some version of headspace analysis. This makes complete sense – particularly as viewed by the analytical chemist. On the other hand, no species of animal finds it necessary to adhere to definitions of 'volatile' when employing olfaction for social communication or foraging. For example, disparlure (Z-7, 8-epoxy-2-methyloctadecane) the Gypsy moth (*Lymantria dispar*) pheromone, would hardly be considered chemically volatile (mw = 282; $bp_{0.25}$ = 146–148°C). Yet disparlure is readily detected by males at distances up to 600 m [4]. More relevant to this discussion, the mammals employed in disease detection studies are equipped with an apparatus (vomeronasal organ) allowing detection of less volatile molecules that might be poorly represented in the headspace.

> "...it is not evident that semi-quantitative data are yielding meaningful patterns in metabolomic discovery."

In order to capture the same volatile signals available to the trained animal, more 'global' approaches to volatile extraction should be explored, even if these techniques are exceedingly more labor intensive than headspace sampling. Traditional liquid/liquid and solid/liquid extraction combined with sample cleanup are rarely employed in volatile metabolomic studies, despite the fact that these 'old fashioned' techniques yield broader coverage of metabolites.

Quantitative analysis

There is no doubt that headspace sampling has the distinct advantage of minimal sample preparation. However, quantitative analysis relies upon efficient introduction of odorants from the sample into the gas chromatograph. Not only are headspace sampling approaches inefficient for compounds with low vapor pressures, headspace sampling can be poorly quantitative because volatile collection is governed by multiple equilibria. Thus, surrogate standards added to the sample are unlikely to mimic analytes in the headspace. Even when analyte introduction into the instrument is well controlled, electron impact (EI) MS is itself subject to poor quantitation. Unless known standards are available to construct external standard calibration curves for each and every analyte (or isotopically labeled internal standards for each analyte are used), these analyses are semi-quantitative at best.

Unlike flame ionization detection (FID) which yields predictable relationships among peak responses based on concentration and carbon number, internal standards do not yield accurate quantitation in GC–MS. This is because EI mass spectrometric chromatographic peak responses are typically not proportional (statistically, calibration curve y-intercepts do not pass through zero). As a result, response factors (concentration or mass divided by peak area) are not constant over even small concentration ranges. For example, a twofold difference in EI mass spectrometric peak response observed for a particular analyte (between two samples, for example) is unlikely to represent a twofold difference in analyte concentration. Indeed, it is still possible to assess if the difference is significant given proper replication and statistical analysis. However, the actual magnitude of that difference can only be accurately assessed through use of standard calibration curves. Significant detector bias also exists between compounds. For example, FID peak ratios of 11 fatty acid methyl esters varied by only 7.5% (RSD), while total ion chromatogram ratios for these same compounds obtained by quadrapole MS demonstrated 23% variability [5]. Bias is exacerbated when selected-ion monitoring (SIM) traces are employed; variability increased to 47% RSD.

Admittedly, it remains uncertain if highly quantitative results are even necessary for initial metabolomic discovery. Alterations of volatiles (e.g., up- or down-regulation of metabolites corresponding to infection) are readily observable from semi-quantitative data. Recognition of such patterns can be highly informative for identifying specific volatiles for biomarker identification. Yet, it is not evident that semi-quantitative data are yielding meaningful patterns in metabolomic discovery.

Experimental concerns

In a purported compendium of human volatiles, 1840 compounds were identified in several human secretions [6]. This astounding number suggests not only that the number of possible odorant combinations is enormous but also that the likelihood of a unique odorant resulting from infection or injury is correspondingly quite small. In fact, production of unique volatiles is rarely reported in volatile metabolomic studies, with the exception of bacterial infections [7]. Thus, disease and healthy (control) states of host populations are commonly represented by different patterns derived from the identical collections of odorants. Classification models such as factor analysis, partial least square regression, linear discriminant analysis, machine learning, artificial neural networks, etc. are routinely employed for pattern recognition in metabolomics investigations. That these powerful techniques have not identified accepted biomarkers suggests that

the data being analyzed are not sufficiently reliable or extensive.

Data compatibility

There is new study on volatile metabolomes published almost every month. In general, the sample sizes are small and the data are not standardized in a manner that renders the peak response data compatible with data from other studies. For example, our data (while very valuable to me) are basically useless to anyone with similar data generated by a different analysis scheme. Briefly, in our scheme biological samples (typically urine) are fortified with l-carvone and subjected to dynamic headspace analysis (employing a trap which is thermally desorbed). EI mass spectral data generated by GC–MS analysis are exported for noise reduction and peak alignment with the freely-available MetAlign TM software [8]. These data, consisting of multiple ion features for all peaks detected in the chromatogram, are then processed with MSClust in order to yield a single SIM response for each peak [9]. Finally, SIM responses of each peak are normalized to the l-carvone SIM response in the sample to yield a multivariate data set for statistical analyses.

The appropriateness of this scheme is not the issue. The greater concern is that these data are not directly compatible with equally valid data resulting from solid-phase microextraction GC–MS analysis employing no internal standard and processed by XCMS/METLIN [10]. This is not to say that my scheme is superior (hopefully it is not inferior) to any other. Nor is it presented here to suggest that it be adopted as the standard. To the contrary, I suggest that samples should be subjected to solvent extraction (whenever feasible) and raw data should be reported as FID peak area responses normalized to response factors of the nearest *n*-alkane added to the sample at a universal concentration. Regardless, research of the volatile metabolome suffers from a data compatability problem.

As compared with the volatile metabolome, there has been considerable progress in biomarker discovery through examination of the 'wider' (small molecules, up to 2 kDa) metabolome (e.g., [11,12]). Surely technical advances in LC–MS/MS have contributed to these successes. However, small-molecule metabolomics has benefitted even more from development of a standardized platform that has no doubt generated an astounding database [13]. A massive database of volatile metabolites from many biological systems could similarly be produced through development of a framework by which volatile data are standardized from analysis to reporting. We are fortunate to be working in an era when 'big data' is not an impediment to successful data mining. An entire discipline of science exists to tackle large data sets. Yet, volatile metabolomic data are not being made available in a meaningful way.

Underlying mechanism: the microbiome?

It is becoming obvious that virtually every perturbation of the host results in alteration of the volatile metabolome. For example, alterations arising from immunization [14], infectious disease [15], chronic disease [16] and brain injury [17] have been characterized in our laboratory. Included in these studies is some evidence of specificity among alterations coinciding with the various insults, giving hope to the concept that volatile metabolites might yield highly specific information regarding health of the host. However, demonstration of specificity would be greatly improved by knowledge of the underlying physiological mechanisms.

> "...volatile metabolomic data are not being made available in a meaningful way."

Among the physiological processes that are likely to contribute to the volatile metabolome, the immune system and the major histocompatibility complex (MHC) in particular, have received tremendous scrutiny. Not only are MHC proteins involved in recognition of foreign molecules in adaptive immunity but also MHC genes are highly polymorphic. As a result, these genes are thought to code for individual odor and have been a frequent target for studying the volatile metabolome. Receiving far less attention, until very recently, has been the microbiome. As more and more information is being gathered about the far-reaching influence of the gut microbiome, it is not surprising to discover that changes in the microbiome have implications for speciation, disease spread and mood [18]. Most important to this discussion, changes in species composition of the microbiome strongly impact urinary metabolites [19].

Volatile metabolites measured in the host are actually part of a 'co-metabolome' arising from exchanges of metabolites between host and microbiome [19]. The microbiome can be considered an ecological community whose species distribution is influenced by resource availability and utilization as predicted by ecological niche theory. Perturbations in host metabolism (caused by disease, infection or injury, for example) might promote an associated change in the microbiome species distribution (favoring those species that are better adapted to the change in resource availability). In turn, changes to the microbiome species distribution result in alteration of the co-metabolome (see excellent review by [20] for a xenobiotic example). Even alterations at subspecies level of gut bacteria might have a significant impact on the volatile metabolome. In our laboratory, we found that gastric infusion of two different strains of *Escherichia*

coli resulted in distinctive alterations of mouse fecal volatiles (employing dynamic headspace analysis with GC-EI-MS, of course!). Integration of host and microbiome metabolism may be crucial to understanding how alterations of the volatile metabolome occur.

Conclusion

Mounting evidence substantiates that the ability of animals to detect volatile signals associated with illness is a fact [2,14,15]. The phenomenon follows logically from the highly evolved system of chemical communication wherein it is adaptive for animals to assess the health status of conspecifics and modify their own behavior in accordance with the information made available to them. In fact, avoidance of odors associated with illness has been oft-studied [21]. While avoidance of these signals is insufficient evidence by itself to conclude the volatile metabolome evolved for this specific purpose, it is quite clear that animal olfaction has undergone evolutionary selection to optimize detection of these signals and transfer this information into action (co-evolutionary trait of the receiver). The question at hand is 'can instrumentation and statistical modeling imitate an evolutionary process eons in the making?'

I contend that the answer to this question is a resounding 'Yes'! However, achievement of this goal will not be realized by a continuing the current practice of analyzing the volatile metabolomes of one biological system after another. This approach, basically compiling the 'natural history' of volatile metabolites, has been useful as a first step, but incompatibility of data among these many studies has limited actual discovery. It is probably more difficult to discover a treatment or condition that does not alter the volatile metabolome. Thus, future research should focus on model systems for exploration of host immunology, metabolism and microbiome. Furthermore, all metabolomic data should be generated under a standardized platform. Not only will novel volatile biomarkers be identified from these studies but biomarker occurrence will also be predicted in systems yet to be studied. As the fine essay by Johnson and colleagues ends: '*…the future prospects of metabolomics lie not only in the unique information it provides, but in its integration into systems biology*' [22]. It cannot be stated any better than that. It is time to stop looking at the volatile metabolome as analysts and start tackling it as scientists.

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