WP 083

HiSorb TD-GC-MS Innovation: Probing Liver Metabolism with Deuterated EVOCs Unveils Potential Novel Enzymatic Pathways and Biomarkers for Disease Diagnosis

Antonio Murgia[1], Yusuf Ahmed[1], Iris Banda[1], Menisha Manhota[1], Daniela Fonseca[1], Olga Gandelman[1], Max Allsworth[1], Billy Boyle[1], Lucy Godbeer[1], Jacob Rudman[1], Connor Clarke[1], Alexandra Martin[1], Giuseppe Ferrandino[1]

Key Points

This study unveils the findings of an *in vitro* experiment investigating the metabolism of deuterated EVOCs by Primary Human Hepatocytes (PHH). The study employs the HiSorb™ platform coupled with Thermal Desorption-Gas Chromatography-Mass Spectrometry (TD-GC-Exploris-MS) for precise analysis.

This approach could be applied to several aspects of medical research, from identifying pathological metabolic alterations as novel biomarkers for diagnostic purposes, to screening potential new drug targets and drug candidates for efficacy in affecting disease associated metabolic pathways.

1. Background and Objectives

Multiple liver specific metabolic pathways generate volatile organic compounds (VOCs) that are detectable in breath. Metabolic alterations due to chronic liver diseases change the spectrum of VOCs exhaled in breath. These changes can be potentially exploited for diagnostic purposes. Studies aimed to study these disease associated changes in VOC production are limited. However, the aim of this study was to establish a new headspace analysis approach for characterisation of VOCs in human primary hepatocytes.

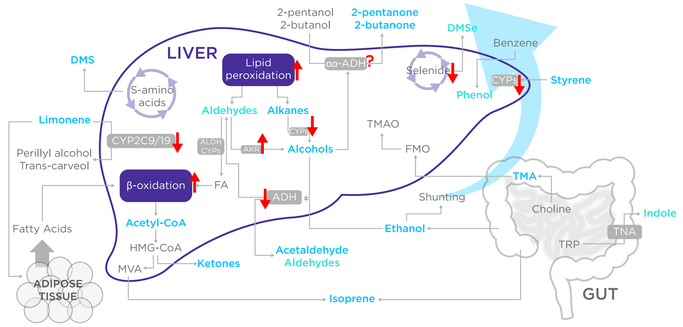

Figure 1: The complex network of established and proposed metabolic pathways from which VOCs originate from, and their alterations in chronic liver diseases.

2. Methods

STAGE 1: Sample preparation

Substrate	Enzyme	Bioproduct(s)
2-Butanol-D5	ADH	2-Butanone-D5
2-Pentanone-D5	ADH	2-Pentanol-D5
Limonene-D5	Unknown	Deuterated Terpenes
Nonanal-D18	AKR1B10	1-nonanol-D18, octane-D18
Benzyl alcohol-D7	ADH	Benzaldehyde-D6

Table 1. List of substrate and expected bioproducts.

- **Sample - substrates and hepatocytes.** Sample (with PHH) 10ng/µL of labelled EVOCs mixture (Methanol: 0.02%) exposed to 1x10⁶ PHH cells/mL
- **Control 1 - substrates only.** Control (without PHH) 10ng/µL of labelled EVOCs (Methanol: 0.02%) in media
- **Control 2 - vehicle and hepatocytes.** Control (Vehicle) Methanol exposed to 1x10⁶ PHH cells/mL
- 4-methylpyrazole: 10mM (ADH inhibitor) 10ng/µL of labelled EVOCs mixture (Methanol: 0.02%) exposed to 1x10⁶ PHH cells/mL
- Tolrestat 10mM: (AKR1B10 inhibitor) 10ng/µL of labelled EVOCs mixture (Methanol: 0.02%) exposed to 1x10⁶ PHH cells/mL

Assessment of cell viability via automated live/dead cell counting

STAGE 2: Headspace extraction

Figure 2. HiSorb vial and probe

EVOCs extraction using HiSorb agitators (37°C, 300 rpm, 2 hours)

Figure 3. HiSorb headspace agitator

STAGE 3: TD-GC-MS analysis

Thermal Desorption (Markes) coupled with Gas Chromatography Mass Spectrometry Orbitrap (Exploris, Thermo): Samples analysed with Stabilwax-DA 30m, 0.25mm, 0.25µm, MS scan mode 30-450m/z.

Figure 4. TD-GC-MS

Targeted analysis performed using Chromeleon software (Version 7.2.10). Areas (counts per min.) of the Quantitation channel of each VOC were integrated based on the 20ppm m/z error limit.

Untargeted analysis performed using Compound discoverer 3.3: 2095 Molecular Features (MFs) were detected and submitted for multivariate statistical analysis.

STAGE 4: MULTIVARIATE ANALYSIS AND EVOCS IDENTIFICATION

The strategy to identify potential bioproducts was performed as follows:

Step 1: Compare substrates + Hepatocytes and Substrates only by performing a t-test, then select features that have a Benjamini-Hochberg (BH) adjusted p<0.05, and a fold change > 2.

Step 2: Compare substrates + Hepatocytes and Vehicle + Hepatocytes by performing a t-test, then select features that have a BH adjusted p<0.05, and a fold change > 2.

Step 3: Filter the features that have been identified in both step 1 and step 2: these are expected to be bioproducts.

Step 4: Identifiation was performed using accurate mass formula annotation and chemical structure prediction and when possible, with the use of co-chromatography.

3. Results

- Viability of PHH was ~80% before and after the incubation in experimental and control conditions.
- Targeted analysis revealed conversion of substrates into bio-products in the presence of PHH, but not in control conditions where PHH or substrates were absent (apart from limonene-D5). Co-treatment with 4-methylpyrazole: (ADH inhibitor) reduced conversion of benzyl alcohol-D7 to benzylaldehyde-D6.
- Untargeted analysis detected 2095 extracted MF of which 3 MF showed similar spectra to <6C deuterated organic acids and 3 deuterated terpenes. 1 MF was identified as non-deuterated 3-octanone.

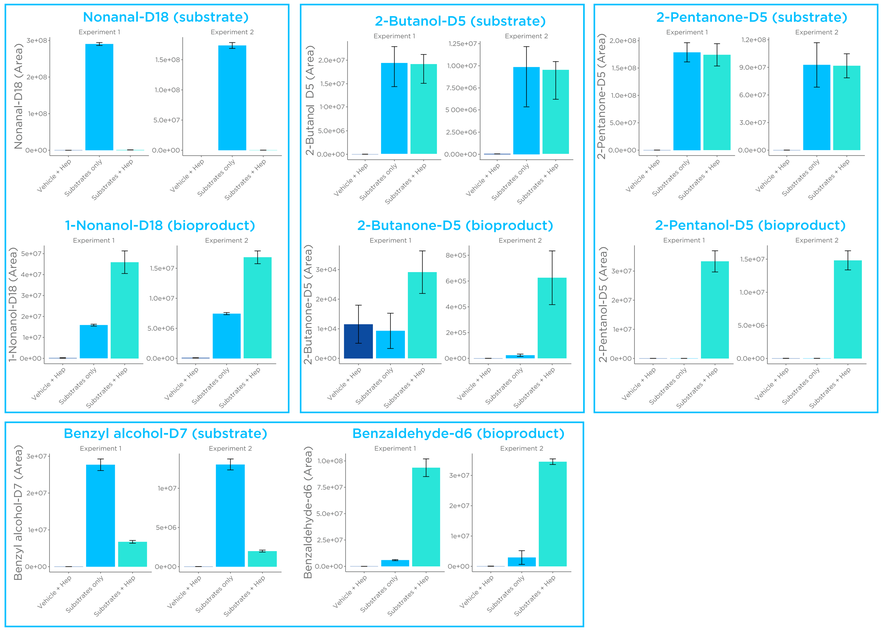

Figure 5: Bar plots of the targeted analysis, showing the area (counts per min.) of the targeted compounds in each sample condition.

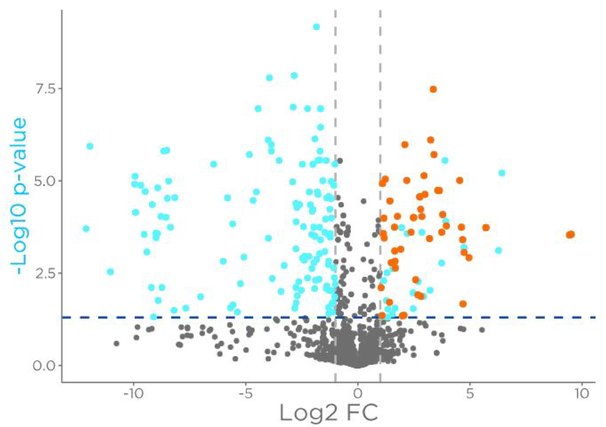

Figure 6: Comparison of substrates + HEP vs. substrates only, represented in a volcano plot.

The X-axis represents the log2 mean ratio fold-change (FC) of the relative abundance of each VOC between the compared conditions.

The Y-axis represents the adjusted p-value of each VOC. Compounds with a fold-change >2 and p<0.05 are highlighted in blue. Orange circles = features upregulated in substrates + HEP vs. Vehicle + HEP representing potential EVOC bioproducts.

MF	RT	m/z	Formula	ΔPPM	Identification
1	7.3	86.07259	C5H10O	1.24	3-octanone
1	7.3	57.033611	C3H5O	2.10	3-octanone
1	7.3	43.054279	C3H7	1.21	3-octanone
1	7.3	99.080406	C6H11O	0.36	3-octanone
2	8.4	44.02425	C2H2DO	2.99	Deuterated Organic acid 1
2	8.4	77.05505	C3H3D3O2	0.09	Deuterated Organic acid 1
2	8.4	91.06910	C4H3D4O2	0.69	Deuterated Organic acid 1
2	8.4	58.036755	C3D3O	1.43	Deuterated Organic acid 1
3	9.1	99.11063	C7H7D4	0.54	Aromatic deuterated compound 1
3	9.1	157.151	C10H11D5O	0.22	Aromatic deuterated compound 1
3	9.1	142.1275	C9H8D5O	0.51	Aromatic deuterated compound 1
3	9.1	69.06683	C5H5D2	0.76	Aromatic deuterated compound 1
4	9.4	58.03676	C3D3O	1.43	Deuterated Organic acid 2
4	9.4	139.153854	C7H3D10O2	0.57	Deuterated Organic acid 2
4	9.4	44.02425	C2H2DO	2.99	Deuterated Organic acid 2
4	9.4	91.0691	C4H3D4O2	0.69	Deuterated Organic acid 2
5	9.7	73.06014	C4H3D3O	0.09	Deuterated Organic acid 3
5	9.7	77.053558	C3HD4O	0.58	Deuterated Organic acid 3
5	9.7	64.047333	C2H2D3O2	1.51	Deuterated Organic acid 3
5	9.7	91.070724	C4H5D3O2	0.14	Deuterated Organic acid 3
6	11.611	80.05745	C6H2D3	0.53	Aromatic deuterated compound 2
6	11.611	92.05759	C7H2D3	1.98	Aromatic deuterated compound 2
6	11.625	92.05755	C7H2D3	1.55	Aromatic deuterated compound 2
6	11.627	114.09453	C7H2D6O	0.85	Aromatic deuterated compound 2
7	12.0	109.0648	C8H5D4	0.09	Aromatic deuterated compound 3
7	12.0	139.1405	C10H9D5	0.73	Aromatic deuterated compound 3
7	12.0	107.0825	C8H7D2	0.33	Aromatic deuterated compound 3
10	12.0	97.0617	C6H5D2O	0.04	Aromatic deuterated compound 3

Table 2: Results from the identification of untargeted features that were statistically increased in PHH + substrate samples (ps≤0.05) compared to vehicle and control samples.

4. Conclusions

All these results combined show that the hepatic metabolism of targeted and untargeted compounds can be evaluated *in vitro* using PHH and headspace analysis. Furthermore, mass spectrometry is an ideal platform for the analysis of deuterated compounds and for potentially identifying new disease-associated VOCs.

COI Disclosure: The authors declare no competing financial interest.