Sample preparation and metabolite extraction:

Repeat this procedure for each sample.

1. Take 250µl of the yeast YPD broth medium and place it in Eppendorf tubes which already contain 750µl very cold (- 80oC) HPLC-grade methanol. (Keep it in case you wish to analyse extracellular metabolites).
2. Aspirate the medium completely.
3. Pour 10ml (adjust volume as per the size of dish/ plate) of 10 mM Ammonium acetate (all over the petri dish, wash cells carefully and gently and then discard the washing solution.
4. Put the plates on dry ice and add 4 ml of 80% (vol/vol) methanol (or Methanol: acetonitrile: water; 4:4:2) (cooled to - 80°C or on dry ice or liquid nitrogen). (adjust volume as per the size of dish/ plate)
5. Incubate the plate at - 80 °C for 20 min. Remove cell plate and keep it on dry ice.
6. Scrape the plates on dry ice with cell scraper.
7. Transfer the cell lysate/methanol mixture to a 15 ml conical tube (or 1.5 ml Eppendorf depending on volumes) on dry ice.
8. Vortex for 5 min at maximum speed and make sure that pellet disintegrates and mixed thoroughly with extraction solvent.
9. Sonicate (in Ultrasonic Bath) the sample tube for 5 min (Note: Put some ice in sonicator water bath to avoid heating of sample during sonication) and vortex briefly after sonication.
10. Centrifuge the tube at 14,000g for 10 min at 4–8 °C to pellet the cell debris.
11. Transfer the metabolite-containing supernatant to a new 15-ml conical tube (or 1.5 ml eppendorf) on dry ice.
12. Spin the tubes at 14,000g for 10 min at 4–8 °C.
13. Transfer the supernatant to a tube on dry ice
14. SpeedVac/lyophilize or dry under nitrogen gas to a pellet using no heat.
15. Submit dried sample in 1.5 ml Eppendorf tube and can be stored at in dried ice. (EMBL, 2017)

Blank negative control: prepare processed blank sample using the same procedure but without a biological sample (use water or buffer instead).

Liquid Chromatography.

This protocol is to be conducted for each sample.

HPLC separations were carried out on three different columns on reverse phase mode for 9.5 min.

1. For amino acids and nucleic bases separation use a Luna Phenyl-Hexyl column (1.0 × 50 mm, particle size 5 µm).
2. Add to the mobile phases used 0.1% acetic acid in H_2O (A) and acetonitrile (B).

3. Start the 9.5-min cycle time starts from 0% B at 0.1 mL/min for 1 min, to a linear increase to 100% B in 0.5 min at 0.1 mL/min, hold at 100% B for 4 min at 0.2 mL/min, and then decrease to and hold at 0% B for 4 min at 0.1 mL/min.
4. For the separation of nucelotides, sugars, sugar phosphates and sugar alchols use an Atlantis T3 OBD column (1.0 × 50 mm, particle size 5 µm).
5. Add to the mobile phase 1% hexafluoroisopropanol (HFIP), 0.015% ammonium hydroxide in water (A) and acetonitrile (B).
6. Start the 9.5-min cycle from 0% B at 0.1 mL/min for 4.3 min, to a linear increase to 100% B in 0.4 min at 0.1 mL/min, hold at 100% B for 3.9 min at 0.15 mL/min, and then decrease to and hold at 0% B for 0.8 min at 0.1 mL/min.
7. For the separation of most organic acids and some nucletotides use a Synergi Polar-RP, 80 Å column (1.0 × 50 mm, particle size 4 µm)
8. Add to the mobile phase 5 mM ammonium acetate in 5/95 acetonitrile/water (A) and 95/5 acetonitrile/water (B).
9. Start the 9.5-min cycle from 95% B at 0.1 mL/min for 3 min, 0% B for 3.9 min at 0.1 mL/min, to a linear increase to 100% B in 0.5 min at 0.15 mL/min. (Wei, Li, & Seymour, 2010)

Mass Spectrometry with electrospray ionisation.

1. Use automatic flow for the ESI. Automation can be used to control tuning in flow injection mode.
2. Use 0.1% formic acid in 50/50 acetonitrile/water (v/ v); flowing at 30 µL/min to deliver compounds into ESI source for both positive and negative tuning.
3. Set Ion Spray (IS) potential for 5000 V in positive mode and 4500 V for negative mode. Use nebulizer gas (GS1) and bath gas (GS2) at 5 psi, curtain gas (CUR) at 12 psi, and collision gas (CAD) at 5 psi. Set source temperature (TEM) to zero and Interface Heater ON. (Wei et al., 2010)

Analysis:

1. Raw data acquisition
- Received from the studies.

2. Generation of a referential database
-Referential database is one containing information on the nominal and exact mass, chemical formula, retention time and a precursor and product m/z values of the metabolites and pathways of interest. In this case, it would the alcohol metabolism pathway. This can be found in previous biochemical studies or biochemical databases.

3. Isolation and identification of metabolites
-Targeted metabolomic studies use LC-MS vendor software e.g., Masslynx, Xcalibur, Analyst, Compass, MassHunter and Chemstation for both isolation and identification of compounds, with the support of the referential database.

4. Data normalization and quantification
-The aim of normalization is to remove confounding variations attributed to experimental sources (e.g. analytical noise or experimental bias) in ion intensities among measurements while preserving the relevant variation (due to biological treatments such as differing alcohol concentrations and gene variants). Student's t-test, analysis of variance, and non-parametric tests like Kruskal-Wallis test can be used to evaluate the significance of the peaks.

5. Statistical analysis steps all-in-one: tools for automated processing
- Use of software such as MRMPROBS, metabolite mass spectrometry analysis tool (MMSAT) and OpenChrom. This program provides a process pipeline from the raw-format import to high-dimensional statistical analysis.

6. Biochemical interpretation.
- This driven by an initial biological hypothesis, final interpretation is usually reduced to confirmation of the predicted alterations. Use of databases such as KEGG to integrate an understanding of pathways back to biology. I predict that the KO and ALD2*2 gene conditions will not processes alcohols well increasing the levels of 4-HNE, suggesting increased levels of cell stress and possibly increased risk of CVD. (Gorrochategui, Jaumot, Lacorte, & Tauler, 2016)

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Additional references for LC-MS video:

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