

## Qualitative and Quantitative Characterization of the Metabolome, Lipidome, and Proteome of Human Hepatocytes Stably Transfected with Cytochrome P450 2E1 Using Data Independent LC-MS

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### Abstract

This application note describes a label-free multi-omics approach that has been applied for the analysis of the transfected human hepatocyte cells by implementing LC-HDMS<sup>E</sup> (LC-DIA-IM-MS), providing both qualitative and quantitative information within a single experiment.

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### Introduction

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Drug toxicity is a major reason for the failure of candidate pharmaceuticals during their development. Therefore, it is important to realize the potential for toxicity in a timely fashion. Many xenobiotics are bioactivated into toxic metabolites by cytochromes P450 (CYP), as shown in Figure 1. However, the activity of these enzymes typically falls in *in vitro* systems. Recently, a transformed human hepatocyte cell line (THLE) became available, where the metabolic activity of specific CYP isoforms is maintained. THLE cells could be an ideal system to examine the potential toxicity of candidate pharmaceuticals. The baseline effect of the addition of CYP2E1 gene, which encodes a member of the cytochrome P450 superfamily of enzymes into THLE hepatocytes, has been characterized to better understand the biochemistry of this model system. In this application note, a label-free multi-omics approach has been applied for the analysis of the transfected human hepatocyte cell by implementing LC-HDMS<sup>E</sup> (LC-DIA-IM-MS), providing both qualitative and quantitative information within a single experiment.

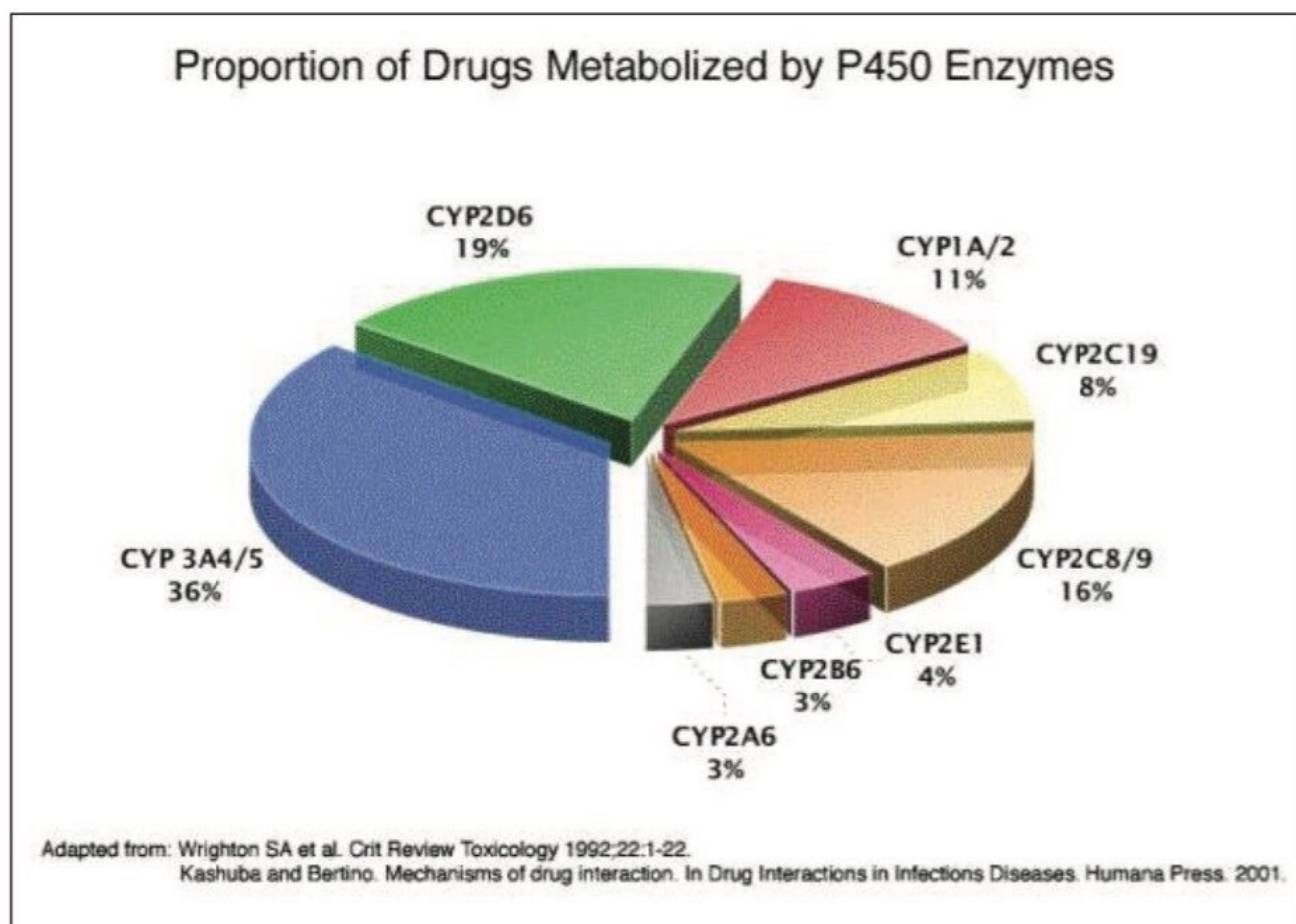


Figure 1. CYPs are the major enzymes involved in drug metabolism, accounting for about 75% of the total metabolism. Most drugs undergo deactivation by CYPs, either directly, or by facilitated excretion from the body.

## Experimental

### Sample preparation

Dedicated and independent sample preparation protocols were applied in order to isolate metabolites, lipids, or proteins, as shown in Figure 2. Three independent replicates of THLE null or THLE+2E1 cells were investigated for all analyte classes. Proteins were recovered and digested with trypsin overnight.

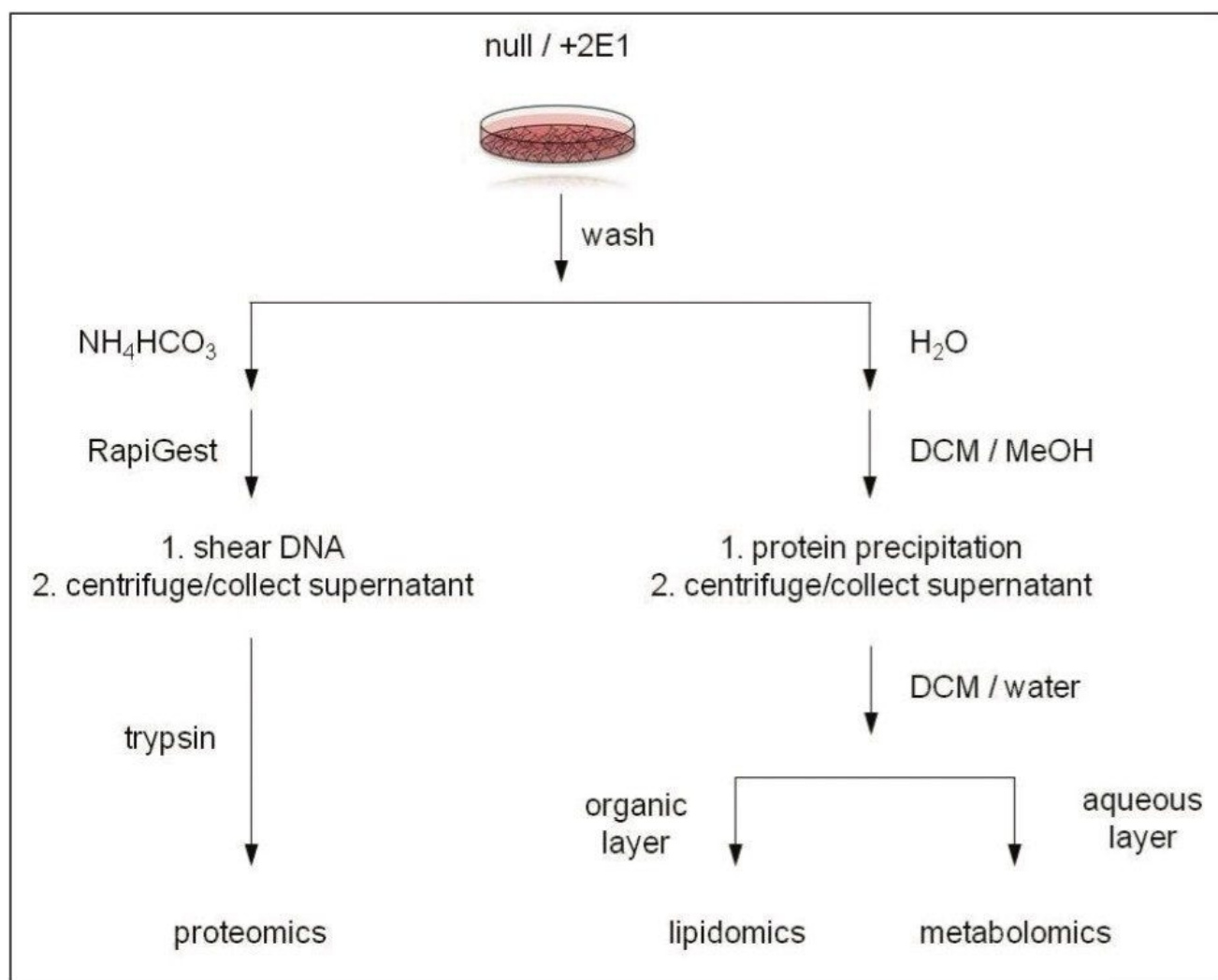
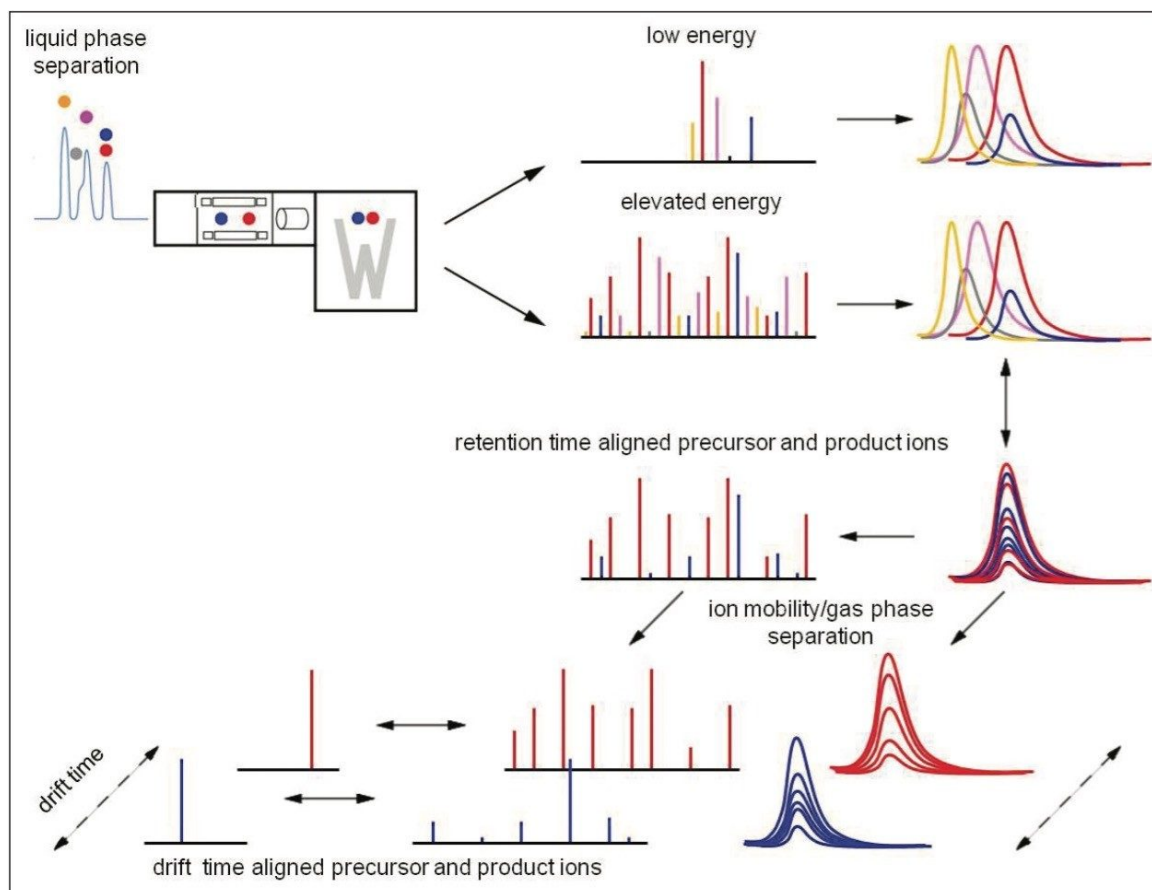


Figure 2. Sample preparation protocol cytochrome P450 2E1 THLE cells for integrated proteomics, lipidomics, and metabolomics LC-MS experiments/studies.

## LC-MS conditions

Waters Omics Research Platform Solution with TransOmics Informatics powered by Nonlinear Dynamics was used for all experiments; generic application-dependent LC conditions were applied throughout. In all instances, MS data were acquired using a data independent analysis (DIA) approach, MS<sup>E</sup>, where the energy applied to the collision cell was switched between a low and elevated energy state during alternate scans. For the proteomics experiments, ion mobility separation (IM) was incorporated into the analytical schema (IM-DIA), HDMS<sup>E</sup>. The principle of an HDMS<sup>E</sup> acquisition is shown in Figure 3. Precursor and product ions were associated using

dedicated algorithms by retention and/or drift time alignment. For structural elucidation, supplementary MS/MS experiments were conducted for the metabolomics and lipidomics studies.



*Figure 3. Retention and drift time principle ion mobility enabled data-independent LC-HDMS<sup>E</sup> analysis (LC-DIA-IM-MS).*

Label-free LC-MS was used for qualitative and quantitative peptide/protein analyses. Experiments were conducted using a 90 min gradient from 5% to 40% acetonitrile (0.1% formic acid) at 300 nL/min, using a nanoACQUITY UPLC System and an ACQUITY UPLC BEH 1.7  $\mu\text{m}$  C<sub>18</sub> reversed phase 75  $\mu\text{m}$  x 20 cm nanoscale LC Column.

For metabolite identification, the LC-MS experiments consisted of a 10 min gradient from 0% to 50% acetonitrile (0.1% formic acid) at 500  $\mu\text{L}/\text{min}$ , using an ACQUITY UPLC System. Here, an ACQUITY UPLC BEH 1.7  $\mu\text{m}$  C<sub>18</sub> reversed phase 2.1 x 10 cm LC Column was used.

The lipid separations were conducted with a CSH (Charged Surface Hybrid) C<sub>18</sub>, 1.7 μm, 2.1 x 100 mm Column, also connected to an ACQUITY UPLC System. Mobile phase A consisted of 10 mM NH<sub>4</sub>HCO<sub>2</sub> in ACN/H<sub>2</sub>O (60/40); and mobile phase B of 10 mM NH<sub>4</sub>HCO<sub>2</sub> in IPA/ACN (90/10). The initial composition of the gradient was 40% B, which was stepped from 43% to 54% B from 2 to 12 min, followed by an additional gradient step from 70% to 99% from 12.1 to 18.0 min. The column flow rate was 400 μL/min and the column temperature maintained at 55 °C.

## Data Acquisition

Data were acquired through data independent analysis (DIA) that utilized a nanoACQUITY UPLC or ACQUITY UPLC System directly interfaced to a hybrid IM-*oa*ToF SYNAPT G2 Mass Spectrometer.

## Bioinformatics

The LC-MS peptide data were processed and searched with ProteinLynx GlobalSERVER v.3.0. Normalized label-free quantification was achieved using TransOmics Informatics Software and additional statistical analysis conducted with Spotfire and EZinfo. The resulting metabolomic and lipidomic data were processed using either MarkerLynx Application Manager or TransOmics Informatics Software, and complementary statistical analysis was conducted with EZinfo.

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## Results and Discussion

Small amounts of the isolated and purified samples were LC-MS analyzed to identify, quantify, and investigate the metabolomic, lipidomic, and proteomic variances between THLE null (CYP2E1 gene absent) and THLE+2E1 cells. Figure 4 shows the type of chromatographic profiles that were typically obtained for the samples, providing chromatographic definition for downstream analysis of the various data streams. In a similar fashion, spectral profiles were obtained for all sample types and in the instance of the proteomics datasets, ion mobility profiles were obtained as well.

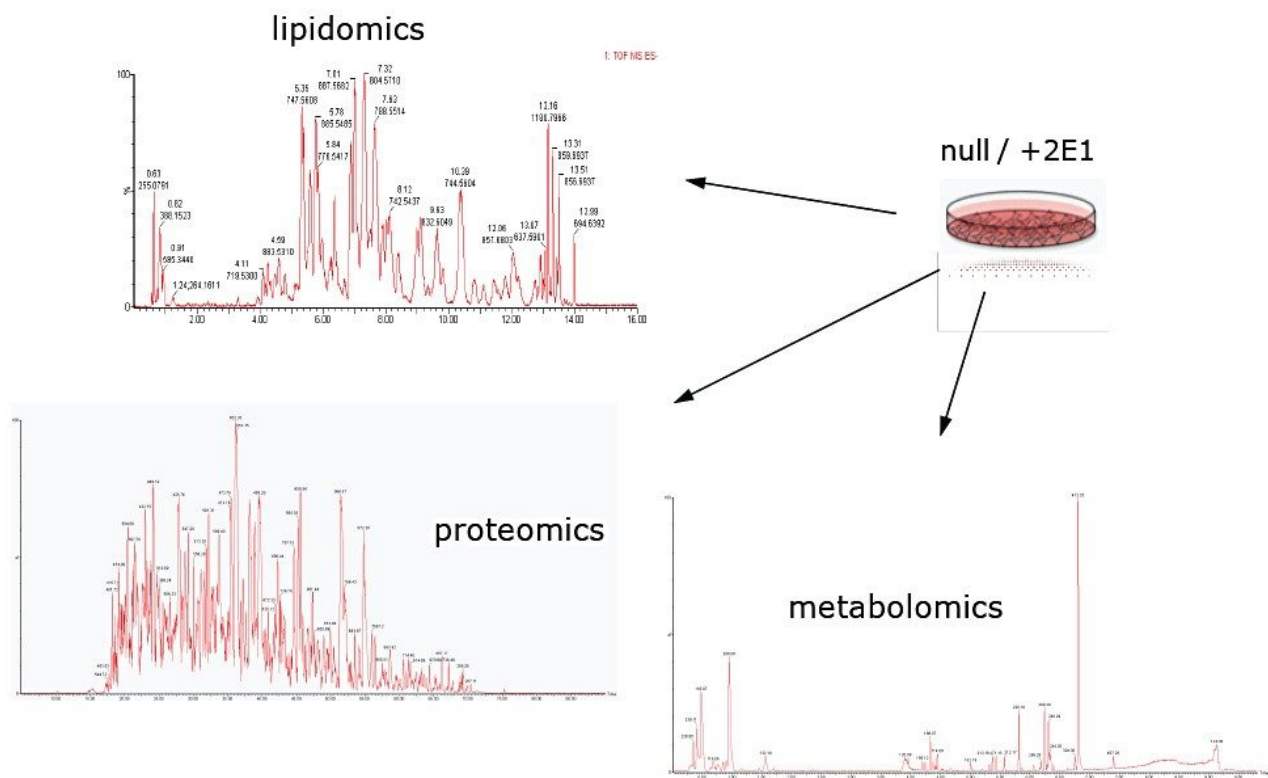


Figure 4. Chromatographic example profiles THLE+2E1 cells. The lipidomics experiments were conducted in ESI(-) mode, whereas the metabolomics and proteomics experiments were carried out in ESI(+) mode.

Principal component analysis (PCA) was used in the first instance to identify and highlight significant differences between THLE null and THLE+2E1 cells; an example is shown in Figure 5. For all experiments, good technical LC-MS measurement replication was observed, with slightly greater biological and/or sample preparation variation. The top pane of Figure 5 illustrates group level analysis of the metabolomics data using TransOmics Informatics Software, whereas the bottom pane of Figure 5 demonstrates analysis at the sample level using EZinfo. Further analysis of the data using MarkerLynx Application Manager indicates significant variance in the metabolic expression of guanine and heteropyrithiamine (data not shown). Similar clustering patterns were observed for the lipid, metabolite, and protein datasets.

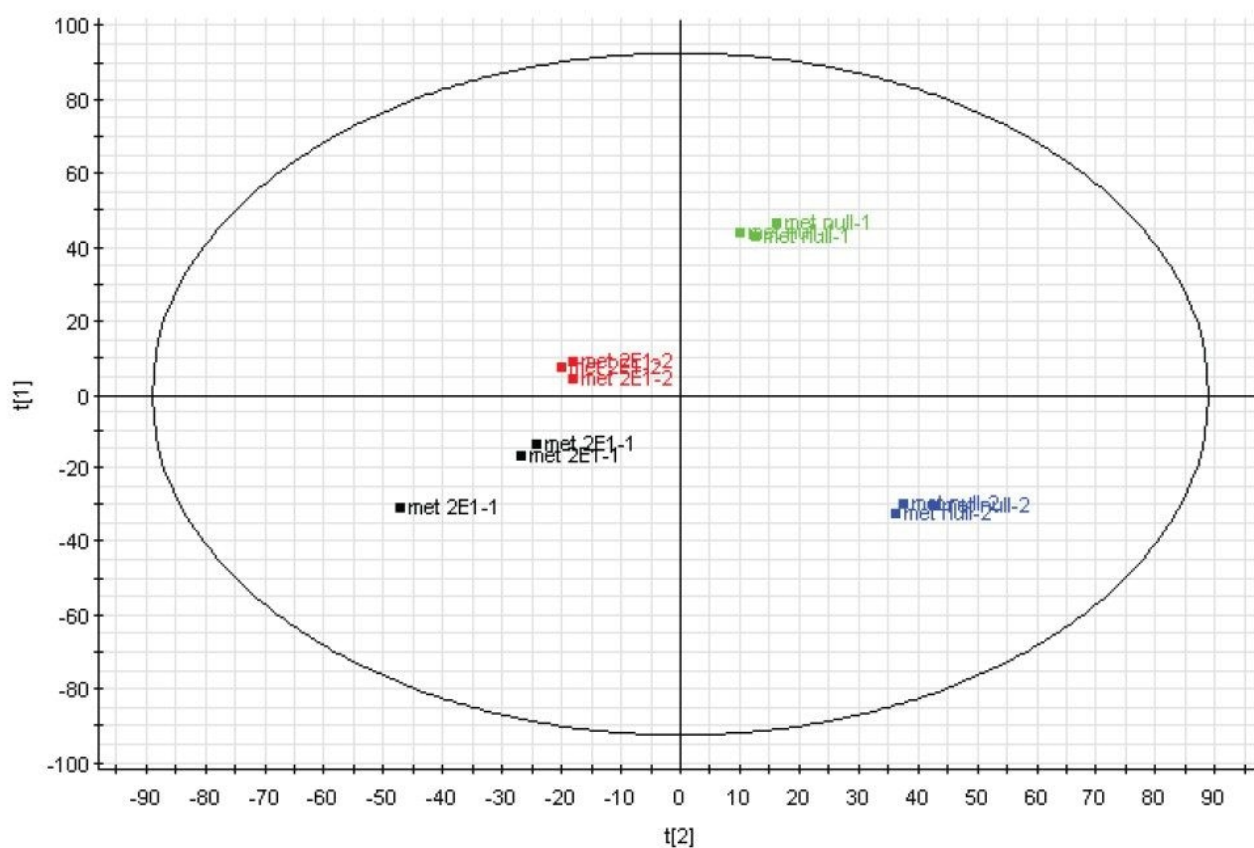
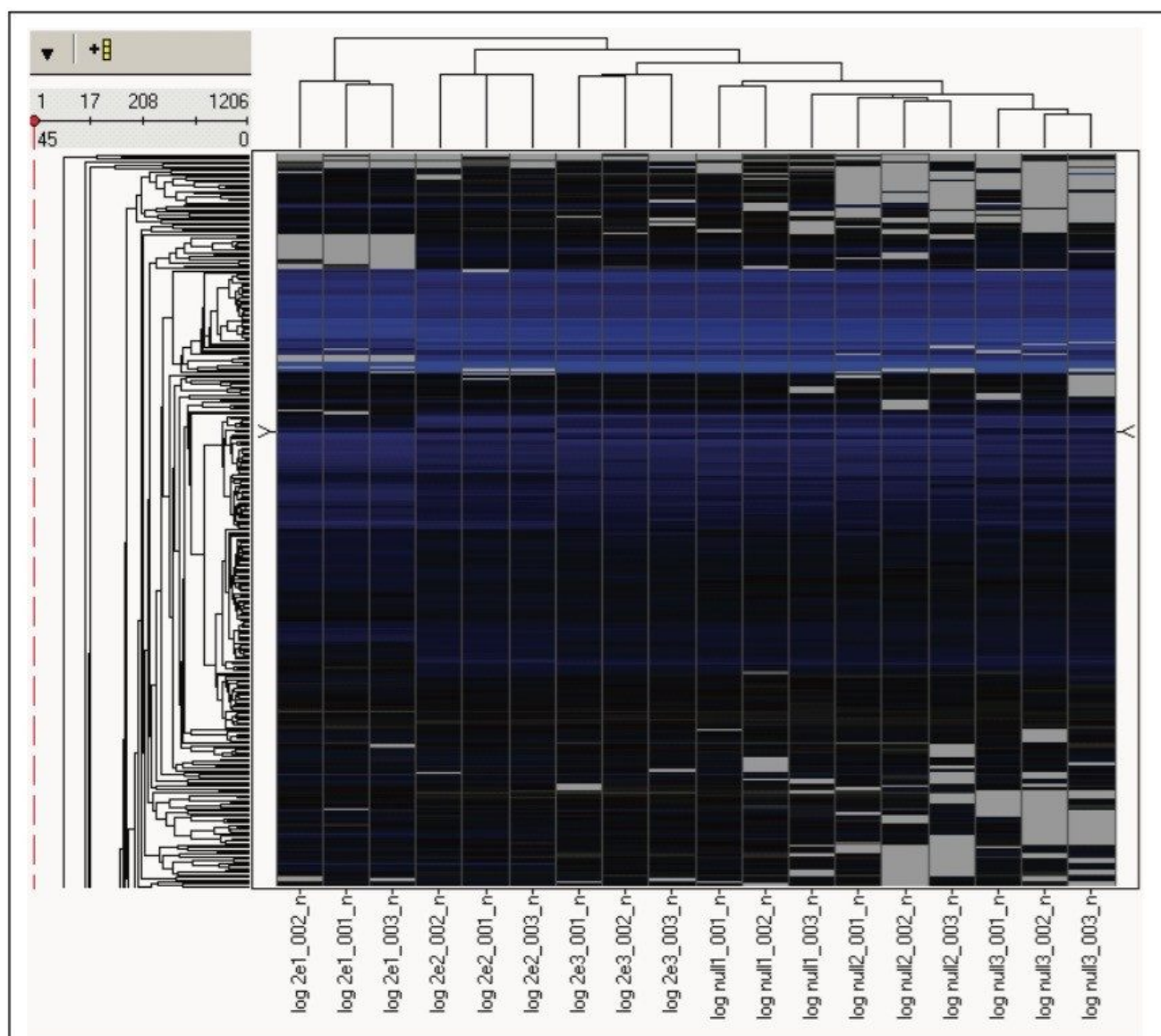


Figure 5. TransOmics Informatics Software (top) and EZinfo (bottom) principal component analysis of the metabolism data, illustrating THLE null versus THLE+2E1 group and sample level differences, respectively.

The estimated protein amounts were normalized and exported to facilitate additional statistical analysis at the protein level. First, hierarchical clustering was conducted, which revealed primary grouping at the technical level and secondary grouping at the sample level, as shown in Figure 6. Next, protein regulation values were calculated as a function of sample group level regulation probability. Only the proteins that were identified for which a regulation probability value could be expressed and found to be common to both samples; *i.e.* THLE null and THLE+2E1 were considered for protein/gene pathway analysis using Ingenuity IPA. The most significantly enriched canonical signalling pathways were EIF2, regulation of EIF4 and p70S6K, mTOR, Actin cytoskeleton, and ILK.

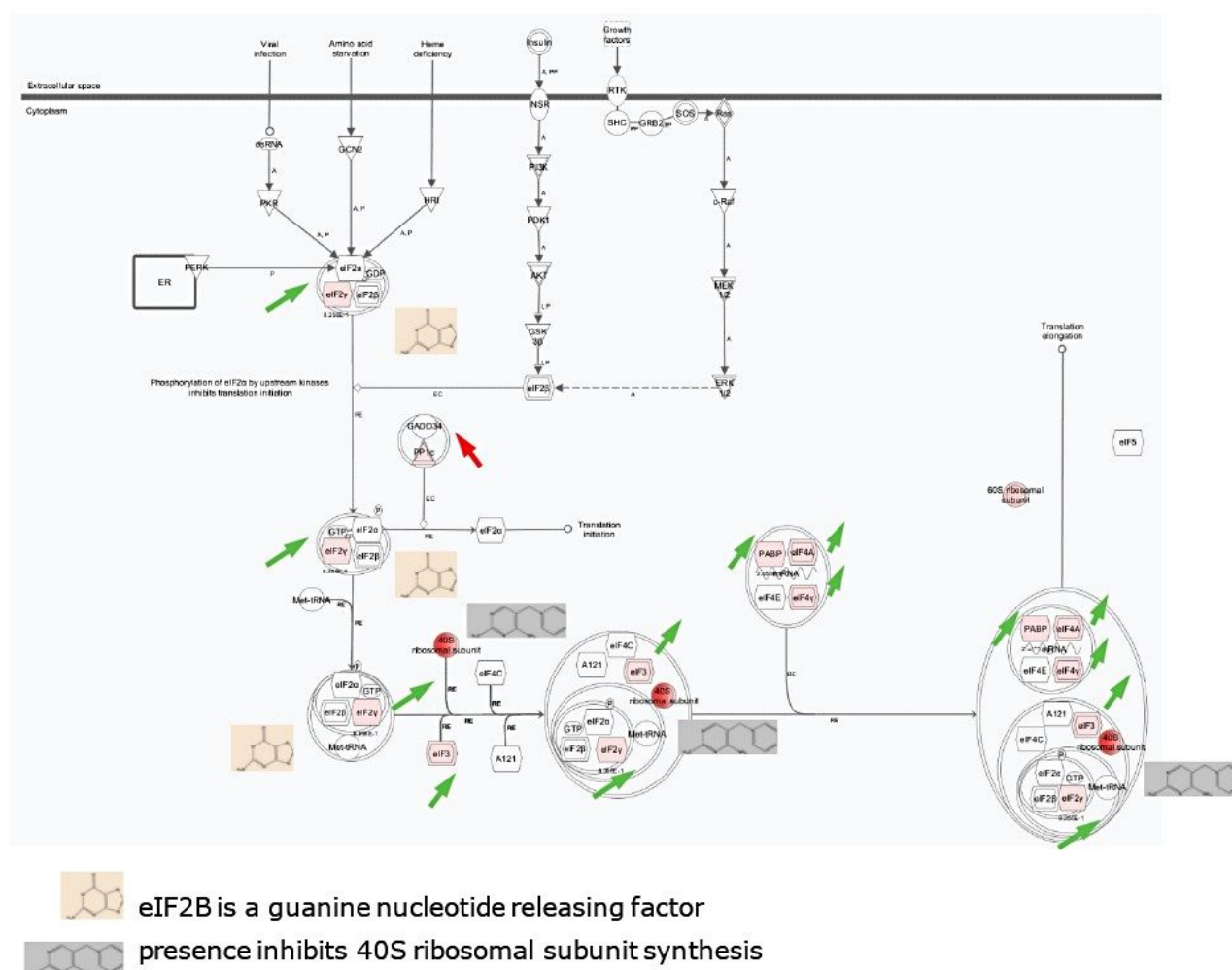




*Figure 6. Unsupervised hierarchical clustering protein data using TransOmics Informatics Software normalized, log-scaled estimated protein amounts as input values.*

The previously mentioned metabolites interplay with the proteins/genes in the EIF2 (eukaryotic initiation factor 2) signalling pathway, as shown in Figure 7. For example, EIF2B is a guanine nucleotide releasing factor required to release Guanosine diphosphate (GDP), so that a new Guanosine-5'-triphosphate (GTP) molecule can bind and activate EIF2. Moreover, the presence of thiamines is known to inhibit the synthesis of 40S ribosomal subunits. These observations are not unexpected since CYPs readily induce oxidative stress when no substrate is

available. Moreover, the EIF2 signalling pathway is one of the primary responders to cellular stress.



*Figure 7. EIF2 signalling pathway, which illustrates up-regulated genes/protein (groups) in green and down-regulated components in red. The size and direction (angle) of the arrows is a measure of the regulation value. The pathway is annotated with two components that were identified as part of the metabolomics track of the experiment/analysis.*

## Conclusion

A label-free multi-omics approach has been applied for the analysis of the transfected human hepatocyte cells by implementing LC/HDMS<sup>E</sup> (LC-DIA-IM-MS), providing both qualitative and quantitative information within a single experiment.

- Various clustering, statistical, and data analysis approaches show protein, lipid, and metabolite data to be complimentary.
- A variety of compounds were identified as contributing towards the metabolite and lipid variance.
- Approximately 20% of the proteins identified were significantly expressed with 10% of the proteins illustrating a p value  $\leq 0.05$  when common to both samples.
- Complementary information obtained from metabolite/lipid and protein analysis has been shown through the use of guanine and heteropyrithiamine within the EIF2 signaling pathway.

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