Analysis of Redox and Bioenergetics Metabolites with Polymeric iHILIC®-(P) Classic HILIC Column and Mass Spectrometry

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Aerobic organisms use oxygen as the terminal electron acceptor in cellular respiration. Molecular oxygen is capable of accepting up to four electrons to become water through the reaction catalyzed by mitochondrial complex IV (1). However, intermediate reactive oxygen species (ROS) are formed by partial reduction of oxygen. Aerobes have evolved both physiological and biochemical strategies to mitigate molecular damage caused by ROS while maintaining oxygen availability for cellular respiration (2). Core metabolism and redox control depend on a number of metabolites, including nucleotides ($NADP^+$/NADPH and NAD^+/NADH) and redox-active amino acids and peptides (particularly glutathione, cysteine, and methionine). For example, reduced glutathione (GSH; γ-L-glutamyl-L-cysteinylglycine) is an important regulator of local hydrogen peroxide signalling by serving as a cofactor of glutathione peroxidases (3). In turn, oxidized glutathione (GSSG) is reduced back to GSH in an NADPH-dependent fashion by GSSG reductase (4). Both cysteine and methionine in proteins may act as redox switches linked to NADPH-dependent repair mechanisms (5,6). NADPH is generated by the pentose phosphate pathway that links to glycolysis, which generates ATP and relies on NAD^+ as a cofactor. Multiple steps of the citric acid cycle generate NADH that supports chemiosmotic ATP synthesis.

Liquid chromatography–mass spectrometry (LC–MS) based metabolomics presents an opportunity to monitor many molecules simultaneously, relying on a combination of chromatographic and mass-based resolution to quantify hundreds to thousands of molecules in a typical experiment (7). However, many of the metabolites noted above could be challenging to profile simultaneously because of poor chromatographic separation or peak shape (8). We sought to develop a simple method using hydrophilic interaction liquid chromatography (HILIC) that could simultaneously profile the metabolites that are important in redox regulation and bioenergetics. By incorporating these metabolites into a nontargeted-compatible and derivatization-free approach, information about redox and bioenergetics can be gained as part of routine profiling.

Experimental

LC–MS System: A Vanquish Horizon binary pump was hyphenated to a Q Exactive HF (Thermo Fisher Scientific), set at 120,000 FWHM, 1e6 AGC target, and 200 ms max IT. Connecting tubings between the column, autosampler, and MS system were 100 µm i.d. PEEK-lined stainless steel MarvelXACT tubing (IDEX Health & Science) instead of original Viper MP35N. The HESI-II probe was held at 320 °C and +3.5 kV with 40 sheath gas, eight auxiliary gas, and one sweep gas flow (arbitrary units).

Column: 150 × 2.1 mm, 5-µm, 200 Å, iHILIC®-(P) Classic (P/N 160.152.0520, HILICON AB), coupled to a 20 mm guard column via a PEEK coupler.

Eluent: A) 15 mM ammonium acetate pH 9.4, B) acetonitrile.

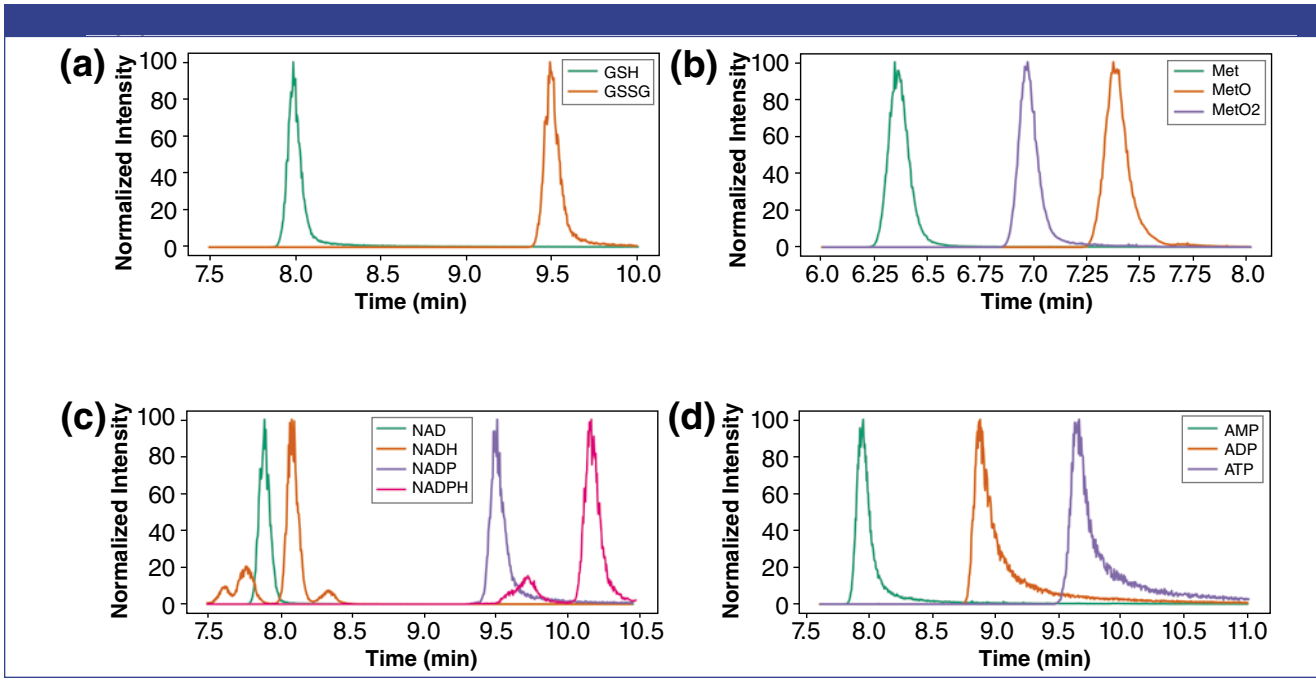

Figure 1: Base intensity-normalized chromatograms of (a) GSH/GSSG; (b) Met/MetO/$MetO_2$; (c) NAD^+/NADH/$NADP^+$/NADPH; (d) AMP/ADP/ATP.

Table 1: Peak areas of reduced and oxidized compounds from reduced standards

	REDUCED	OXIDIZED	OXIDIZED %
GSH	6.82×10^{7}	2.16×10^{4}	0.03
METHIONINE	2.87×10^{8}	5.89×10^{5}	0.21
NADH	2.76×10^{7}	ND	NA
NADPH	8.32×10^{6}	ND	NA

* Metabolites were quantified in positive mode as their $[M+H]^+$ ions with 5 ppm mass accuracy. The result is based on a semi-quantitative comparison of peak areas.

Gradient Elution: 0–15 min, gradient from 10–90% A; 15–17 min, 90% A; 17–25 min, 10% A (column re-equilibration)
Flow Rate: 200 µL/min
Column Temperature: 40 °C
Injection Volume: 2.5 µL

Metabolite Samples: Individual metabolites were prepared fresh from reference standards in water (50 µmol/L). We focused on the following 14 metabolites: oxidized/reduced nicotinamide adenine dinucleotide (NAD^+/NADH), oxidized/reduced nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH), adenosine mono-/di-/triphosphate (AMP/ADP/ATP), glutathione/glutathione disulfide (GSH/GSSG), methionine/methionine sulfoxide/methionine sulfone (Met/MetO/$MetO_2$), and cysteine/cystine (Cys/CySS). We also analyzed human plasma Standard Reference Material 1950 (MilliporeSigma) and metabolite extract of *E coli* (Cambridge Isotope Labs), which were either reconstituted with a solution of 1:1:1 acetonitrile–methanol–water + 8.33 µM D5-hippuric acid or extracted with addition of 2 vol 1:1 acetonitrile–methanol + 12.5 µM D5-hippuric acid followed by vortexing and incubation on ice for 30 min. Samples were then centrifuged at 20,000 *g* and 4 °C for 10 min. The supernatant was then applied for HILIC–MS analysis.

Results and Conclusion

The newly developed method provides excellent peak separation and overall good peak quality for all of the metabolites as shown in Figure 1.

We analyzed all of the reduced metabolite standards to determine if autoxidation was rapidly occurring (Table 1). Peak areas of most oxidized species from injections of reduced standards were <1% that of the reduced species at pH 9.4. However, in the case of the Cys standard, only oxidized CySS was detectible. When using neutral pH instead of 9.4, Cys was preserved and detected (though considerable autoxidation was still noted). Cys may be unstable at alkaline pH as a result of enhanced deprotonation to the nucleophilic thiolate form ($pK = 8.3$) (3).

We then determined whether the 12 metabolites shown in Figure 1 were well represented in standard reference materials for untargeted metabolomics experiments. Human plasma SRM 1950 and the unlabelled *E. coli* metabolite extracts were used as probes. Across the two matrices, signals for all metabolites except $NADP^+$ were observed (Table 2).

This work demonstrates the feasibility of profiling core metabolites related to redox metabolism and bioenergetics, which might be incorporated into a nontargeted workflow for more extensive profiling. We note that thiol metabolites are labile and nontargeted profiling of these should be experimentally validated with derivatization-based methods (9). A HILIC method with an acidic mobile phase may offer better stability for thiol compounds as well.

Table 2: Peak areas of metabolites in reference samples

	SRM 1950	*E. COLI*
GSH	1.06×10^{5}	9.89×10^{8}
GSSG	ND	8.92×10^{7}
METHIONINE	7.22×10^{7}	6.75×10^{7}
METO	5.75×10^{6}	1.48×10^{7}
$METO_2$	5.42×10^{5}	ND
NADH	ND	8.29×10^{4}
NAD^+	ND	1.42×10^{8}
NADPH	ND	2.01×10^{5}
$NADP^+$	ND	ND
ATP	ND	5.44×10^{6}
ADP	ND	1.46×10^{7}
AMP	ND	5.11×10^{7}

* Metabolites were quantified in positive mode as their $[M+H]^+$ ions with 5 ppm mass accuracy.

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