Improved workflow for mass spectrometry–based metabolomics analysis of the heart

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MS-based metabolomics methods are powerful techniques to map the complex and interconnected metabolic pathways of the heart; however, normalization of metabolite abundance to sample input in heart tissues remains a technical challenge. Herein, we describe an improved GC-MS–based metabolomics workflow that uses insoluble protein–derived glutamate for the normalization of metabolites within each sample and includes normalization to protein-derived amino acids to reduce biological variation and detect small metabolic changes. Moreover, glyce- gen is measured within the metabolomics workflow. We applied this workflow to study heart metabolism by first comparing two different methods of heart removal: the Langendorff heart method (reverse aortic perfusion) and in situ freezing of mouse heart with a modified tissue freeze-clamp approach. We then used the in situ freezing method to study the effects of acute β-adrenergic receptor stimulation (through isoproterenol (ISO) treatment) on heart metabolism. Using our workflow and within minutes, ISO reduced the levels of metabolites involved in glycogen metabolism, glycolysis, and the Krebs cycle, but the levels of pentose phosphate pathway metabolites and of many free amino acids remained unchanged. This observation was coupled to a 6-fold increase in phosphorylated adenosine nucleotide abundance. These results support the notion that ISO acutely accelerates oxidative metabolism of glucose to meet the ATP demand required to support increased heart rate and cardiac output. In summary, our MS-based metabolomics workflow enables improved quantification of cardiac metabolites and may also be compatible with other methods such as LC or capillary electrophoresis.

The beating heart sustains mammalian life by circulating blood through every organ to supply oxygen, nutrients, and heat distribution. The heart is the biggest consumer of ATP in the body (1), hydrolyzing ~20 times its mass in ATP each day (2, 3). Maintaining ATP production under environmental stress is crucial for normal cardiac homeostasis; deviations in metabolism can have deleterious consequences ranging from cardiomyopathy (4) to heart failure (5). To support metabolic demands, the heart uses multiple substrates including carbohydrates (6, 7), lipids (6, 7), glycogen (8), and amino acids (9, 10) as energy sources. However, precisely how cardiac metabolic networks are dynamically regulated to maintain cardiac contraction remains a key question in the field.

Targeted metabolomics approaches allow the simultaneous detection of hundreds of metabolites covering all major metabolic pathways (11, 12). Modern metabolomics techniques combine chromatographic separation with high resolution MS to allow two-dimensional assignment of metabolites with high sensitivity, accuracy, and confidence. For example, Dr. Oliver Fiehn established a library of over 1000 metabolites that can be detected using GC-MS, which has been adapted for the routine interrogation of many biological systems and is commercially available through Agilent Technologies (13, 14). Using targeted metabolomics for biomarker discovery (15), analysis of perturbed metabolic network (16), and inborn error of metabolism (17, 18) has been demonstrated extensively in cancer research (19, 20) and is beginning to be adapted for cardiovascular research (21–23).

The heart requires a constant supply of energy to sustain contractile function. Understanding how production of ATP is regulated is critical to understand how metabolic flexibility is used to meet daily physiological demand and how alterations in energy metabolism contribute to the severity of cardiac disease. One drawback in the metabolic profiling of the heart is the lack of a uniform and accurate way to normalize metabolite abundance against sample input. As a result, current studies require large samples sizes (n ≥15) to reliably detect changes in metabolite abundance in animal models (16, 24, 25). Because small changes in individual metabolite levels can lead to pathological consequences caused by feedback loops intrinsic to the metabolome of the heart, an improved metabolomics workflow is needed to reduce the coefficient of variance and to increase statistical power in sample cohorts. We hypothesize that...
greater precision can be achieved through a refined method of normalization against sample input.

Herein, we describe an improved workflow for metabolomic analysis of heart samples. After extraction of polar metabolites, we performed mild hydrolysis of the insoluble fraction that contains proteins and glycogen to their monomeric forms, i.e., amino acids (26) and glucose (27). The hydrolyzed insoluble material is recorded on the GC-MS and is used for the normalization of polar metabolites within the same sample. We applied this workflow to study metabolic perturbations of three traditional heart isolation techniques: first, direct in situ freeze-clamping (direct) using a modified tissue clamp prechilled in liquid nitrogen \( (\text{LN}_2) \); second, the Langendorff hung heart method (referred to herein as hung) with aortic cannulation for reverse perfusion of the coronary artery; and third, traditional heart excision following PBS wash and snap freezing in \( \text{LN}_2 \) (rinse). We then proceeded to use the in situ freeze-clamp method to examine the acute metabolic responses of the heart to \( \beta \)-adrenergic stimulation.

**Results**

**Normalization to protein-derived glutamate reduces biological variance**

Current methods of metabolomics data normalization include the use of wet weight (28), dry weight (29), or protein concentration from bicinchoninic acid (BCA) analysis (30). All three methods are sensitive to intrinsic biological and technical variation and offer only modest improvement when assessed by unsupervised multivariate analysis (31, 32). We hypothesized that accurate GC-MS measurement of amino acids released by mild hydrolysis of the insoluble fraction from each sample would significantly reduce biological variance.

To optimize mild hydrolysis conditions used within the heart metabolomics workflow, a panel of 18 amino acid standards were tested for stability under three distinct hydrolysis conditions: \( 2 \text{ N} \text{ HCl for 2 h} \), \( 6 \text{ N} \text{ HCl:12 N TFA for 2 h} \), and overnight incubation in \( 6 \text{ N} \text{ HCl} \). All three methods resulted in protein hydrolysis and were suitable for amino acid analysis. However, when used with heart tissue, only \( 2 \text{ N} \text{ HCl} \) preserved glycogen-derived glucose (Fig. 1A and B, and Table S1).

We next examined whether \( 2 \text{ N} \text{ HCl} \) hydrolysis coupled with GC-MS or BCA analysis generated a more accurate measure of biomass (Fig. 1C). Although both methods provided accurate determination of protein concentration from in vitro HEK293 cells (Fig. 1C), only measurement of protein-derived glutamate by GC-MS provided an accurate measure of heart tissue weight. In particular, BCA proved unreliable when using 10–50 mg of heart tissue, an optimal range for metabolomics analysis (Fig. 1C).

Metabolite extraction from whole heart tissue is a challenging task because of a tissue matrix that prevents an even penetration of solvents. Here, we adapted the new method of \( \text{LN}_2 \)-assisted magnetic pulverization, resulting in heart tissue milled to 5-\( \mu \)m particles, allowing for efficient solvent extraction (28). We have employed this cryomilling method previously and shown robust reproducibility during metabolite extraction from whole tissue (Fig. 2A) (37, 38).

To test this workflow, 20 mg (tissue weight) of cryomilled heart tissue powder were extracted for polar metabolites using 50% ice-cold methanol (Fig. 2). The resulting insoluble fraction, which includes proteins and glycogen, was hydrolyzed to amino acids and glucose. Both polar and hydrolyzed insoluble fractions were derivatized by \( N \)-methyl-\( N \)-trimethylsilyl trifluoroacetamide, and free metabolites were separated and recorded using an Agilent 7800B GC coupled to a 5977B MS detector (39, 40) (Fig. 2A). Protein-derived glutamate from GC-MS analysis was used for the final normalization for each sample, and this method compared with the traditional method of normalization to tissue weight. Using the coefficient of variance (COV) as a measure of relative biological variation (41), metabolites normalized to protein-derived glutamate show up to a 70%
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A

Polar Metabolite extraction → Extract → Immunoaffinity → Polar fraction

MSTFA derivatization + GCMS analysis → Insoluble fraction

Acid hydrolysis → Glucose, Amino acids → Glycogen, Protein

B

Log_{10}(Relative abundance)

<table>
<thead>
<tr>
<th>COV-1</th>
<th>Lle</th>
<th>Thr</th>
<th>Pro</th>
<th>Gly</th>
<th>Suc</th>
<th>Fum</th>
<th>Asp</th>
<th>B-Ala</th>
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<tr>
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<td>0.52</td>
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<tr>
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<td>0.14</td>
<td>0.20</td>
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<td>0.27</td>
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<td>0.21</td>
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<td>0.42</td>
<td>0.39</td>
</tr>
</tbody>
</table>

C

Peak assignment

/Norvaline/W.W

/Norvaline/P/G

Treated

Control

Outlier
reduction in COV compared with metabolites normalized to tissue weight (Fig. 2B). We then performed double-blinded principle component analysis (31) using the metabolomics data presented in Table S2 either normalized to tissue weight or protein-derived glutamate. We observed distinct separation between two different groups in the data set normalized to protein-derived glutamate. By contrast, normalization to tissue weight improved clustering when compared with only using peak assignment data; group separation remained ambiguous (Fig. 2C).

**Different methods of heart extraction result in unique metabolic profiles**

The myocardium is metabolically resilient and rapidly switches among carbon substrates upon varying environmental stimuli such as stress (42), hypoxia (43), trauma (44), and anesthesis (45), all of which are conditions that present as variables during tissue harvest from laboratory mice. Metabolic enzymes remain active post-mortem (46–48), and metabolic changes can occur in milliseconds (49). Rapid preservation of tissue that represents the accurate metabolome remains a challenging task for the metabolomics workflow. Trauma and stress pre-euthanasia can induce metabolic changes in mice prior to tissue collection. Furthermore, delayed tissue freezing can induce an ischemic-like phenotype arising from a lack of oxygen to the harvested tissue (50, 51).

Three different heart extraction methods were tested for their application to study the heart metabolome within our revised workflow: direct, hung, and rinsed. The mice in all three groups were anesthetized to a uniform deep plane (HR of <300 bpm) and randomly assigned to three different groups. Using the FiehnLib metabolomics library, we assigned over 150 metabolites from each of the three preparation methods. Metabolites were normalized to protein-derived glutamate before subsequent analyses. Using double-blind principle-component analysis (PCA) with all available metabolic features, we found that both direct and hung heart groups clustered well within each group but showed clear separation on the two-dimensional PCA plot, suggesting differential end-point metabolome profiles when using these two methods (Fig. 3A). The rinsed group showed inconsistent results and did not cluster well in PCA plot (Fig. 3A), suggesting that this method is not suitable for returning precise metabolomics analysis. Clustering heat-map analysis further confirmed that direct and hung methods have unique metabolic profiles (Fig. 3, B and C).

To identify major changes in metabolism between the direct and hung methods, we mapped central carbon metabolism such as glycolysis, the Krebs cycle, pentose phosphate pathway, amino acid metabolism, and glycogen metabolism. We found no difference in early glycolysis metabolites such as glucose, glucose-6-phosphate (G6P), and fructose-6-phosphate (Fig. 3D); however, 3-carbon glycolytic metabolites are significantly altered between the two harvest methods, with phosphoenolpyruvate (PEP) and pyruvate being significantly lower in the hung compared with the direct method (Fig. 3D and Table S3).

We then examined the Krebs cycle, a metabolite hub for energy production and amino acid biosynthesis and of particular interest in the field of heart metabolism. The Krebs cycle metabolite pools are sustained by: 1) the oxidative reactions that produce NADH for ATP synthesis, and 2) anaploretic reactions that connect glycolysis with the Krebs cycle metabolites independent of NADH (52). We found decreases in citrate and α-ketoglutarate in the hung method compared with direct freeze-clamping, whereas succinate, fumarate, and malate are much higher in the hung group (Fig. 3D). Citrate and α-ketoglutarate are primarily enriched by the oxidative cycle from acetyl-CoA produced by pyruvate dehydrogenase, whereas succinate, fumarate, and malate are supplied by the anaploretic reaction of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme. Coupled with reductions in PEP and pyruvate in the hung method, substrates for pyruvate carboxylase and phosphoenolpyruvate carboxykinase activity, our data suggest that anaploretic reactions are over represented in the hung method, whereas the oxidative phosphorylation pathway is emphasized by the direct method. Because cardiac ATP demand is fulfilled primarily by oxidative phosphorylation, which contributes ~95% of the heart’s ATP requirements (53–55), we adopted the direct freeze-clamp method for the remainder of these studies.

Amino acid metabolism plays major roles in heart physiology, especially tolerance to ischemia (56) and postsurgery recovery (57). The effects of different heart harvest workflows on amino acid metabolism is relatively unknown. To our surprise, most amino acids remain relatively consistent between these two distinct methods (Fig. 4), although only alanine and aspartate showing significant differences. Changes in alanine and aspartate were expected because both amino acids are de novo synthesized from glycolysis and anaplerosis; thus, these results provide additional evidence that the Krebs cycle is differentially represented by the two methods of extraction.

Glycogen has been shown to be a vital metabolite of the heart (58, 59), and accumulation may help protect against anoxia (60) and ischemia (61). Glycogen is a nutrient source post-mortem in the heart, with glycogen stores rapidly consumed after euthanasia (62–64); therefore, it is a good indicator of sample quality following tissue collection. We found comparable amounts of glycogen in both methods, suggesting that neither method requires the mobilization of glycogen for bioenergetics, hence

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**Figure 2. Statistical analysis of biological variance and clustering in metabolomics data set.** A, schematic workflow of GC-MS metabolomics. Frozen tissues were pulverized to 5-µm particles using magnetic assisted tissue cryomilling. Tissues were subsequently extracted with 50% methanol to release free polar metabolites. The insoluble fraction is further processed by mild acid hydrolysis to produce monomeric amino acids and monosaccharides. Metabolites in both polar and insoluble fractions were separated and detected by GC-MS. B, COV of representative metabolites either normalized to tissue weight (yellow arrowhead) or protein-derived glutamate (green arrowhead). C, principle component analysis shows improved clustering in metabolomics data sets normalized to protein-derived glutamate. MSTFA, N-methyl-N-trimethylsilyl trifluoroacetamide; P/G, protein-derived glutamate; T/W, tissue weight; W/W, wet weight; Suc, succinate; Fum, fumarate; Oxa, oxaloacetate; Mal, malate; Lle, isoleucine; Thr, threonine; Pro, proline; Gly, glycine; Asp, aspartate; B-Ala, Beta-alanine; CCA, citraconic acid.
Figure 3. Metabolomics analysis of different heart extraction methods. A, principle component analysis shows tight clustering between the direct and hung groups, whereas the rinsed group did not cluster well. B, clustering heat-map analysis shows separation between the direct and hung groups. C, graphical representation of the hung and direct methods. D, differences in relative abundance of metabolites in the glycolytic and Krebs cycle pathways between the hung and direct methods. E, schematic of over-represented pathways between hung and direct methods. Red arrow, over-represented pathway in hung method; green arrow, over-represented pathway in the direct method.* 0.01 < p < 0.05; ** 0.001 < p < 0.01.

Figure 4. GC-MS metabolomics analysis of glycogen and amino acid pools between direct and hung heart extraction methods. *, 0.01 < p < 0.05; **, 0.001 < p < 0.01. GABA, gamma aminobutyric acid.
preserving endogenous glycogen accumulation (Fig. 4 and Table S3).

**Isoproterenol stimulates oxidative metabolism for the production of ATP**

The major physiological acute mechanism the myocardium employs to adapt to a sudden demand for increased work is catecholaminergic stimulation of cardiomyocyte expressed β-adrenergic receptors (β-ARs). Isoproterenol (ISO) is a potent β-AR agonist that acutely increases heart work manifested as an increased HR. To isolate rate effect from ISO-stimulated β-AR signaling, we anesthetized mice to a uniform deep plane defined as HR < 300 bpm similar to above. Basal HR was noted, and 3 min following ISO challenge (or not) HR was again recorded, the hearts were harvested by direct freeze clamp, and normalized metabolites were used to study metabolic perturbations after isoproterenol stimulation.

As expected, the ISO and control groups were discretely segregated by PCA and clustering heat-map analyses using the improved metabolomics workflow with protein-derived glutamate-dependent normalization (Fig. 5, A and B). Isoproterenol stimulation resulted in the reduction of metabolites in glycolysis and the oxidative portion of the Krebs cycle, whereas the pentose phosphate pathway and anaplerotic Krebs metabolites remained unchanged. These findings coupled with a 6-fold increase in adenosine nucleotide levels suggest that an increase of oxidative glucose metabolism is responsible for increased ATP production in the ISO-stimulated heart.

Although we observed decreased G6P, total glucose levels remain unchanged in isoproterenol-stimulated hearts (Fig. 5C and Table S2). This suggests that hexokinase activity is unaltered by isoproterenol stimulation, and an alternate pool of substrate(s) supplies G6P to maintain glycolysis activity follow-
Cardiac metabolic pathways are integrated and adaptive to stress by dynamically altering substrate utilization to maintain cardiac contraction, thereby ensuring normal heart physiology and health. Understanding the heart metabolome and how flux through metabolic pathways is altered during cardiac adaptation could reveal mechanisms of disease pathology and how to exploit metabolic change for therapeutic benefit. Currently, in vivo methods such as magnetic resonance imaging or magnetic resonance spectroscopy permit the most direct analysis of cardiac metabolism but lack resolution, sensitivity, and coverage needed for detailed interrogation of heart metabolism. For a more comprehensive view of the heart metabolome, terminal extraction of the heart is required to map detailed metabolic networks under different physiological conditions. Although metabolite extraction from model cell lines is relatively straightforward, complex tissue metabolite extraction remains a technical challenge. Because of the high number of labile metabolites, inefficient cardiac extraction can compromise the interpretation of metabolic data. Because metabolomics experiments are hypothesis-generating in nature, identifying metabolite extraction methods that accurately represent heart physiology is crucial for downstream experimental designs.

In this study, we introduced an improved workflow utilizing metabolomics to study heart metabolism with four innovative advances. First, frozen heart tissues are pulverized in LN₂ to 5-μm particles to allow maximum and efficient metabolite extraction. Second, monomeric forms of insoluble biomass such as protein-derived glutamate and glycogen-derived glucose from each sample are recorded in the same workflow by GC-MS (other amino acids such as alanine or glycine can also be used). Third, normalization to protein-derived amino acid significantly reduces biological variance and increases the power to detect small metabolic changes. Fourth, glycogen is measured as a metabolite within the metabolomics workflow. These improvements can be adapted to other chromatography methods such as LC or capillary electrophoresis. In the case of anticipated changes to protein levels in response to treatment, chromosomal DNA derived thymine can be used as an alternative normalization candidate.

We employed our workflow to study metabolic differences between distinct heart harvest methods. Both hung and direct methods rely on anesthesia but differ in procedures before metabolites are preserved in LN₂. Based on our pathway analysis, we found a major shift from lower glycolysis to the anaerobic pathway of the Krebs cycle when using the hung heart method. Decreased G3P, PEP, and pyruvate can be attributed to increased anaplerosis that would convert these metabolites to malate, fumarate, and oxaloacetate. Increases in succinate can be a symptom of mild ischemia of the heart; however, we did not notice any increase in lactate in our analysis, suggesting that these metabolic changes are not due to ischemia but rather may be the result of cellular signaling events within the hung heart. One possibility is that the continuous perfusion...
of glucose-rich Krebs–Henseleit solution promotes adaptive metabolic signaling. The ability of glucose to alter cellular metabolism, such as the Crabtree effect, is well-documented in different tissue types and is known to result in changes to glycolysis and the Krebs cycle (72). Taken together, these data suggest that both hung and direct heart approaches yield reproducible data, and the specific method of heart extraction used should be dictated by the nature of scientific question to be addressed rather than by a one-size-fits-all approach.

Isoproterenol is a Food and Drug Administration–approved β-adrenergic receptor agonist for the treatment of heart block and bradycardia (73). Although it is known that ISO induces adenosine nucleotide production (74, 75), the present study provides the first description of acute ISO-mediated changes in central carbon metabolism from heart tissue maintained within its native interstitium, particularly the interstitial fluid environment with its physiological capacity to promote protective tissue stability. Thus, compared with the hung heart preparation, the direct approach provides a more physiological condition for metabolomics analyses because of the maintenance of circulating plasma. Using the in situ LN2-chilled heart-clamp heart-extraction method (direct), ISO was found to cause a reduction in metabolite levels in glycolysis and the first half of the Krebs cycle coupled with a 6-fold increase in cellular adenosine nucleotides. These data support the notion that isoproterenol drives ATP production through oxidative glucose metabolism. To our surprise, we found rapid depletion of glycogen in isoproterenol-stimulated hearts. The reduction in total glycogen correlated with the graded degree of effect of isoproterenol stimulation. These data fit with the metabolic enzymes known to be targeted following adrenergic receptor stimulation in the heart (76, 77) and liver (78, 79). These data show β-adrenergic receptor hormonal control is “anticipatory”: ATP is generated because β-adrenergic receptor signaling normally also results in increased contraction and HR (cardiac output). Thus, glycogen would be a buffer to allow acute ATP generation as the heart adapts to this new work state and starts to utilize additional fuels. Isoproterenol stimulation is a chemical induction stand-in for the fight or flight response, and these data suggest that heart glycogen could be a critically important metabolite fueling this process; however, this hypothesis requires further research and validation.

In summary, we improved on traditional tissue metabolomics workflow through the implementation of a simple yet effective method to normalize against sample input. We used this workflow to examine the metabolome following different methods of heart extraction and identified interesting metabolic features unique to hung heart and freeze-clamp methods. We then used the in situ freeze-clamp method to study the effects of isoproterenol on heart metabolism. Our analysis suggests that the heart utilizes its own glycogen reserves to support the increased ATP demand following acute β-AR stimulation. We anticipate that this method can easily be adapted for other tissue types or tumors and can be implemented in metabolomics approaches for both biomarker discovery and mechanistic interrogations.

**Experimental procedures**

**Animals**

Male Black Swiss mice (56 weeks) were purchased from Charles River (Wilmington, MA) and housed in a climate-controlled environment with a 14-h light/10-h dark cycle (lights on at 0600 h) with water and solid diet provided *ad libitum* throughout the study. The Institutional Animal Care and Use Committee at the University of Kentucky approved all of the animal procedures carried out in this study under Public Health Service Assurance A3336-01.

**Langendorff heart preparation**

The hearts were excised, and the aorta cannulated and retrogradely perfused on a Langendorff apparatus. Retrograde perfusion buffer consisted of oxygenated Krebs–Henseleit (118 mmol/liter NaCl, 5.3 mmol/liter KCl, 2 mmol/liter CaCl2, 1.2 mmol/liter MgSO4, 0.5 mmol/liter EGTA, 25 mmol/liter NaHCO3,10 mmol/liter D-glucose, 0.5 mmol/liter pyruvate, and 10 mmol/liter HEPEs) < 60 s after cannulation. Constant pressure regulated aortic reverse perfusate flow. Hearts were monitored for constant supraventricular origin of rhythmic beating for 15 min, and approximately the apical two-thirds of the ventricular chambers were subject to immediate flash-freezing.

**Rinsed heart preparation**

Prior to heart excision, the mice were anesthetized with 5% isoflurane (O2 at 1.5 liters/min). Respiratory response was monitored to ensure deep sedation. The hearts were excised with a single rapid dissection that allowed capture of approximately the apical two-thirds of the ventricular chambers, quickly rinsed in Milli-Q water, and immediately flash frozen in LN2 (<30 s total).

**Freeze-clamp heart preparation**

Hearts from anesthetized mice (5% isoflurane; O2 at 1.5 liters/min) were harvested by a single rapid dissection at a level that allowed capture of approximately the apical two-thirds of the ventricular chambers, freeze-clamped by placing the heart directly on the swivel pad of a liquid LN2-cooled C-clamp (TEKTON, 14-inch welding C-clamp), immediately clamped shut, and immersed in LN2. The samples were placed in cryostorage (~90 to ~196 °C) until further analysis. For ISO treatment, anesthetized mice were placed on an electrocardiogram platform, and HRs were recorded using a Philips Affinity Ultrasound machine. After stabilization of HR, the mice were either not treated (control) or injected with ISO (30 mg/kg, intraperitoneally). Approximately, 3 min after ISO treatment, a stable, elevated HR was reached, the HR was recorded, and hearts were harvested using freeze-clamping.

**Sample preparation**

The hearts were removed from cryostorage and pulverized to 5-μm particles in LN2 using a Freezer/Mill® cryogenic grinder (SPEX SamplePrep model 6875D). 20 mg of each pulverized tissue was extracted in 1 ml of 50% methanol containing 20 μM l-norvaline (procedural, internal control) and separated into
Improved workflow for metabolomics analysis

polar (aqueous layer) and protein/DNA/RNA/glycogen pellet by centrifugation at 4 °C and 15,000 rpm for 10 min. The pellet was subsequently washed four times with 50% methanol and once with 100% methanol to remove polar contaminants. The polar fraction was dried at 10⁻³ mBar using a SpeedVac (Thermo) followed by derivatization.

Pellet hydrolysis

Hydrolysis of the protein/glycogen pellet and the amino acid standards were performed by first resuspending the dried pellet in deionized H₂O followed by the addition of equal part 2 N HCl. The samples were vortexed thoroughly and incubated at 95 °C for 2 h. Additional hydrolysis methods included addition of 200 μl of 6 M HCl and 2 volumes of 6 M HCl to 1 volume of 12 M HCl for overnight incubation at 95 °C or at 95 °C for 2 h, respectively. All reactions were quenched with 100% methanol with 40 μM l-norvaline, the samples were incubated on ice for 30 min, and the supernatant was collected after centrifugation at 15,000 rpm at 4 °C for 10 min. The collected supernatant was subsequently dried by vacuum centrifuge at 10⁻³ mBar. For method comparison, a Pierce BCA protein assay was performed utilizing standard assay protocols found in the kit.

Sample derivatization

Dried polar and glycogen samples were derivatized by the addition of 20 mg/ml methoxamine hydrochloride in pyridine and incubation for 1.5 h at 30 °C. Sequential addition of N-methyl-N-trimethylsilyl trifluoroacetamide followed with an incubation time of 30 min at 37 °C with thorough mixing between addition of solvents. The mixture was then transferred to a V-shaped amber glass chromatography vial and analyzed by GC-MS.

GC-MS quantitation

An Agilent 7800B GC coupled to a 5977B MS detector was used for this study. GC-MS protocols were similar to those described previously (38, 80), except a modified temperature gradient was used for GC: initial temperature was 130 °C, held for 4 min, rising to 6 °C/min to 243 °C, rising at 60 °C/min to 280 °C, and held for 2 min. The electron ionization energy was set to 70 eV. Scan (m/z, 50 – 800) and full scan mode were used for metabolomics analysis. Mass spectra were translated to relative metabolite abundance using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software matched to the FiehnLib metabolomics library (available through Agilent) for retention time and fragmentation pattern matching with a confidence score of >80 (13, 14, 19, 81). Quantitation was performed using the software Mnova, Mestrelab with a primary ion and at least two or more matching qualifying ions. Relative abundance was corrected for recovery using the L-norvaline standard and adjusted to protein input.

Statistics

Statistical analyses were carried out using GraphPad Prism. All numerical data are presented as means ± S.E. A p value less than 0.05 using a Student’s t test was considered statistically significant. PCA and clustering heat-map analyses were performed using the ClustVis package for R (available through Github) (36). For PCA and heat-map analysis, untransformed metabolomics data were used, and unit variance scaling was employed for row numbers and singular value decomposition with imputation for PCA clustering. Correlation and tightest cluster first options were used for heat-map visualization (82). All available metabolomics data points were used for multivariate analysis.


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References

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