A comparison of extraction methods for measuring TCA metabolites in porcine and murine cortical bone

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INTRODUCTION: Cortical bone, which makes up approximately 80% of the human skeleton, experiences both decreased fracture resistance and bone matrix quality in aging. The properties of bone matrix are regulated by bone cells, including the osteocyte. In prior work, untargeted metabolomics revealed pathways by which the metabolism of osteocyte-rich cortical bone tissue changes in aging. Some of these pathways implicate Tricarboxylic Acid Cycle (TCA) metabolism. However, targeted metabolomics for TCA metabolites in cortical bone is currently limited by several unanswered questions. A key question is the selection of the extraction protocol to isolate metabolites before detection using Mass Spectrometry (MS). Three candidate extraction procedures include 70:30 methanol: acetone (**MeOH Ace**), modified Bligh-Dyer (**BD**) and modified Matyash **(Mat)**.[1] The BD and Mat methods were modified to decrease exposure to harmful reagents, consistent with their use for other non-bone tissues[2–4]. The purpose of this study was to compare the extraction methods for detecting TCA metabolites in porcine and murine samples. Further, since mice are commonly used models to study bone fragility in aging, we also asked whether the detection of TCA metabolites depends on the long bone studied (i.e., femur, tibia, humerus) or on the sex of the mouse.

METHODS: Porcine: A previously frozen porcine male femur was acquired from a local butcher shop. Cortical bone from the femur midshaft was dissected and periosteum and marrow were removed; samples were 0.05 g of grated and powdered bone. **Murine:** Right and left femora, tibiae and humeri from 12 male and 12 female C57/BL6 mice (20–24 weeks) were utilized. Animal procedures were approved by the institutional review board. For each animal, contralateral limbs were assigned to different extraction methods (i.e., repeated measures). A total of 48 bones were studied per extraction method. Bones were dissected, ends removed, and marrow removed. Bones were then pulverized and weighed. **Porcine and Murine:** The **MeOH Ace** method extracts metabolites in 7:3 v/v MeOH. The **BD** method extracts metabolites in 2/2/1.8 v/v/v chloroform, methanol, and water. The top aqueous layer containing the metabolites was transferred to a new tube. A second extraction was then performed. The **Mat** method extracts metabolites using 2.6/2.0/2.4 v/v/v MTBE/MeOH/ H_2O. After phase separation, the middle layer containing metabolites was transferred to a new tube. A quality control with no bone was included for each extraction. All samples were dried down via vacuum concentrator and stored at –80 °C. Samples were resuspended in 50:50 acetonitrile water, vortexed, then 15 µL from each were transferred to analysis vials. A pooled sample was made from each of the three extraction methods. Metabolites were analyzed using a Waters SYNAPT G2-Si Mass Spectrometer in negative ion mode, targeting 17 central energy metabolites (pyruvic acid, lactic acid, fumaric acid, succinic acid, oxaloacetic acid, malic acid, alpha-ketoglutarate, glutamine, glutamic acid, phosphoenolpyruvate, fructose and glucose, citric acid, fructose-6-phosphate, 6-phosphogluconate, sedoheptulose-7-phosphate, NADH, HS-CoA). A pooled standard was prepared for the 17 metabolites (100 µg/mL) and a calibration curve was made from 6 standard concentrations (0.1 - 25 µg/mL). The mobile phase consisted of 0.1% ammonium hydroxide and 0.1% acetyl nitrile. An Acquity UPLC BEH C18 column was utilized for retention of polar metabolites, with an injection volume of 2 µL. An amino acid standard followed by a blank was run after every 10 biological samples. Metabolites were quantified from parent and fragmented ions. Concentration data were normalized by the bone sample weight, then metabolites were log transformed if necessary to satisfy ANOVA assumptions. For porcine data, ANOVA assessed the effect of extraction method. For murine data, mixed-model ANOVA assessed the effects of extraction method, limb, sex, and their interactions. Following significant main effects or interactions ($p<0.05$), Tukey tests were used for post-hoc tests.

RESULTS: Porcine: 7 of the 17 metabolites (citric acid, fructose or glucose, fumaric acid, glutamic acid, glutamine, lactic Acid, oxaloacetate) were detected overall. For porcine bone, the MeOH Ace, BD, and Matyash methods detected 2, 7, and 4 TCA metabolites, respectively, in at least 1/2 of the samples (**Figure**). Intensity of metabolites was higher in BD for 3 out of 7 metabolites detected (oxaloacetate, fumaric acid, citric acid) and similar to Mat in 4 out of 7 (glutamic acid, fructose & glucose, glutamic acid, glutamine, lactic acid). Both BD and Mat outperformed MeOH Ace in terms of number of metabolites detected and intensity. **Murine:** 10 out of 17 metabolites were detected. For murine bone, the MeOH Ace, BD, and Matyash methods detected 6, 8, and 9 TCA metabolites, respectively, in at least 1/2 of the samples (**Figure**). Extraction method significantly impacted the intensity of all detected metabolites. For all detected metabolites, the intensity was higher for Mat than MeOH Ace. The intensity was higher for Mat than BD for some, but not all, metabolites. Extraction method interacted with sex for pyruvic acid, oxaloacetic acid, glutamine and citric acid. 2-way interactions showed that Mat males and females had the highest difference in means compared to the other groups. Skeletal site was a significant main effect for 6 of the 10 detected metabolites. For fumaric acid, oxaloacetic acid and phosphonyl pyruvate, femora had significantly higher mean intensity than humeri. For glutamic acid and Citric Acid, femora had a higher mean than tibiae. For fructose & glucose, humeri had a higher mean than tibiae.

DISCUSSION: For detecting TCA metabolites with the greatest intensity, multiphase extraction methods (BD, Mat) outperformed the single-phase method (MeOH Ace) in the porcine model, likely due to better removal of proteins and lipids. In the murine model, Mat outperformed the other extraction methods for detection and intensity of metabolites. There was a lack of sex differences observed in Mat and MeOH Ace extractions, but the BD method produced sex differences for some metabolites. The skeletal site was also found to matter for the intensity of metabolites detected, demonstrating the need for consistent methodology across studies.

SIGNIFICANCE: This work establishes sample preparation protocols for targeted TCA metabolites of cortical bone from two animal models, enabling more accurate study of bone metabolism and informing future research on the impact of aging on cellular metabolism and bone fracture resistance.

REFERENCES: 1. Sostare+, Chim. Acta., 2018. 2. Carlson+, Osteo. Cart., 2020. 3. Lillefosse+, J. Proteome Res., 2014. 4. Gonsalves+, Sci Rep., 2020.

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IMAGES AND TABLES:

Fraction of Samples with Specific TCA Metabolites: Porcine (16 samples) and Murine (48 samples)

Metabolite	Extraction method			Metabolite	Extraction method		
	70:30 Methanol Acetone	Modified Bligh-Dyer	Modified Matyash		70:30 Methanol Acetone	Modified Bligh-Dyer	Modified Matyash
Citric Acid	0/16, 38/48	8/18, 34/48	4/16, 45/48	Fructose 6 Phosphate	0/16, 1 /48	0/16, 3 /48	0/16, 4 /48
Fructose or Glucose	16/16, 46/48	16/16, 47/48	16/16, 47/48	Malic Acid	0/16, 4 /48	2/16, 10 /48	1/16, 37 /48
Fumaric Acid	0/16, 14/48	9/18, 21/48	1/16, 39/48	NADH	0/16, 0 /48	0/16, 0 /48	0/16, 0 /48
Glutamic Acid	0/16, 43/48	13/16, 41/48	12/16 46/48	Phosphoenolpyruvate	0/16, 20/48	0/16, 18 /48	0/16, 17 /48
Glutamine	0/16, 40/48	12/16, 38 /48	14/16, 44/48	6-Phospho gluconate	0/16, 0 /48	0/16, 0 /48	0/16, 0 /48
Lactic Acid	9/16, 40 /48	16/16, 36 /48	16/16, 45/48	Pyruvic Acid	1/16, 32 /48	0/16, 32 /48	0/16, 45 /48
Oxaloacetate	0/16, 20/48	7/16, 27 /48	3/16, 45/48	sepoheptulose-7-phosphate	0/16, 0 /48	0/16 , 0/48	0/16, 0 /48
Acetyl-CoA	0/16, 0 /48	0/16, 0 /48	0/16, 0 /48	Succinic Acid	0/16 ,1/48	0/16, 2 /48	0/16, 1 /48
Alpha-Ketoglutarate	0/16, 0/48	0/16, 0 /48	0/16, 0 /48	Metabolite detected in > ½ of porcine samples	Metabolite detected in > ½ of murine samples		Metabolite detected in > ½ of porcine & murine samples