Optimization of Synovial Fluid Metabolite Extraction Methods for Untargeted Metabolomics

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INTRODUCTION: Osteoarthritis (OA) is a degenerative disease traditionally characterized by the degradation of articular cartilage, but also including changes in metabolism within the joint tissues, inflammatory activity, and cellular stress[1]. OA is a common cause of chronic pain and disability in the US, but there are currently no FDA-approved biomarkers or established methods for early detection, which could allow for reversal or limitation of symptoms[2]. Untargeted metabolomic profiling provides a robust snapshot of cellular activity within a sample, including tissues and fluids, making it an advantageous tool to study disease pathogenesis and define new molecular endotypes of OA. Synovial fluid (SF), found within the joint cavity, is rich with molecules produced by many joint tissues making it an optimal sample type for studying OA as it reflects the metabolic state of the joint[3]. Optimal extraction of SF-derived metabolites is challenging because proteins and lipids are notoriously difficult to remove and strongly interfere with downstream instrumentation performance. The goal of this study is to optimize the extraction method to provide high quality metabolomic profiles from SF. Future studies will build upon this to examine metabolomic differences related to factors such as Kellgren-Lawrence (KL) grade and patient-assessed pain using SF samples from the STEpUP OA consortium. SF from this consortium has been used in proteomic analysis for molecular OA endotypes[4,5].

METHODS: A total of 6 different metabolite extraction methods were performed on vacuum concentrated human SF preliminary extracts (Table 1). These samples had already undergone preliminary extraction but still contained proteins and lipids. Protocols 1-3 all involved sample resuspension in varying solvents, incubation at -80°C overnight (0°C for 15 minutes for the 1st method), followed by centrifugation at 16,100 x g for 10 minutes. Supernatant was collected and samples were vacuum concentrated. The 1st protocol used 100% acetonitrile and 1% formic acid for resuspension, and water was added to the supernatant following centrifugation. For the 2nd protocol, a 70:30 methanol:acetone precipitation was performed, and samples were resuspended in cold 100% acetone and the previous steps were repeated. The 3rd protocol extracted with 100% acetone. The 4th protocol used 3kDa molecular weight cutoff (MWCO) spin columns: samples were resuspended in water, applied to equilibrated MWCO spin columns, and centrifuged at 14,000 x g until nearly all sample had passed through the filter. Effluent was collected and 1:1 acetonitrile/water was added, followed by vacuum concentration. We also tried modifications where samples were resuspended and applied to the spin column in 50:50 methanol/water or 90:10 water/methanol. For the 5th protocol, the Modified Matyash method[6], ice-cold methanol was added to samples and vortexed, followed by the addition of MTBE and vortexing. Water was added, followed by vortexing and incubation on ice for 1 hour. Samples were centrifuged at 5,000 rpm for 10 minutes, and the aqueous layer was collected and vacuum concentrated. The 6th protocol used on-line chromatography, where samples in 50:50 acetonitrile/water were injected directly onto a solid-phase extraction column and then eluted onto a reverse-phase analytical column on an Agilent 6538 Q-TOF. Across all other extraction methods, SF samples were resuspended in 50:50 acetonitrile/water for injection onto a Synapt XS Q-TOF. Samples were run on a HILIC column for optimal separation of polar metabolites, and some samples were run on a RP column in addition, to better assess protein and lipid content. Raw data was examined in MassLynx and converted to mzML file format with MSConvert, then processed in MS-DIAL. MetaboAnalyst was used for Principal Component Analysis (PCA), to assess metabolomic variation across extraction methods (Figure 2).

RESULTS: The first four extraction methods all showed varying issues with high levels of molecules not ideal for untargeted metabolomics. The acetonitrile/formic acid method did not effectively eliminate proteins as well as other methods, while the methanol/acetone method appeared to overload the column, possibly with lipids, resulting in poor chromatographic separation of metabolites (Figure 1). The MWCO spin column method resulted in an especially high amount of plastic and did not remove sufficient protein. Modified MWCO spin column extractions appeared to possibly be slightly better at removing proteins, but the presence of substantial plastic persisted. Samples processed with the Modified Matyash method showed reduced lipid content, but high salt cluster content remained, as in the other methods. The on-line SPE chromatography method remains preliminary but shows the strongest potential for effectively removing proteins and lipids and providing high quality metabolomic data.

DISCUSSION: Untargeted metabolomics on biological samples poses substantial challenges in sample cleanup while minimizing the loss of metabolites. Successful removal of proteins and lipids is essential for accurate characterization of the SF metabolome, and an optimized metabolite extraction protocol will also benefit future SF metabolomic studies. Using metabolomics to examine SF samples from a consortium that has utilized proteomics takes a systems biology approach with potential for characterizing OA endotypes and pathophysiology. Different extraction methods show variations in effectiveness for removing varying problematic molecules, like proteins, and providing quality metabolomic data. Currently, the on-line SPE method shows the best potential for sufficient extraction, but several steps remain to ensure its reliability. This method appears to be best because it minimizes processing steps thus reducing the risk for variability due to human error and appears fairly effective at removal of proteins and lipids. As of submission, this method has been performed on 2 different mass spectrometers and only n=2 SF samples, so these findings remain preliminary and further experimentation is needed to finalize.

SIGNIFICANCE/CLINICAL RELEVANCE: An SF metabolite extraction that allows for optimal metabolite detection will be used for untargeted metabolomic profiling of STEpUP OA consortium samples to identify potential molecular endotypes of OA and characterize changes in the SF metabolome based upon clinical factors such as KL grade and pain. This approach to characterizing disease pathogenesis provides insight that is highly individualized and applicable to studying other diseases.

REFERENCES: [1]Loeser *et al* Arthritis Rheum. 2012 22392533, [2]Carlson *et al* Biochem Biophys Res Commun. 2018 29551687, [3]Kim *et al* Joint Bone Spin. 2017 27461192, [4]Perry *et al* medRxiv 2024 24308485, [5]Deng *et al* PLoS One 2024 39556578, [6]Sostare *et al.* Analytica Chimica Acta. 2018 30292307

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IMAGES AND TABLES:

Table 1.

Protocol	n	Male	Female	Unknown
1: Acetonitrile w/ Formic Acid	4	3	1	0
2: Methanol/Acetone	44	24	18	2
3: Acetone	8	4	4	0
4: Spin Column (including modified methods)	32	15	14	3
5: Modified Matyash	17	6	5	6
6. On-line Chromatography	2	1	0	1

Table 1. Sample sizes across SF metabolite extraction protocols, with sex information.

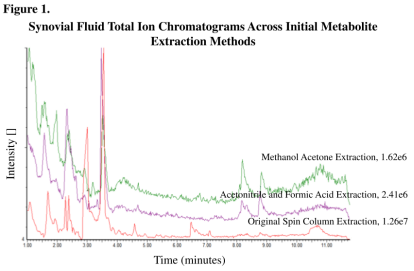

Figure 1. Total ion chromatograms for methanol/acetone, acetonitrile/formic acid, and original MWCO spin column extraction methods on synovial fluid, using HILIC chromatography.

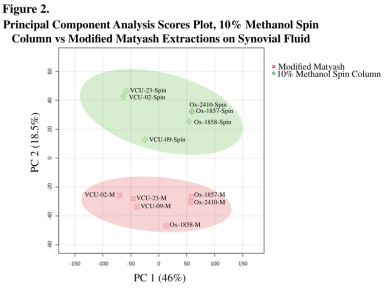

Figure 2. PCA plot for 10% methanol MWCO spin column and Modified Matyash extraction methods on synovial fluid.