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Analyzing the stability of metabolites in long-term cold storage

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Introduction

Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), is a high-resolution analysis method used across multiple fields, including metabolomics, proteomics, and other biochemistries. Samples used for this type of analysis are typically stored in a −80°C freezer either before or after analysis. A series of studies began in 2019 (McKetney 2021) codenamed MASTRE, where war fighters were put through specific tests and stress events, then had their saliva tested for the presence or change of various bioindicators under acute stressors (Figure 1). Protein, metabolite, and lipid samples were extracted and stored in long-term cold storage. The purpose of this is to analyze the effects of long-term cold storage, and other harsh conditions on LC-MS samples, and validating their integrity after years spent in storage.

Materials & Methods

The MASTRE study initially stored samples on dry ice after collecting them in the field. Samples were then separated into lipid, metabolite, and protein fractions, and stored in −80°C freezers after analysis. The original metabolite samples were suspended in 9:1 water: acetonitrile (ACN) (McKetney, 2021). In this study, four samples, sorted by MPA (soldier identification number), were taken from each condition. The conditions for the study are as follows: CAR (Cortisol Awakening Response), D0-D4; TSMA (Tactical Stress Marksmanship Assessment), TP1-TP6; SUPRA (Small Unit Performance Analytics), Pre & Post; AM Daily (sample taken at awake time, daily), D2-D4; Infil/Exfil (Infiltrating and Exfiltrating a mission), Pre & Post.

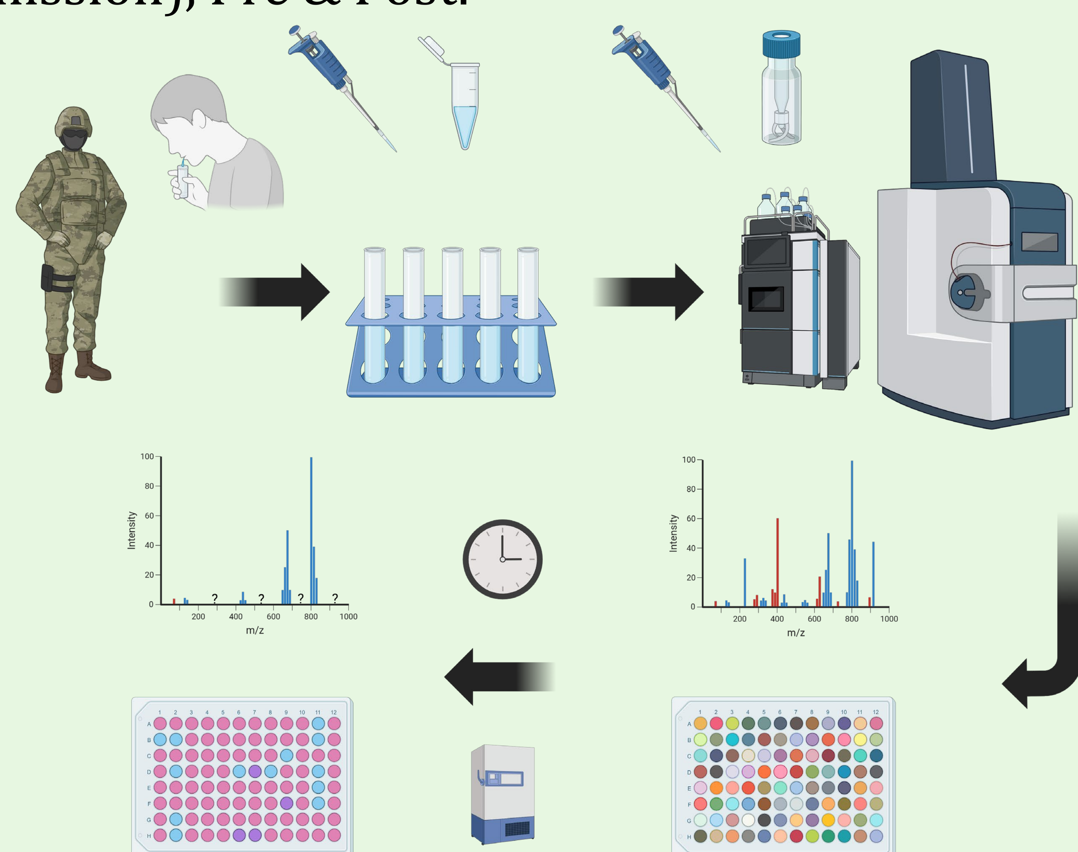


Figure 1 (left): A diagram created with BioRender.com showing the workflow of the full study, including the original study and the transition into the current study. Sample extraction methods and analysis methods are shown in this diagram.

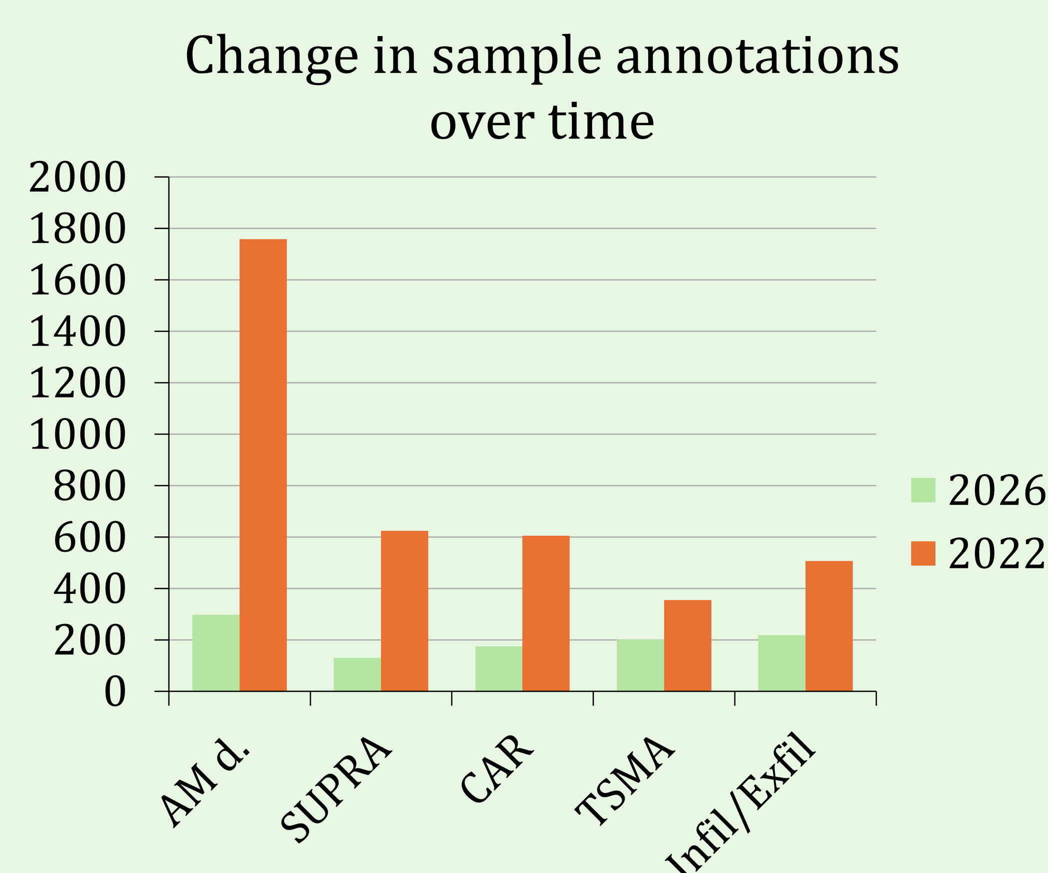
Materials & Methods (continued)

Samples were dried in a SpeedVac dryer and then reconstituted in a 2:2:1 ACN:methanol:water solution. Samples were diluted to a 5:1 dilution of their original volume due to the instrument's threshold of 50 μL. For each experiment the Splash II mix (Avanti Lipids), Mobile Phase, Blank, and Quality Control (QC) were included in the running list. The Splash II mix is a solution used to check the stability of the chromatography. The mobile phase is a solution specific to a column consisting of mostly ACN and buffer, the column in this study used a HILIC stationary phase. The blank is the reconstitution solution used, we use this to eliminate background noise in the mass spectrum. The QC solution consists of 5–10 μL aliquots of each sample. LC-MS data was acquired using a ThermoFisher Vanquish and Q Exactive Plus, following standard lab protocol (McBride 2019). After the LC-MS process was complete, the chromatography was manually checked in Compound Discoverer for compound validity.

Results

Compounds annotated in both new and old runs were quantified and compared (Graph 1). The key indicator of compound degradation aside from annotations, was the increase of breakdown products of larger, common compounds, namely ADP (Graph 2). ADP is closely related to ATP, which is an important component of energy metabolism, which can be used as a biomarker for influxes in different biological pathways. ADP and ATP easily break down into its nucleoside and purine backbones (Figure 2). This breakdown was quantified in multiple datasets to confirm sample degradation (Graph 2).

Graph 1 (right): Annotations are compounds identified by LC-MS via databases. Graph 1 is a bar chart showing the number of sample annotations in the original study versus this study. Annotations decreased by 6-fold in most extreme cases, signifying substantial sample degradation.



Results (continued)

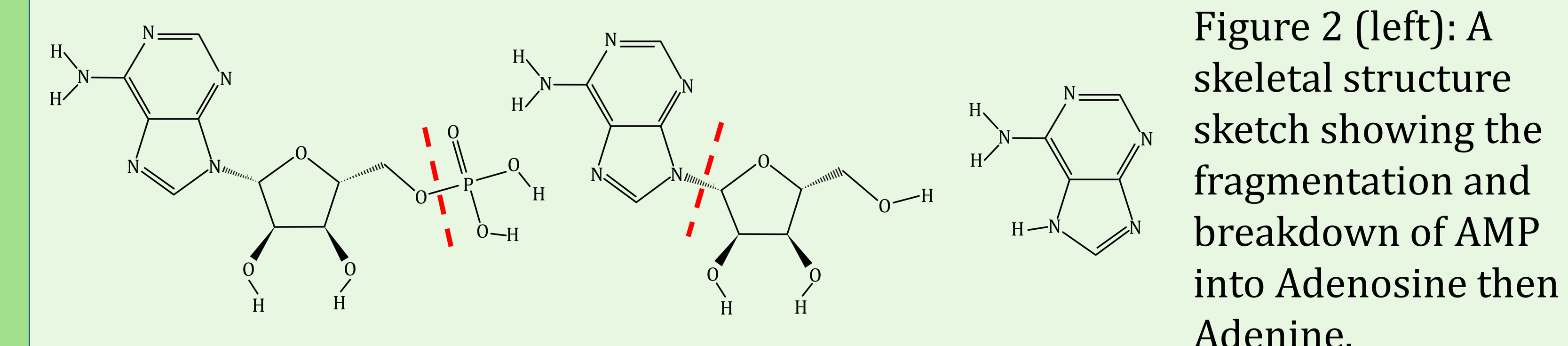
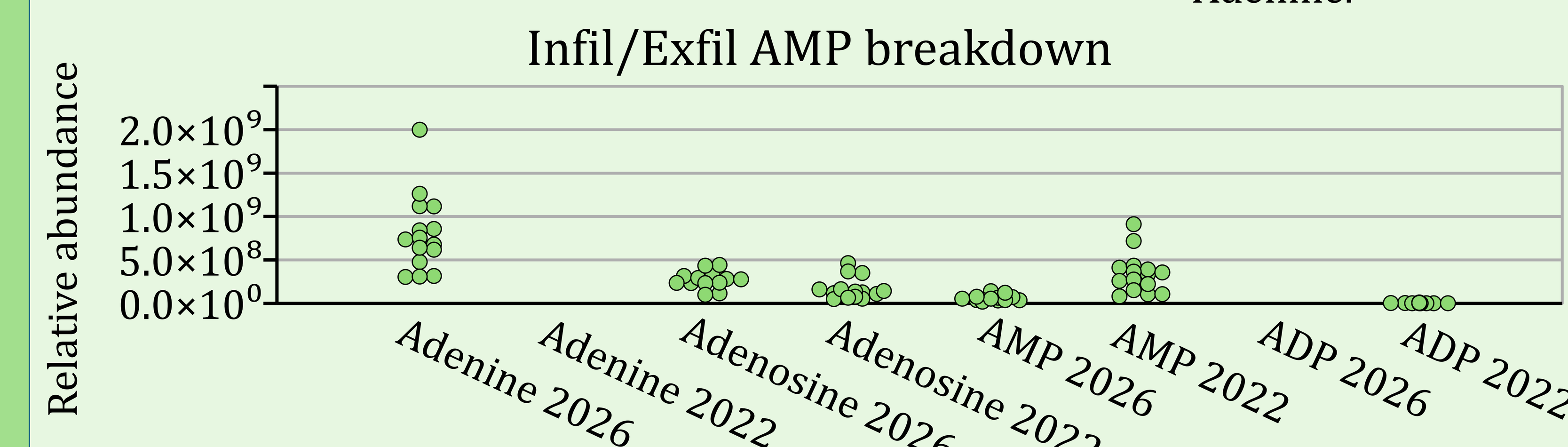


Figure 2 (left): A skeletal structure sketch showing the fragmentation and breakdown of AMP into Adenosine then Adenine.



Graph 2 (above): A univariate scatter plot showing the quantitative differences between ADP and its breakdown products during the past study and the recent study. ADP and adenosine have broken down into adenine over time. A large decrease in AMP/ADP was observed while an increase in adenosine and adenine was observed. ADP had a relative abundance of 2.9×10^{6} in 2022.

Conclusion

This study found that metabolic samples degrade over time in cold storage. Punctures in the septum during initial LC testing exposed the samples to atmospheric gases, which reacted with metabolites and accelerated decomposition. Variance in the data confirms that sample integrity was lost over time, shown by the decrease in annotation count (Graph 1). Because metabolic samples are highly sensitive to breakdown, they must be handled quickly and carefully to ensure reliable data. Repeated use of samples can skew results, especially in studies relying on unstable metabolites. Future studies could extend this work by examining the long-term stability of lipid samples, which may exhibit different degradation patterns.

References

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