INTERFACING CHROMATOGRAPHIC, MASS SPECTROMETRY, AND IONIZATION TECHNIQUES FOR BETTER PROFILING ENDOGENOUS AND EXOGENOUS METABOLITES IN HUMAN MILK

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Introduction

In this study, we used liquid chromatography coupled with high and low-resolution mass spectrometry (LC-MS) to broaden the scope and increase sensitivity in detecting known and unknown metabolites in breast milk Standard Reference Material *(SRM) 1953 (Organic Contaminants in Non-Fortified Human Milk)* and *SRM 1954 (Organic Contaminants in Fortified Human Milk)*.

The unidentified spectra of metabolites will be searched against NIST Tandem MS Library 2020 by Direct or "Hybrid" [3] searches.

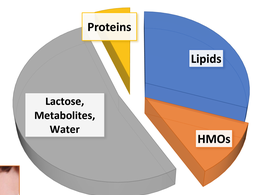

Figure 1. Composition of human milk.

Objectives

- ❑ Building a reference MS/MS library of metabolites from breast milk [1].
- ❑ Collecting unidentified spectra for expanding data on currently annotated recurrent unidentified spectral libraries [1,2].

Materials & Methods

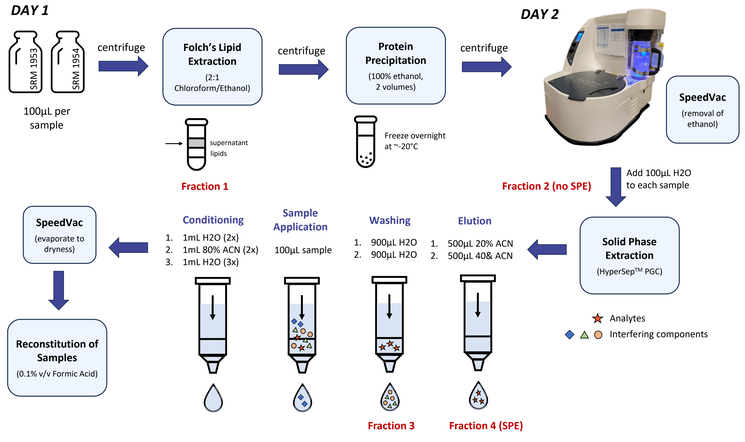

Figure 2. Sample preparation protocol modified for the identification of metabolites in Human Breast Milk SRM 1953 and 1954 [2]. A total of eight samples were prepared for LC-ESI-MS analysis. Porous Graphitic Carbon (PGC) SPE cartridges were used to concentrate the metabolites in the sample.

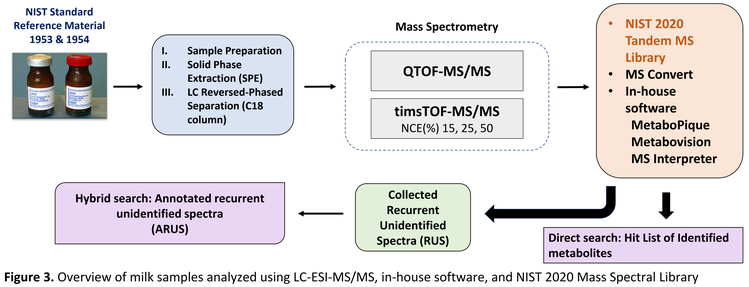

Figure 3. Overview of milk samples analyzed using LC-ESI-MS/MS, in-house software, and NIST 2020 Mass Spectral Library to generate a comprehensive list of metabolites for further characterization.

Results and Discussion

NIST Human Milk Standard Reference Materials

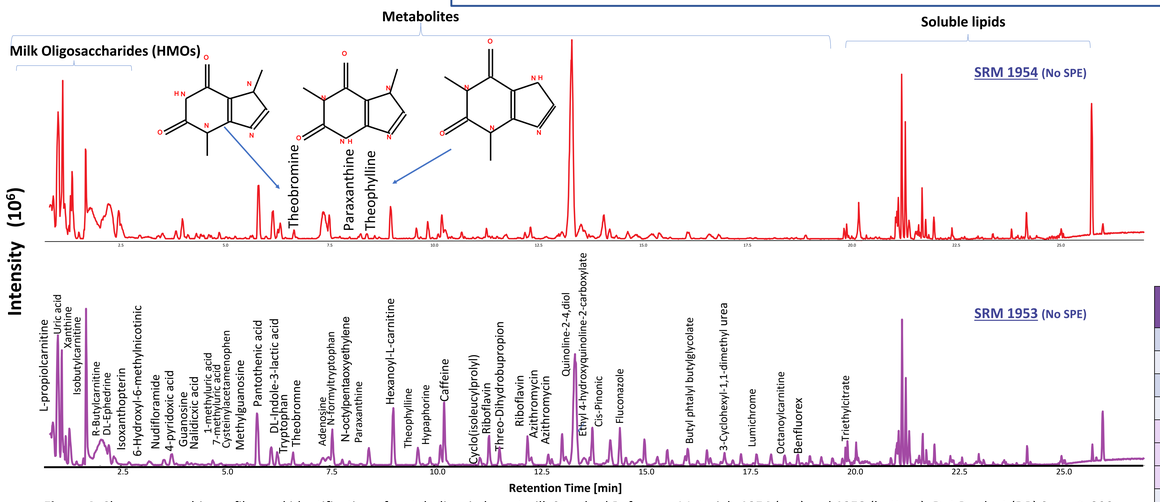

Figure 4. Chromatographic profiles and identification of metabolites in breastmilk Standard Reference Materials **1954** (top) and **1953** (bottom). Dot Product (DP) Score ≥ 800 with NCE 25%

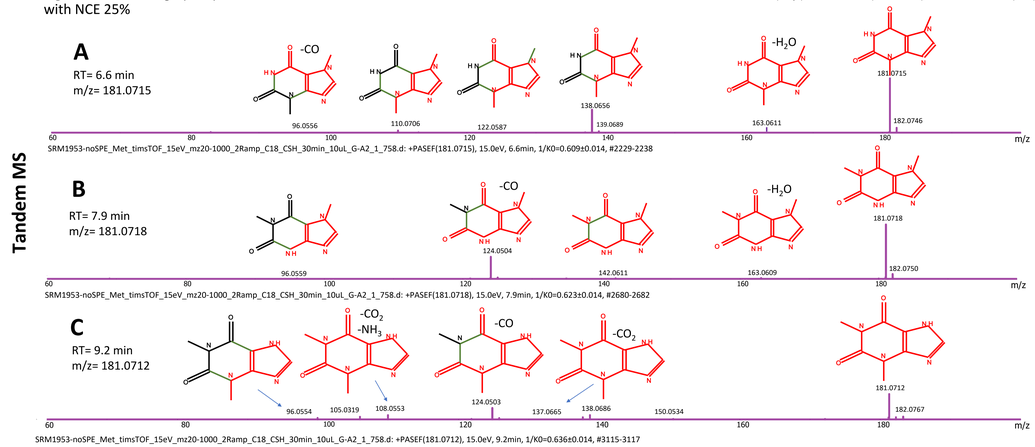

Figure 5. timsTOF-MS/MS of (A) **Theobromine** (B) **Paraxanthine** (C) **Theophylline** identified in breastmilk SRMs. An Illustration of fragment annotation using the NIST MS Interpreter.

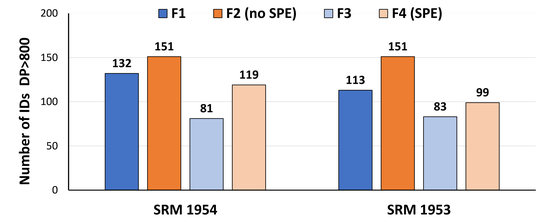

Figure 6. Tandem MS Library-based direct search identification of metabolites in human milk.

Table 1. Examples of identified metabolites abundant in breast milk SRM(s) 1954 & 1953.

◊Metabolite	Retention (min)	Precursor m/z (Exp.)	Adduct Type	*Predicted CCS (Å²)	*Experimental CCS (Å²)
D-Psicose	0.8	145.0484	[M+H-H2O]+	136.200	--
DL-Ephedrine	2.8	148.1107	[M+H-H2O]+	133.900	--
3-Methylxanthine	4.6	167.0552	[M+H]+	135.203	127.896
Uric acid	1.2	169.0344	[M+H]+	136.170	131.000
Theobromine	6.6	181.0715	[M+H]+	137.906	134.367
Paraxanthine	7.9	181.0718	[M+H]+	138.467	135.625
Theophylline	9.2	181.0712	[M+H]+	137.695	137.580
7-methyluric acid	4.2	183.0499	[M+H]+	131.013	--
1-methyluric acid	4.7	183.0499	[M+H]+	131.769	--
Hypaphorine	9.1	188.0689	[M+H]+	156.200	--
4-Pyridoxic acid	4.3	184.0587	[M+H]+	139.149	135.797
Caffeine	10.2	195.0863	[M+H]+	141.007	141.900
Pantothenic acid	6.1	220.1168	[M+H]+	149.659	148.126
R-Butyrylcarnitine	2.4	232.1529	[M+H]+	150.991	--
Nalidixic acid	4.5	233.0966	[M+H]+	150.200	145.251
Hexanoyl-carnitine	9.7	260.1837	[M+H]+	164.802	--
Fluconazole	14.3	307.1097	[M+H]+	168.800	163.378

*Human Metabolome Database (HMDB) https://hmdb.ca/metabolites/HMDB0001860
◊Dot Product ≥800

Discussion

- ➢ There are approx. 269 identified metabolites (DP >400) in SRM 1953 & 1954. There are only 117 breastmilk metabolites reported in the HMDB.
- ➢ Theobromine, paraxanthine and theophylline are examples of isomeric metabolites found in milk SRMs by library-based mass spectral searching. The 3 compounds are separated well by retention time. The fragmentation patterns of three isomeric metabolites (Fig. 4) were confirmed using the NIST MS Interpreter.
- ➢ Collision cross section (CCS) values (Table 2) are also different for the 3 isomers and constitute an important third dimension for identification.

Conclusions & Outlook

- ❖ Library-based mass spectral matching technique has proven effective in identifying untargeted metabolites in breast milk.
- ❖ Identified & annotated 269 metabolites in breast milk SRMs using high-resolution mass spectrometry, in-house bioinformatics tools & NIST 2020 Tandem MS Library.
- ❖ MS Interpreter has been a very useful tool in confirming the identity of metabolites found in human milk.
- ❖ For future work, 10K recurrent spectra are obtained from breast milk SRMs acquired from LC-timsTOF-MS/MS.
- ❖ "Hybrid" searching, extensive manual verification, and building a reference library of annotated metabolites for human milk.

References

[1] Simón-Manso, Y., et al. (2019). Mass Spectrometry Fingerprints of Small-Molecule Metabolites in Biofluids: Building a Spectral Library of Recurrent Spectra for Urine Analysis. *Analytical chemistry*, *91*(18), 12021. https://doi.org/10.1021/acs.analchem.9b02977

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[3] Burke, M. C., et al. (2017). The Hybrid Search: A Mass Spectral Library Search Method for Discovery of Modifications in Proteomics. *Proteome Res.* 2017, 16, 5, 1924–1935 https://doi.org/10.1021/acs.jproteome.6b00988