A METABOLOMICS APPROACH TO CHARACTERIZE RAW, PASTEURIZED AND ULTRA-HIGH TEMPERATURE MILK USING UPLC-QTOF-MS AND MULTIVARIATE DATA ANALYSIS

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INTRODUCTION

In this study UPLC-QTOF-MS integrated with multivariate data analysis was applied to investigate the impact of heat treatment on milk composition and its nutritional quality. Bovine milk is heat treated to pasteurized milk (185.0˚F, 15 s) and ultra high temperature (UHT) milk (278.6˚F, 15 s) prior to human consumption and before it is processed into a variety of dairy products. Metabolomics studies in milk poses significant analytical challenges as bovine milk is a complex mixture of proteins, lipids, vitamins, carbohydrates and metal salts. The high resolution mass spectrometry (HRMS) based metabolite profiling workflow used in this study provides identification and quantification of wide range of milk metabolites.

Three different milk samples (fresh, pasteurized and UHT milk, 10 replicates) and a pooled composite sample (1:1:1 ratio) were investigated. Samples from different groups were centrifuged to remove the top fat layer and the proteins in skimmed milk were precipitated. The supernatant was analyzed in a random order using UPLC-QTOF-MS in electrospray positive and negative modes.

Figure 1. Raw, pasteurized and UHT milk samples used in this metabolomics experiment.

METHODS

UPLC Conditions:

LC system : ACQUITY UPLC I-Class
Column temperature : 40 °C
Sample temperature : 10 °C
Injection volume : 5 mL
Flow rate : 0.50 mL/min
Mobile phase A : 0.1% Formic acid in water
Mobile phase B : 0.1% Formic acid in ACN
Column : HSS T3, 2.1*100 mm, 1.8 µm

Time	Flow	%A	%B	Curve
Initial	0.5	99	1	Initial
10.0	0.5	5	95	6
12.0	0.5	5	95	6
13.0	0.5	1	99	6
13.1	0.5	99	1	6
15.0	0.5	99	1	6

MS Conditions:

MS system : QTOF-MS
Ionization mode : ESI+/ESI-
Capillary voltage : 1.0 KV
Cone voltage : 30.0 V
Cone gas : 50 L/h
Source temperature : 150 °C
Desolvation temperature : 550 °C
Desolvation gas flow : 1000 L/h
Acquisition mode : MS^E
Low CE : 4 eV
High CE : 10-50 eV
Acquisition Range : 50-1200

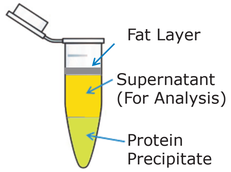
Informatics Software:

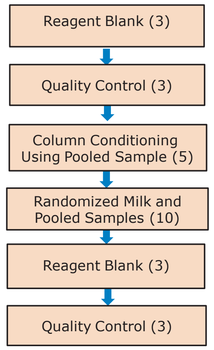
Data Acquisition: MassLynx
Data Processing: Progenesis QI

RESULTS AND DISCUSSIONS

Experimental Design for Non-Targeted Metabolomics

- A 2 mL sample aliquot was centrifuged (4,000 x g, 20 minutes, 4˚C) to remove the top fat layer.
- To 1 mL sample of skimmed milk, 3mL of pre-cooled methanol was added and centrifuged (14,000 ×g, 15 minutes, 4˚C) to precipitate proteins.
- Ten technical replicates of raw, pasteurized, UHT and a composite milk sample (1:1:1 mixture) were analyzed by UPLC/QTOF-MS in ESI+ and ESI− modes.





Chromatograms (ESI+) MS^E Acquisition (Precursor and Fragment Ion)

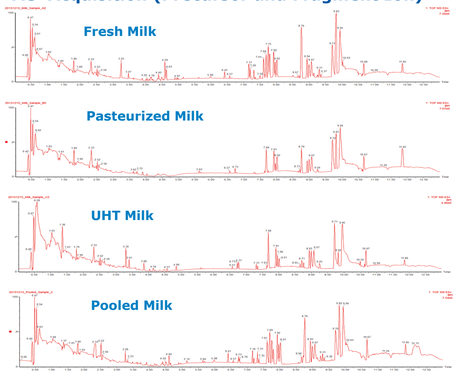
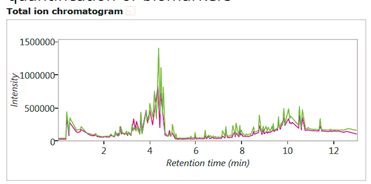


Figure 2. Chromatograms of raw, pasteurized, UHT and pooled milk samples using UPLC/QTOF-MS in ESI+ ionization mode.

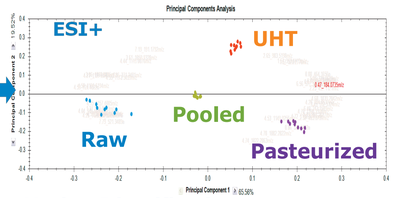
Peak Alignment and Peak Detection

Progenesis QI provides consistent peak alignment and peak detection for accurate quantification of biomarkers



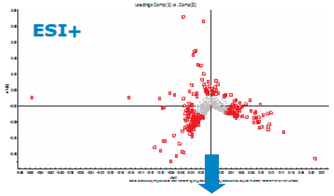
Principal Component Analysis

PCA plot shows good clustering of replicate milk and composite samples to illustrate statistically relevant dataset



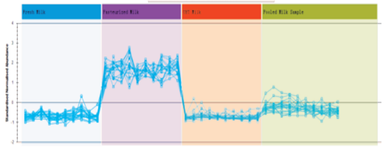
Marker Selection:

Loadings Plot helps identify prominent markers in different groups of samples which can be tagged for elucidation



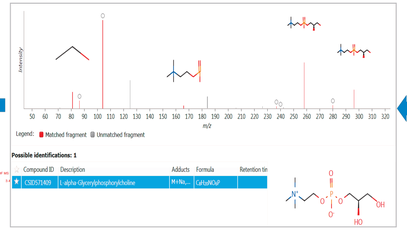
Marker Abundance Profile:

Prominent markers isolated from the S-plot and correlation analysis can be compared across the sample groups



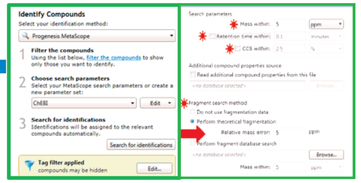
Marker Identification:

Accurate mass precursor and fragment ion data retention time and CCS values (with IMS) can be used for marker identification



Structure Elucidation:

Progenesis has tools for structural elucidation, determination of elemental composition and search capabilities using chemical databases such as metascope, chemspider, NIST, ChEBI and HMDB



Chromatograms (ESI-) MS^E Acquisition

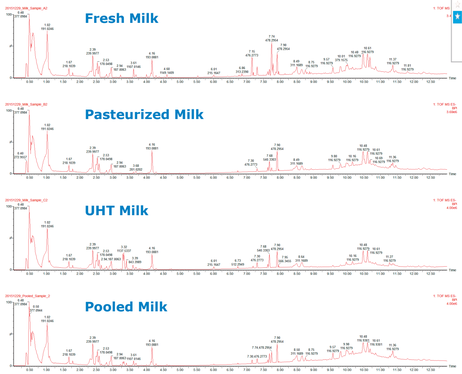


Figure 3. Chromatograms of raw, pasteurized, UHT and pooled milk samples using UPLC/QTOF-MS in ESI− ionization mode.

CONCLUSIONS

- **A combination of UPLC separation, QTOF-MS detection and informatics (Progenesis QI) was used to identify metabolites in different milk samples to evaluate the impact of heat treatment on milk metabolites.**
- **Method development and proof of principle is demonstrated. Several metabolites that show significant differences between raw, pasteurized and UHT milk were identified for further validation using targeted UPLC-MS/MS methods.**

Description	Compound (m/z)	Adducts	Formula	Score (out of 60)	Mass Error (ppm)	Isotope Similarity	Max Fold Change
Glycerophosphocholine	257.1032n	M+K, M+Na	C8H20NO6P	50.8	1.48	95.04	65.43
Cytosine	112.0510m/z	M+H	C4H5N3O	43.9	3.79	98.47	20.88
6-Hydroxynicotinic acid	140.0343m/z	M+H	C6H5NO3	54.6	0.86	97.99	1423.50
Xanthylic acid	364.0414n	M+Na, M+K, M+H	C10H13N4O9P	40.3	-1.71	95.00	16.89

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