

THE FRAMEWORK PROGRAMME FOR RESEARCH AND INNOVATION

# HORIZON 2020

LC-MS Tools in The Campaign Against Food  
Fraud in Infant Formula

Di Wu, PhD.  
ZJTH

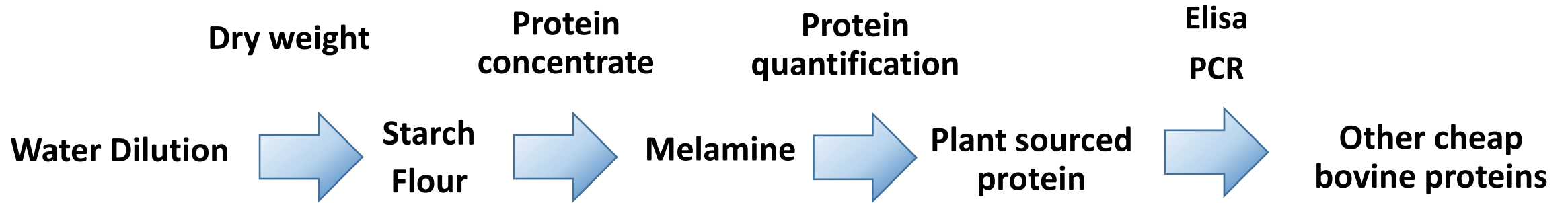


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# Economic Motivated Adulteration

- Logic development of adulterations in milk



- Even though authenticity issues have been considered less hazardous since it does not always end up in risking human health, but in some cases, it can develop into destructive and notorious events. Infants are especially more vulnerable with risks from insufficient or imbalanced nutrition intake due to their under developing digestion and immune system.



# Effects of Post Melamine Scandal

- A catastrophe towards local dairy industry: Chinese parents completely lost their trust over Chinese made infant products.



## Parents outraged as Chemist Warehouse buys into Chinese baby formula racket

CHEMIST Warehouse has begun offering direct shipping to China, fuelling parents concerns about the black market in baby formula.



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# Why is It Possible for Melamine Scandal to Explode in China?

- Kjeldahl nitrogen method is insufficient in specificity
- Fanatic and wild growth of local dairy industry
- The gap between supplement and demand of fresh milk
- Loophole inside the governmental surveillance and regulatory systems



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# International Standards of Proteins for Infant Formula

- **EU- 2006/141/EC** Protein concentration : **1.8-3.0 g/kcal**
- Human breast milk amino acid content as reference.
- **USFDA- 21 CFR 106/Docket No. FDA-2013-N-0067**
- Protein concentration : **1.8-4.5 g/kcal。**
- **Australia and New Zealand- Standard 2.9.1**
- Protein concentration: **0.45-0.7 g/kcal**
- **Protein quantification via Kjeldahl nitrogen method is still using as the only standard parameter for infant formula**

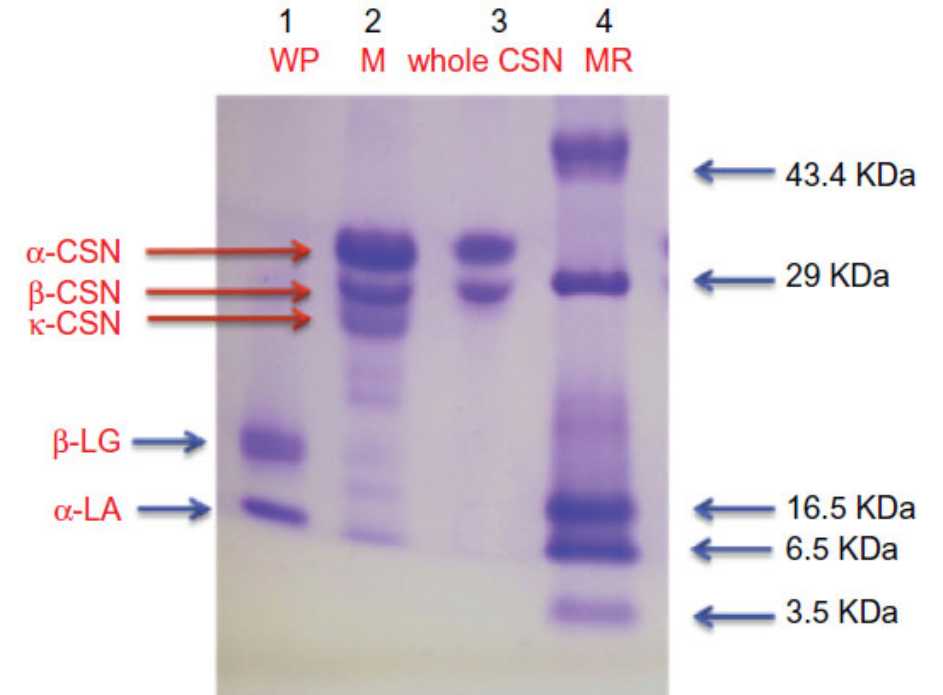


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# Chinese Standards of Proteins for Infant Formula

- **GB 10765** is the standard of proteins for Infant formula in China
- Protein concentration : 1.88-2.93 g/kcal
- It clearly stated **the whey protein content should be >60%**
- **GB/T 5413.2** is the corresponding quantification method of whey protein via **SDS-PAGE-1997**



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# Recent AOAC SDS-CGE Method for Whey Protein



**EUCHINASAFE**  
**中欧食品安全**



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# Five key products



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## T3.1.2 Dairy-Infant formula

- Involved: ZJTH, QUB, CFSA, Danone (China), Nestle (China), Yili, USP



# Potential Fraud Issues in Infant Formula

- **None-protein**

- Nitrogen-rich compounds
- Melamine like
- Carbamide like
- Purine like
- Others like



- **Protein**

- Cheap animal protein
- Cheap milk protein
- **Plant protein**
- Soy bean
- Pea
- Other proteins



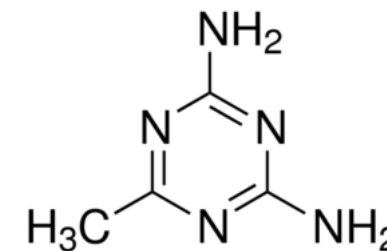
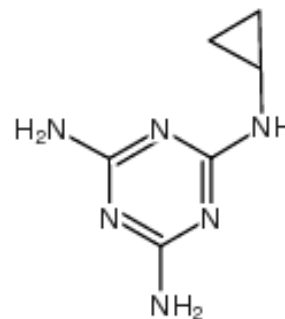
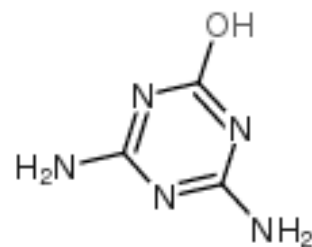
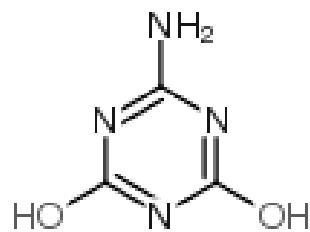
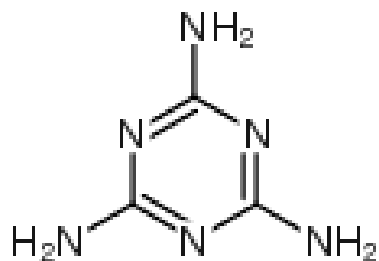
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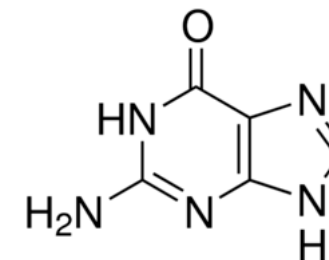
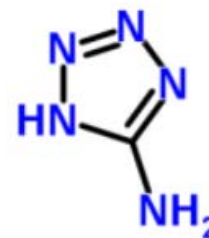
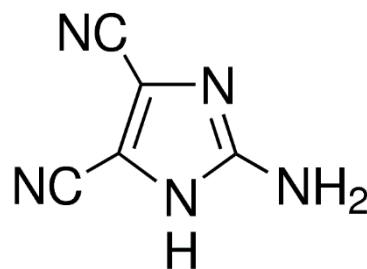
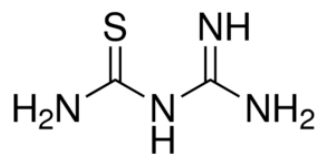
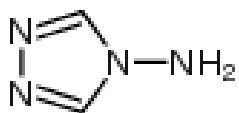
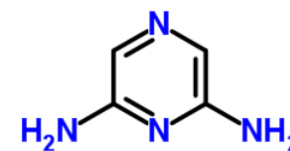
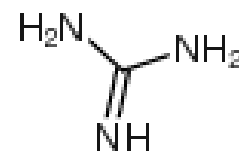
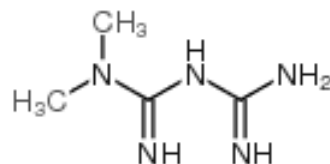
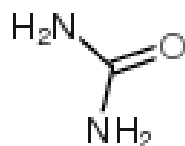
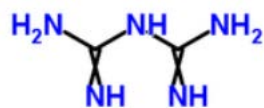
# Nitrogen-rich compounds LC-MS Database

- **USP: FCC11 -GUIDANCE STANDARD FOR UHPLC-MS/MS SCREENING OF NITROGEN-CONTAINING ADULTERANTS IN MILK INGREDIENTS (16 Amino Acids+ 25 N-rich compounds)**
- **SN/T 5071-2018 出口食品中19种非蛋白含氮化合物的测定 液相色谱-质谱/质谱法 (19 N-rich compounds, 18 contained in USP FCC except Aminotriazinone)**
- **Frank N, Bessaire T, Tarres A, Goyon A, Delatour T. Development of a quantitative multi-compound method for the detection of 14 nitrogen-rich adulterants by LC-MS/MS in food materials. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 2017 Nov;34(11):1842-1852. (14 compounds quantitative methods in several food matrix)**
- **Our Goal: A database consisted of 50-70 compound together with sample prep and UHPLC-MS fast screening/Qualification methods semi-Quantification.**

# Nitrogen-rich compounds of 55 compounds



## Melamine-like

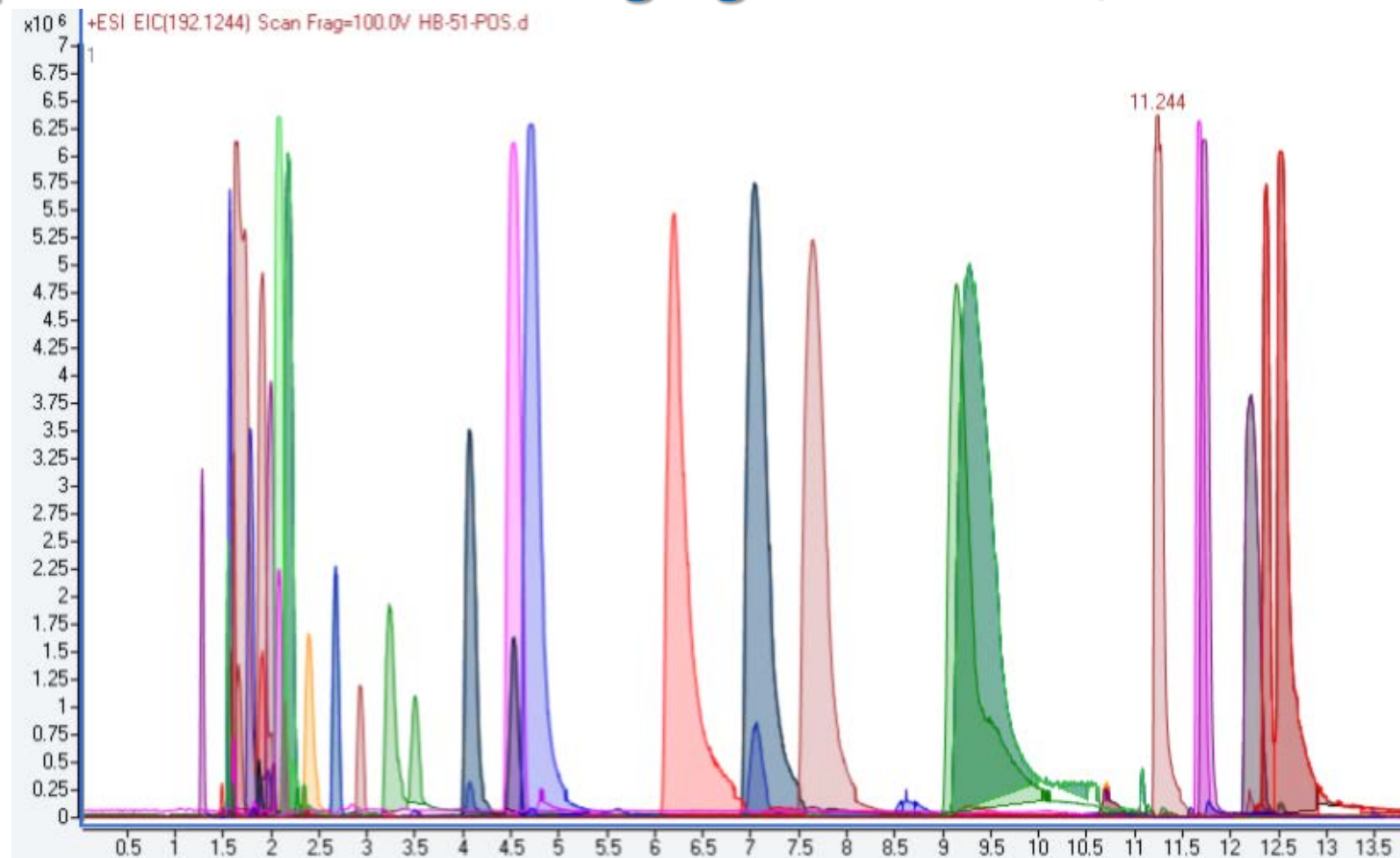


## None-Melamine-like

# Nitrogen-rich compounds database using Agilent 6545 Q-TOF



Agilent 6545  
LC-Q-TOF

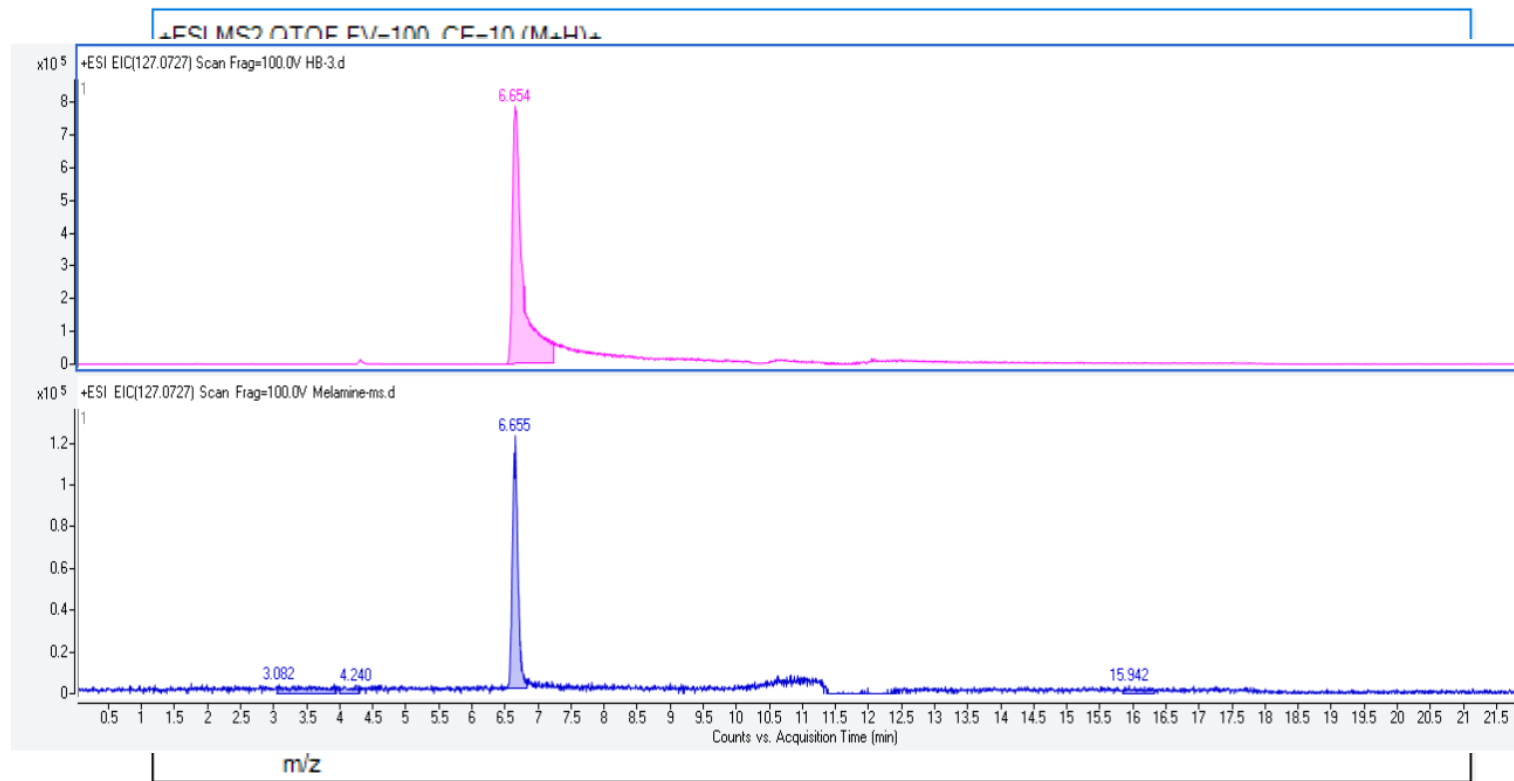
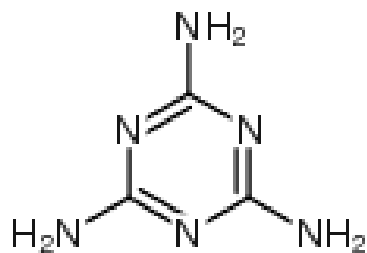


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# Melamine

Melamine  
C<sub>3</sub>H<sub>6</sub>N<sub>6</sub>  
m/z = 126.06539

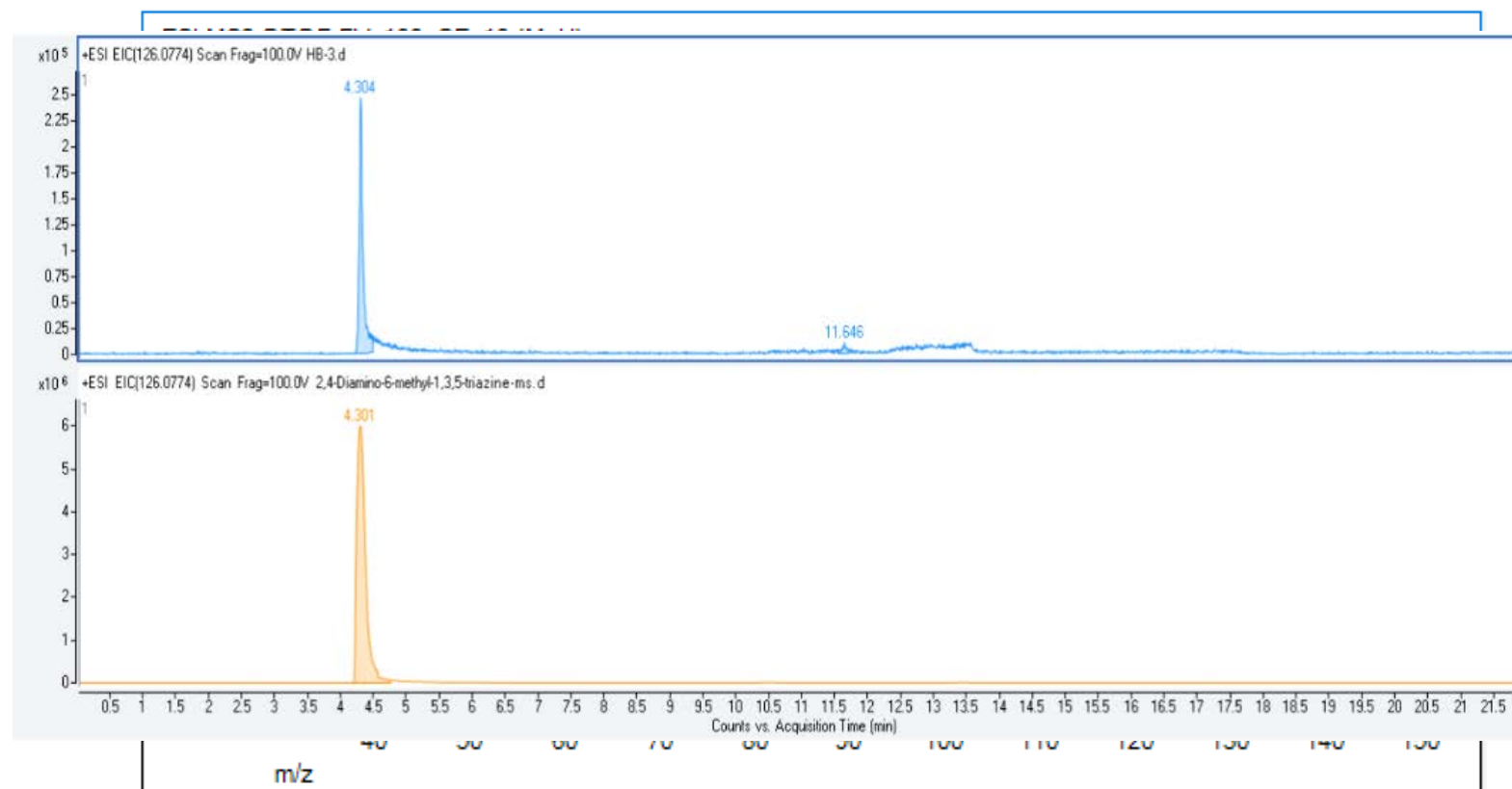
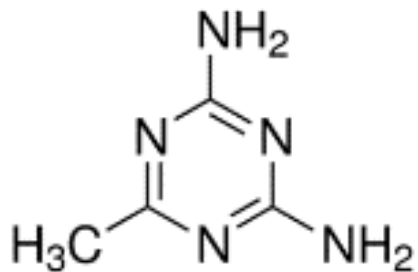


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# Melamine Like Compounds

2,4-Diamino-6-methyl-  
1,3,5-triazine  
 $C_4H_7N_5$   
 $m/z=125.07015$

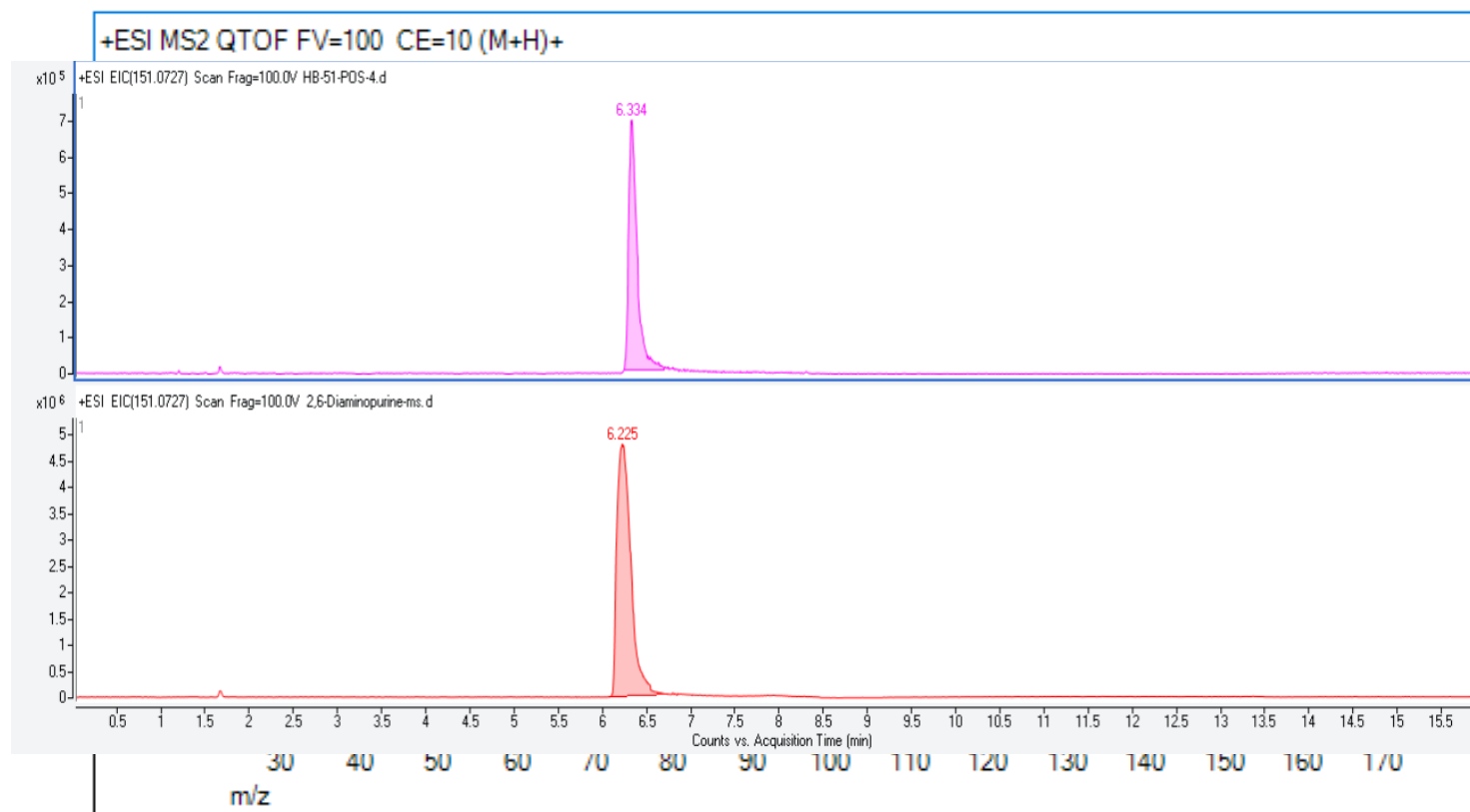
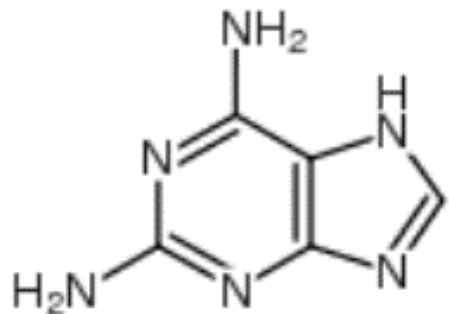


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# None-Melamine Like N-rich Compounds

2,6-Diaminopurine  
C<sub>5</sub>H<sub>6</sub>N<sub>6</sub>  
m/z=150.06539

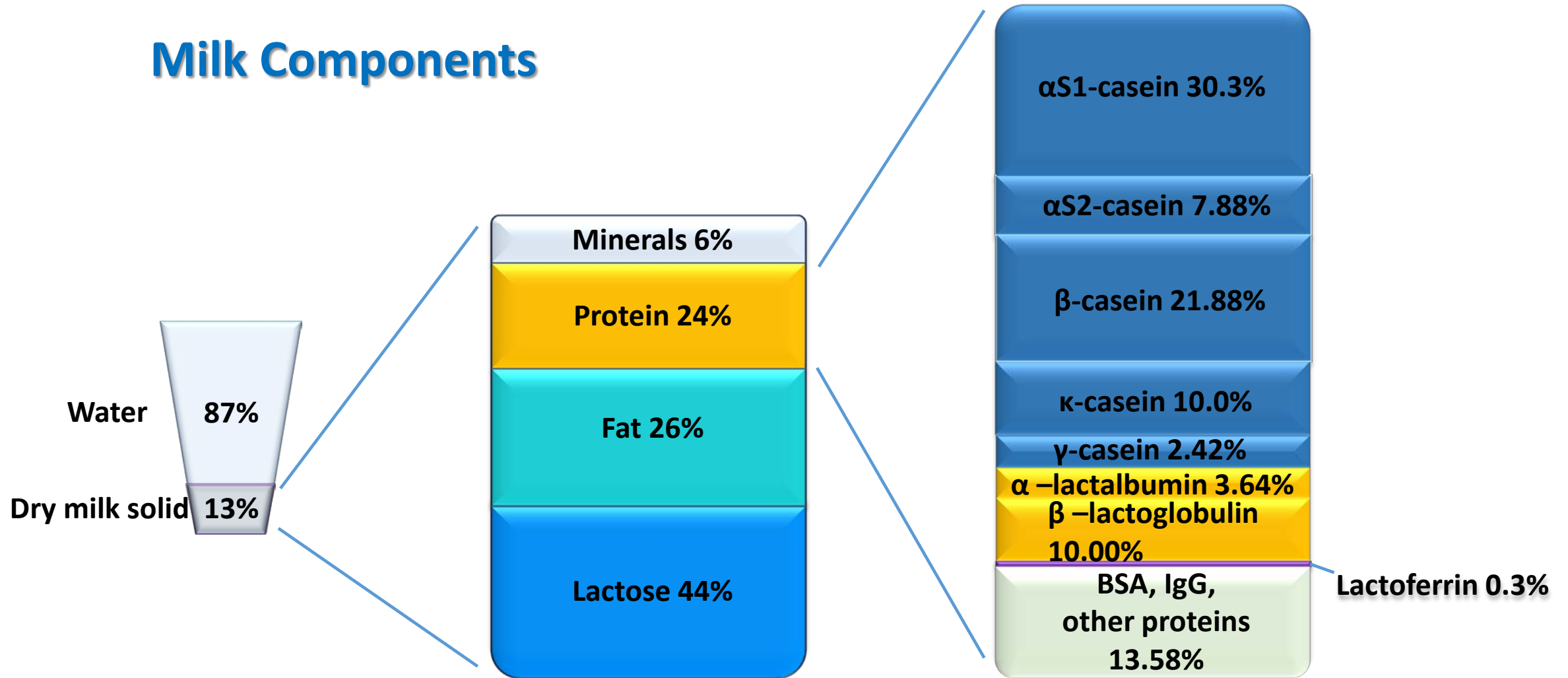


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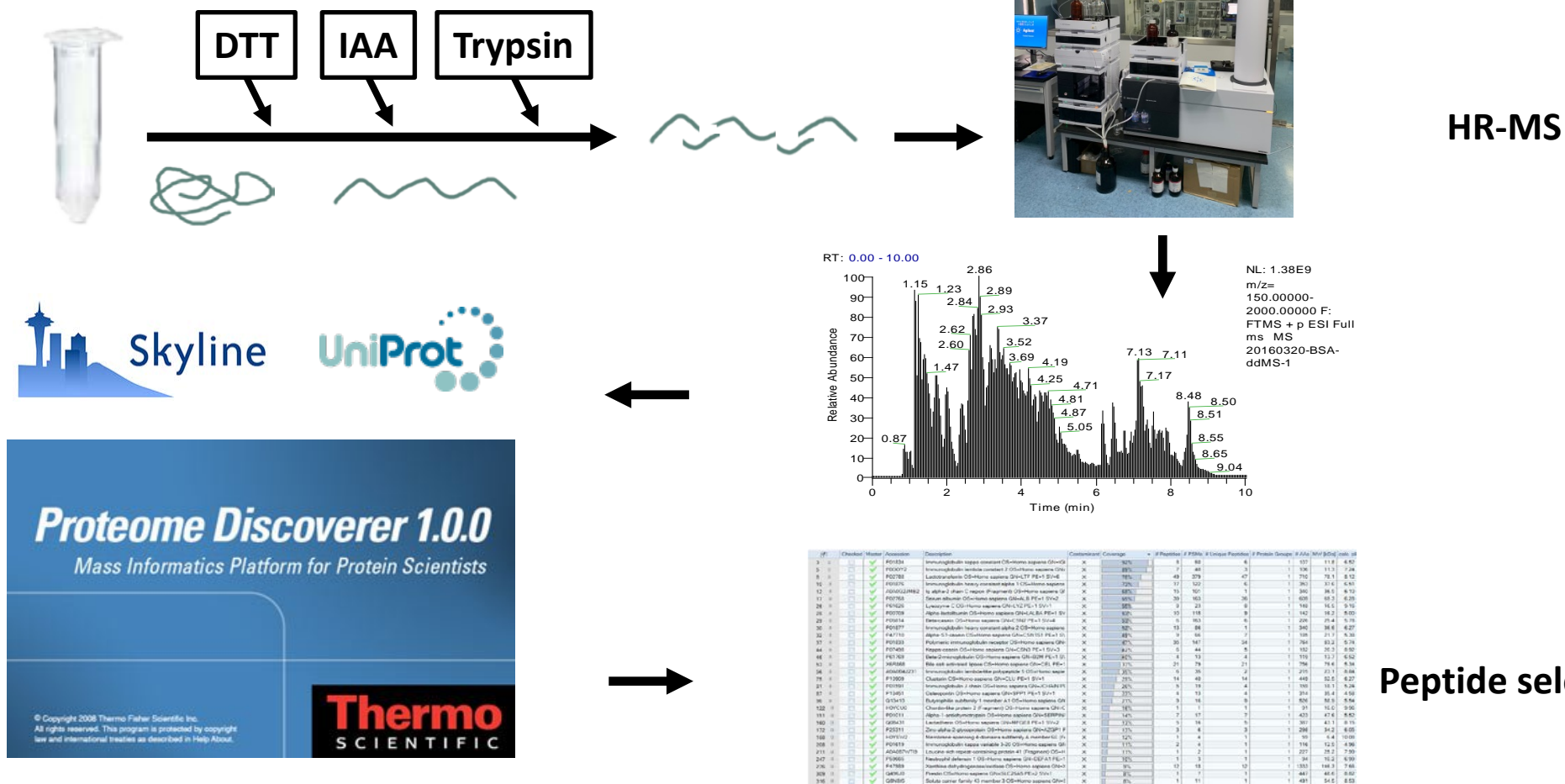




# Milk Components



# Workflow of Targeted Proteomics

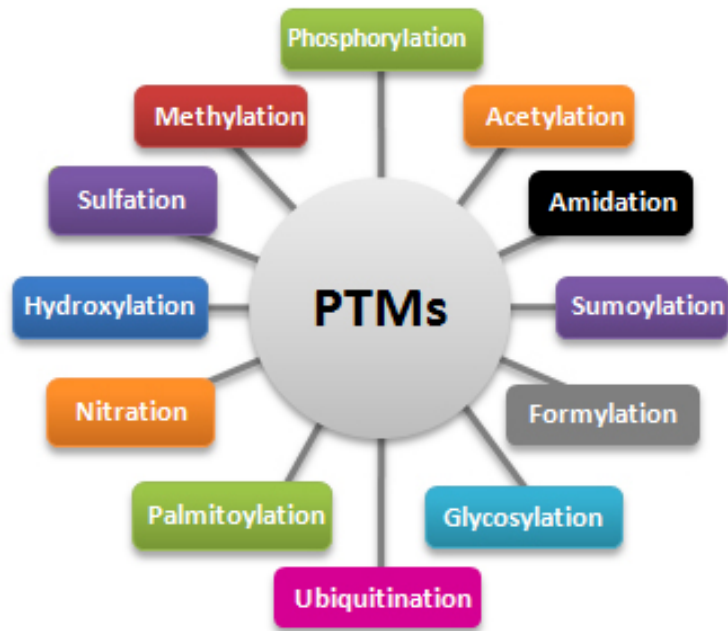


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# Why Peptides(Bottom-Up) not Intact Proteins(Top-down)?

- Post-translational modifications (PTMs)



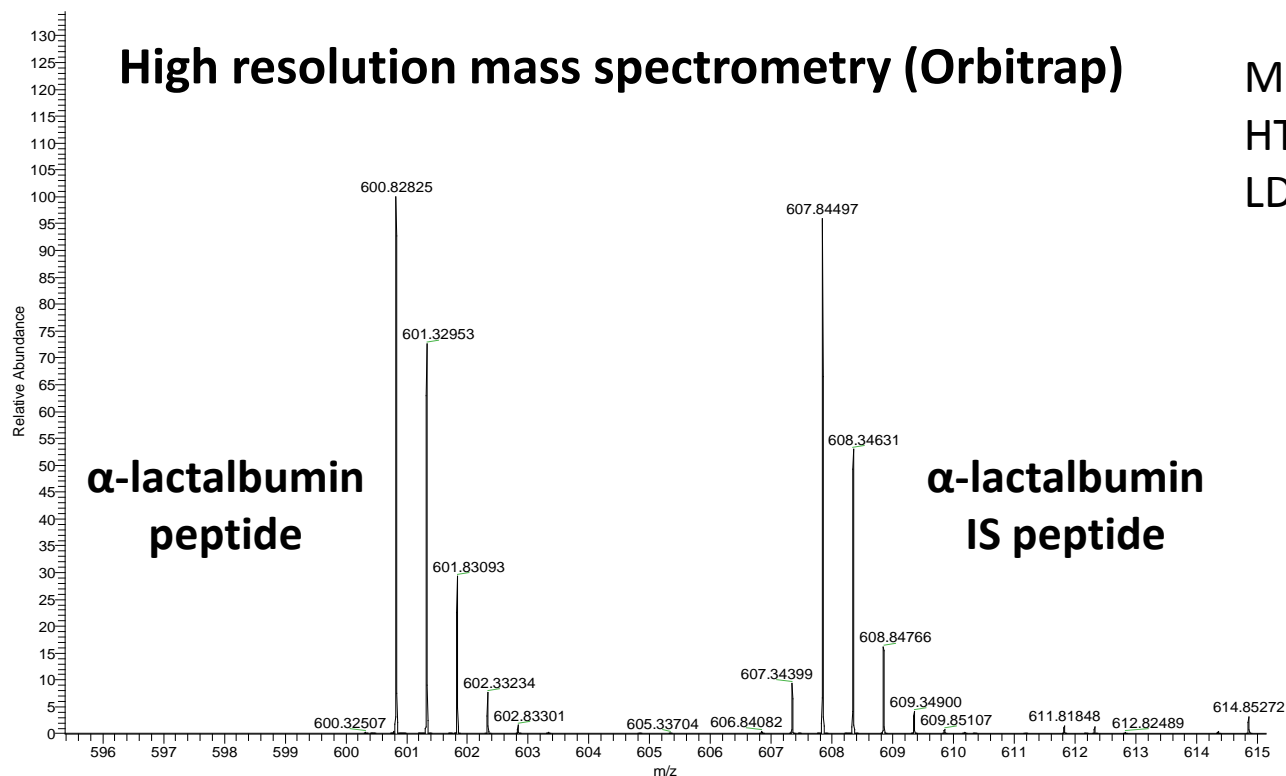
- Multiple Charges (Change in  $m/z$ )
- Multiple Isotopes
- Poor performance of MS detector for large molecules ( $>5000$   $m/z$ )
- Matrix effect in quantification



# Quantification Using Stable Isotope Dilution (SID) Assay

20150215-002 #320 RT: 2.65 AV: 1 NL: 6.86E7  
T: FTMS + p ESI Full ms [150.00-2000.00]

## High resolution mass spectrometry (Orbitrap)



## $\alpha$ -lactalbumin sequence:

MMSFVSLLLVGILFHATQAEQLTKCEVFRELKDLKGYGGVSLPEWVCTTF  
HTSGYDTQAIQVQNNDSTEYGLFQINNKIWCKDDQNPSSNICNISCDF  
LDDDLTDDIMC**VKKILDKVGINYWL**AHKALCSEKLDQWLCEKL

**VKKILDKVGI\*NYWL\*AHKALCSEKL**

Trypsinization

**VGI\*NYWL\*AHK**

Amino acid with \*: U-  $^{13}\text{C}$ ,  $^{15}\text{N}$  labeled

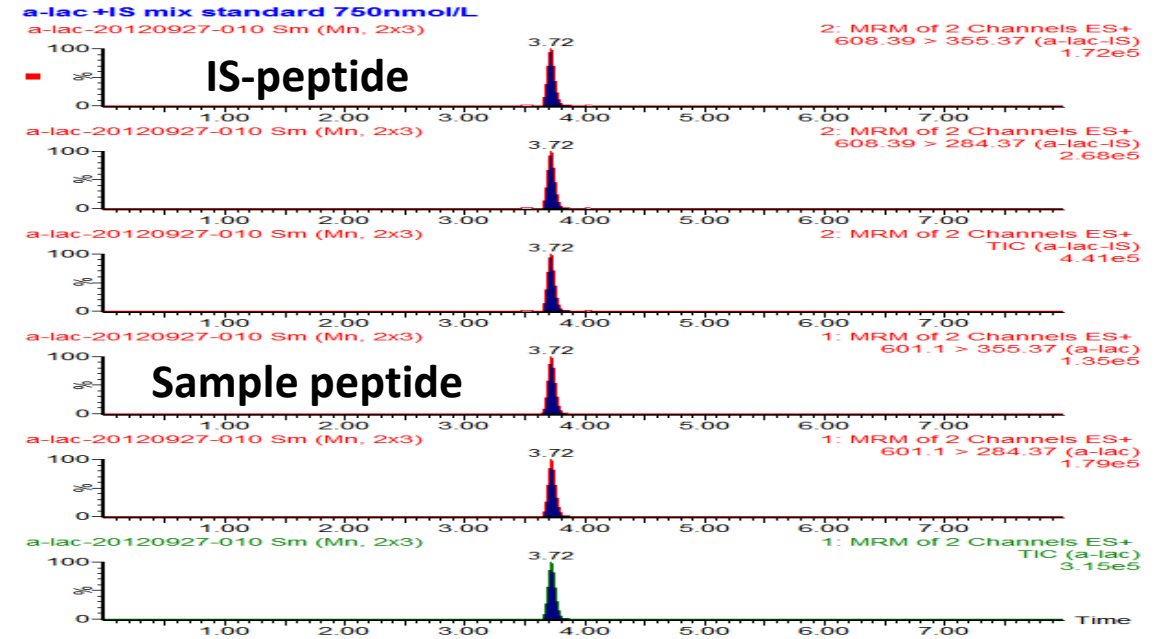
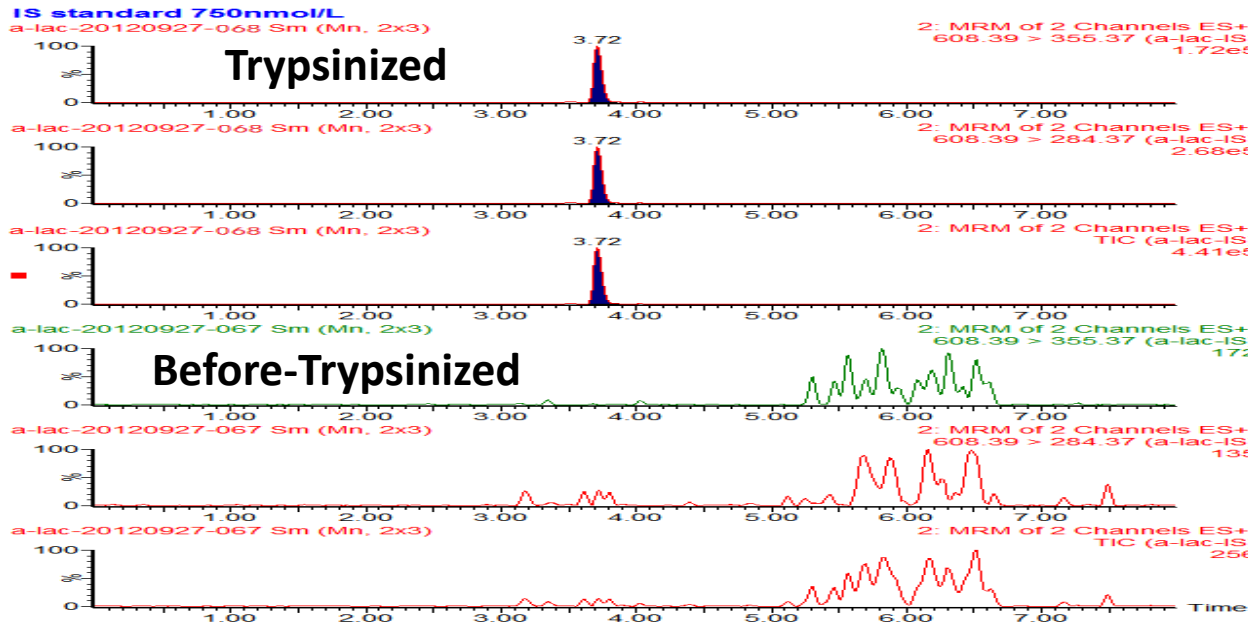


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# Quantification Using Stable Isotope Labeled Peptide

## Low resolution mass spectrometry (TQ-XS)

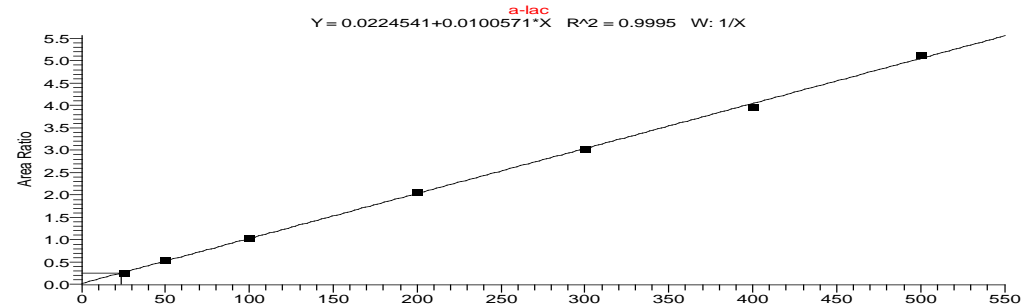
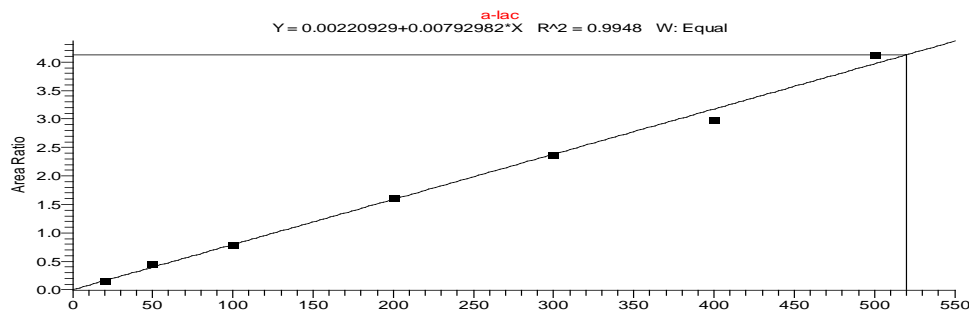


# Linear Range of SID Assay

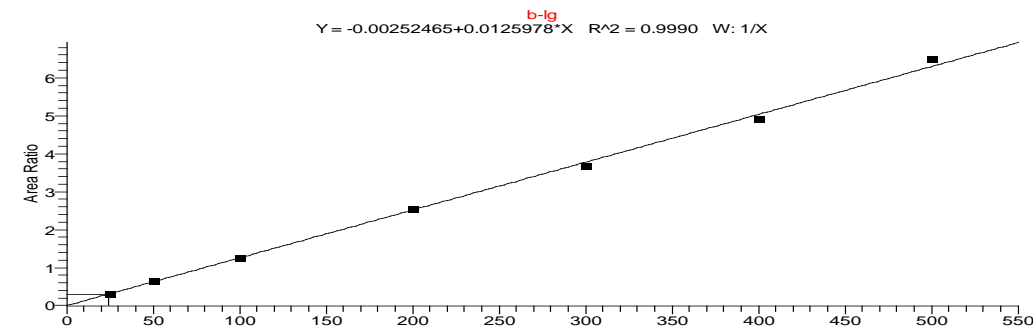
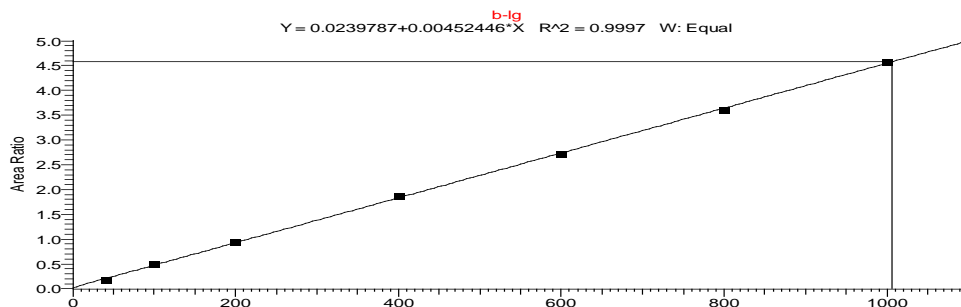
## Full scan

## SIM scan

$\alpha$ -lactalbumin



$\beta$ -lactoglobulin

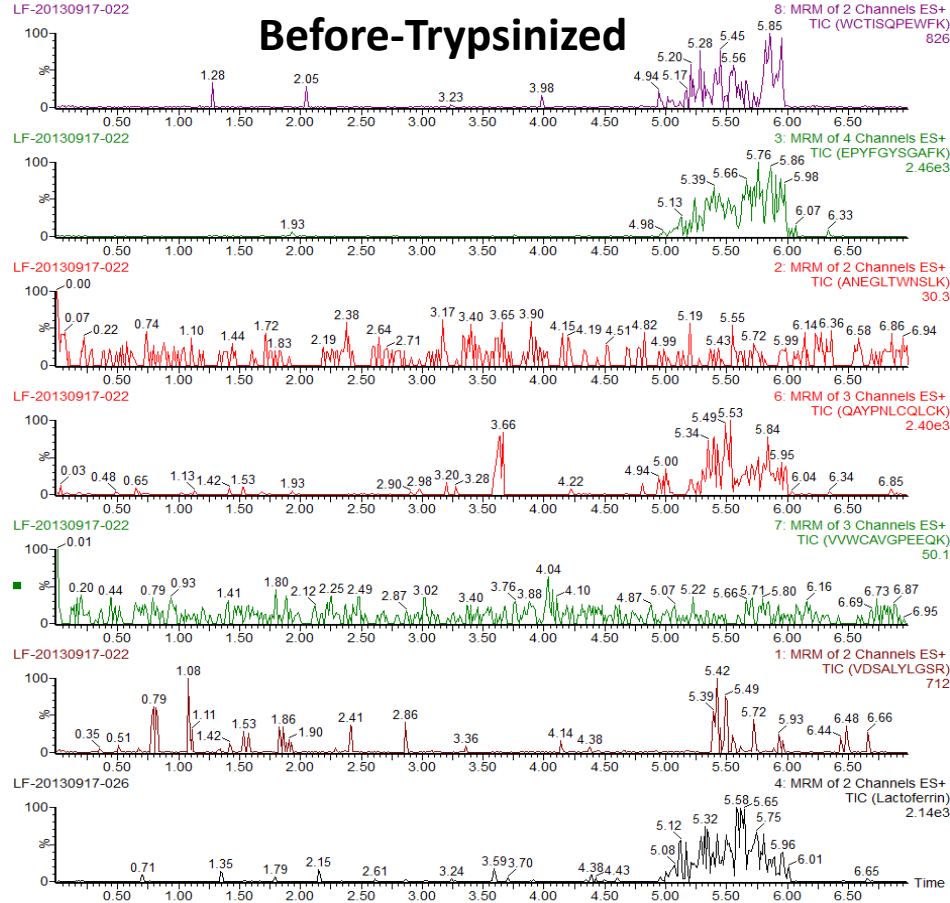


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# Lactoferrin

LF STDs 15ug/ml before enzymolysis



WCTISQPEWFK

EPYFGYSGAFK

ANEGLTWNSLK

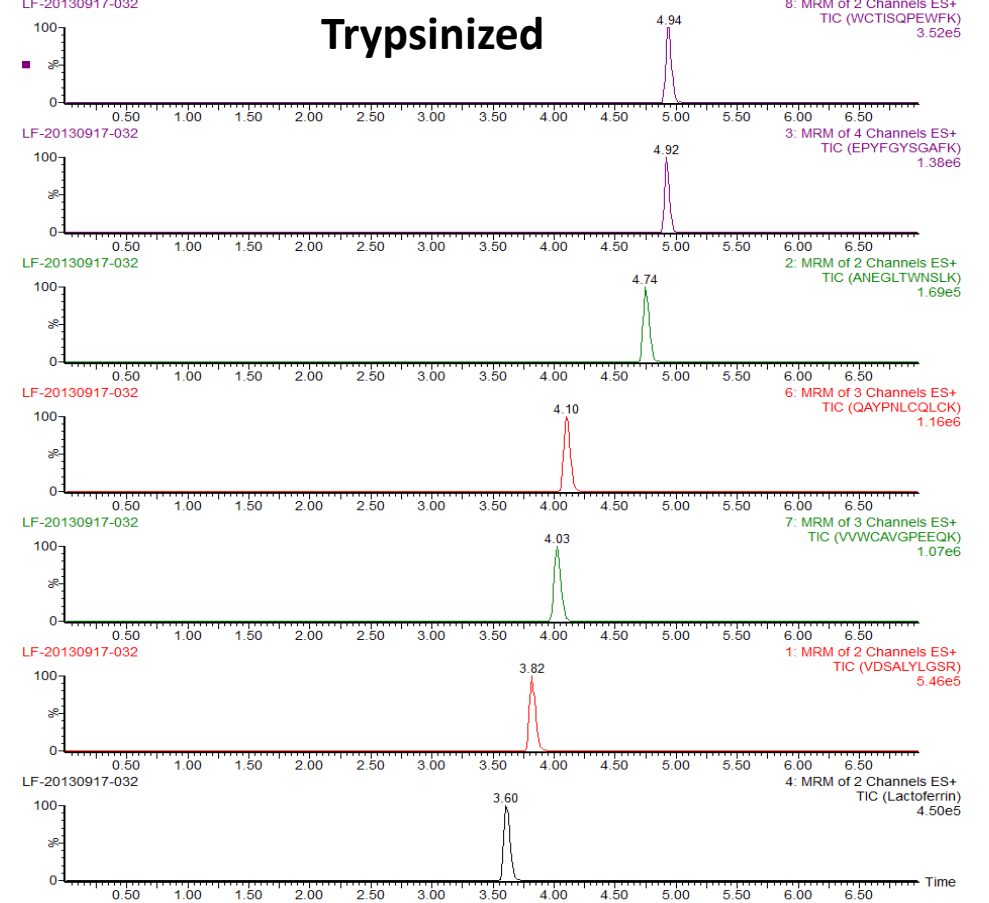
QAYPNLCQLCK

VWCAVGPEEQK

VDSALYLGSR

LPRVAAEIYGTK

LF STDs 15ug/ml



Trypsinized



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# Different milk protein components between cow and sheep/goat

## $\alpha$ -lactalbumin

ALBA\_BOVIN MMSFVSLLLV GILFHATQAE QLTKEVFR LKDLKGYGGV SLPEWVCTTF  
 ALBA\_SHEEP .....Q.....D.....A.  
 ALBA\_CAPHI .....QK.....D.....A.  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ALBA\_BOVIN HTSGYDTQAI VQNNDSLEYG  
 ALBA\_SHEEP .....V.....A.....  
 ALBA\_CAPHI .....V.....A.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ALBA\_BOVIN DDDLTDIMC VKKILDKVGI  
 ALBA\_SHEEP .....V.....A.....  
 ALBA\_CAPHI .....V.....A.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

LACB\_BOVIN MKCLLLAL-- ALTCTGAQALI  
 LACB\_SHEEP .....GL..A..V..I..  
 LACB\_CAPHI .....GL..A..I..I..  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

LACB\_BOVIN DAQSAPLRVY VEELKPTPEG  
 LACB\_SHEEP .....V.....A.....  
 LACB\_CAPHI .....V.....A.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

LACB\_BOVIN KIDALNENKV LVLDTDYKKY  
 LACB\_SHEEP .....T.S.E.....D.....  
 LACB\_CAPHI .....T.S.E.....D.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

LACB\_BOVIN LEKFDKALKALPMHIFLSFN  
 LACB\_SHEEP .....A.....A.....  
 LACB\_CAPHI .....A.....A.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA2\_BOVIN SKKTVDMEST EVFTKTKLKT EEEKNRLNPL KKISORYOKF ALPOYLKTVY  
 ASA2\_SHEEP .....I.....Y.....W.....D  
 ASA2\_CAPHI .....I.....Y.....W.....D  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA2\_BOVIN OHOKAMKPWI QPKTKVIPYV RYL  
 ASA2\_SHEEP .....T.....NA.....  
 ASA2\_CAPHI .....T.....NA.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

## $\alpha$ S1-casein

CASB\_BOVIN MKVLILACLV ALALARELEE LNVPGEIVES LSSSEESITR INKKIEKFQS  
 ICASB\_SHEEP .....O.....V..T.....H.....  
 ICASB\_CAPHI .....I.....V..P.....V.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

CASB\_BOVIN EEQQQTEDEL QDKIHFFACT QSLVYFFPGF IPNSLPCNIP PLTQTFVVVP  
 ICASB\_SHEEP .....A.....A.....T.....L.....  
 ICASB\_CAPHI .....A.....A.....T.....L.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA1\_BOVIN KVNELSKDIG SESTEDOAME DIKOMEAESI SSSEEIVPNS VEOKHIQKED IQPLPPTV MFPPQSVLSL SQSKVLPVPO KAVYPQORDM  
 ASA1\_SHEEP .....E.....A.....K.G.S.....A.....Y.....R.....T.V.....T.....  
 ASA1\_CAPHI .....N.....I.....A.....K.G.S.....A.....Y.....R.....T.V.....T.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA1\_BOVIN VPSERYLGYL EQLRLRKKYK VPOLEIVPNS AEERLHSMKE GIHAQOKEPM LGEVIRGPF EIIIV  
 ASA1\_SHEEP .....N.....K.....O.....NP.H..O..  
 ASA1\_CAPHI .....N.....K.....O.....NP.H..O..  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA1\_BOVIN IGVNRELAYF YPELFRGFYQ LDAYPSGAWY YVPLGTOYTD APFSFDIPNP  
 ASA1\_SHEEP .....O.....O.....L.....L.....  
 ASA1\_CAPHI .....O.....O.....L.....L.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA2\_BOVIN MKFFIFTCLL AVALAKNTME HVSSEESI- ISOETKQEK NMAINPFSKEN  
 ASA2\_SHEEP .....HK.....P.N.....I.....H.R..K  
 ASA2\_CAPHI .....HK.....P.N.....F..I.....H.R..K  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA2\_BOVIN LCSTFCKEVV RNANEEYSI GSSEESAEV ATEEVKITVD DKHYOKALNE  
 ASA2\_SHEEP .....T.S.E.....D.....R.....P..I.....  
 ASA2\_CAPHI .....T.S.E.....D.....R.....P..I.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

ASA2\_BOVIN INQFYOKFPO YLOYLYOGPI VLNPWDOVKR NAVPITPTLN REQLSTSEEN  
 ASA2\_SHEEP .....P.....G.F..V.....G.F..V.....  
 ASA2\_CAPHI .....P.....G.F..V.....G.F..V.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

## $\alpha$ S2-casein

ALTLPLFLG AQEQNOEQPI RCEKDERFFS DKIAKYIPIQ  
 .....R.....C.....D.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

YQOKPVA LINNOFLPYF YYAKPAAVRS PAQLLOWQVL  
 .....R.....R.....V.....T.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

PTMARHP HPHLSFMAIP PKKNODKTEI PTINTIASGE  
 .....L.....D.....D.....V.....A.....  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*

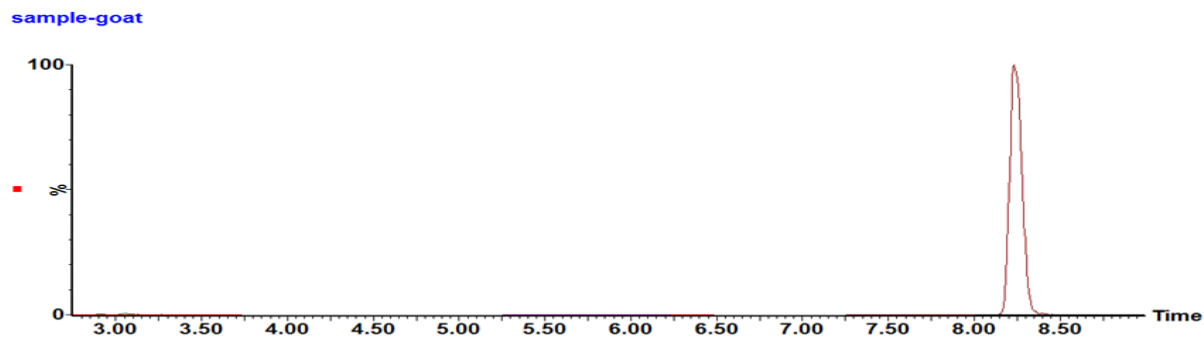
ESTVATLE DSPEVIESPP EINTVQVTST AV  
 /NA.DNP A.S.S.A.A. T.A.....E.  
 /N..DNP A.S.S.A.A.S T.A.....E.  
 Identity \*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*\*\*\*\*:\*



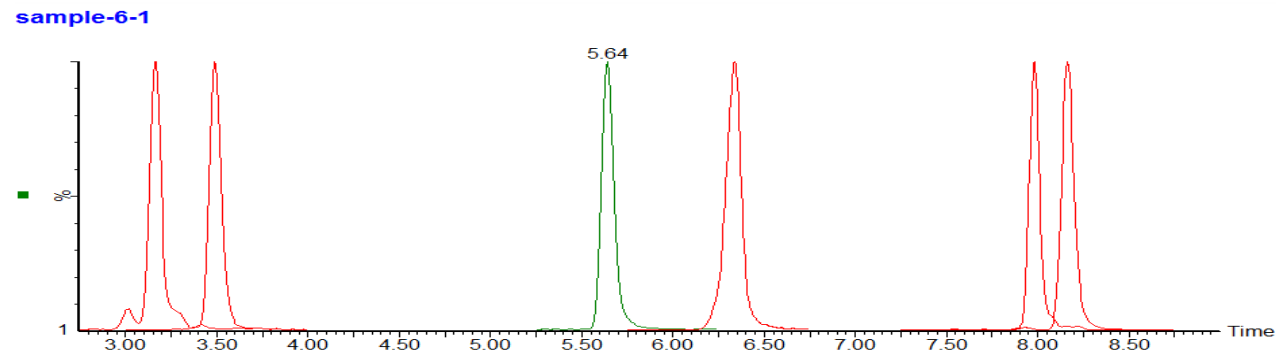
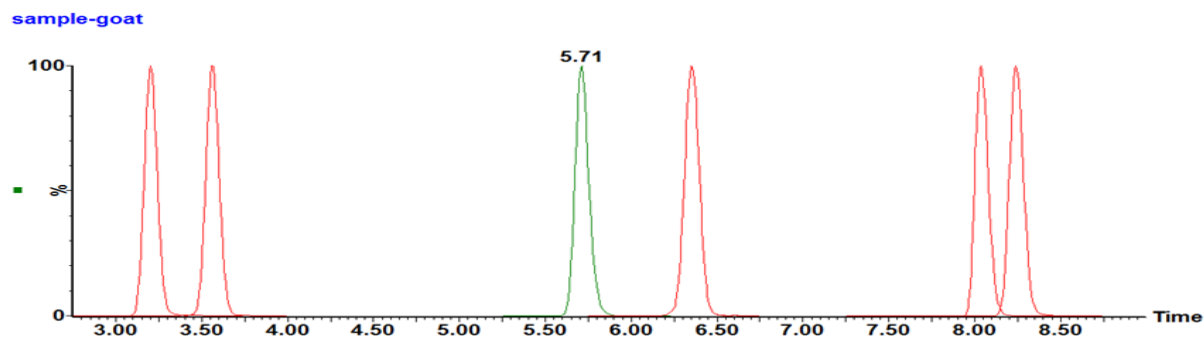
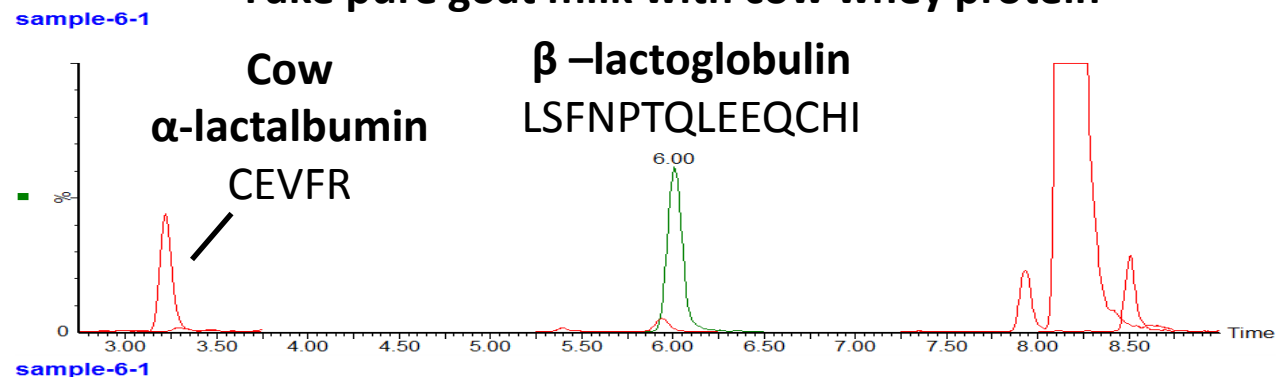


# LC-MS applied in milk authentic analysis

## Real pure goat milk



## Fake pure goat milk with cow whey protein

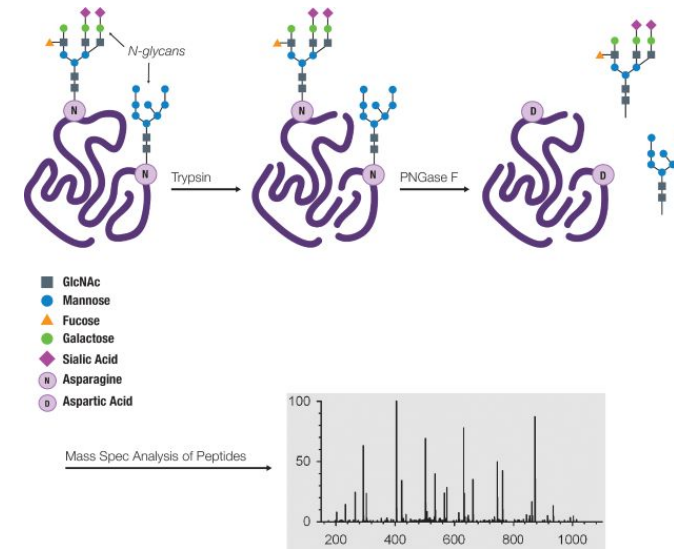


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# Issues We are Still Facing

- Glycosylation of proteins of Maillard reaction- Ketosamines and Acrylamide
- PNGase F
- Highly variable proteins: IgGs
- Large sizes proteins: BSA
- Metal ion- conjugation proteins
- Hydrophobic : Milk fat globule membrane protein



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# Future Plans

- LC-MS method developing for minor functional protein contents in cow milk
- Database for potential adulterate nitrogen compounds and plant protein
- Other possible enzymes replacing trypsin in conventional proteomic studies
- Chinese human breast milk nutrition studies- A formula most suitable for Chinese babies
- Allergen and mycotoxin detection in dairy products
- Milk nutrition studies of other mammalian species ( Yak, camel, buffalo, horse, donkey,)
- Rapid screening method via portable spectrometry



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# Acknowledgement



**中华人民共和国科学技术部**

Ministry of Science and Technology of the People's Republic of China



**国家自然科学基金委员会**

National Natural Science Foundation of China



European  
Commission



**中国博士后科学基金会**

China Postdoctoral Science Foundation



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