Identification of Proteins in Raw Materials and Finished Goods by LC/MS/MS

UNPA Dietary Supplement Analytical Summit

November 6, 2015

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History/Current Practice to Identify Proteins

- Total nitrogen content methods have long been used to assay proteins
- Kjehldahl and Dumas methods are most common
- Result Interpretations assume source of nitrogen comes from proteins
- Assumption has been exploited:
  - 2007 scandal with melamine – nitrogen-rich, but not a protein – spiked into pet food, infant formula, etc
  - Inexpensive amino acids can be added to increase nitrogen content
- Methods do not distinguish between different types of proteins
- These methods are still accepted and widely used today

Other Methods to Identify Proteins

- Organoleptic (subjective)
- Relative comparison of amino acids by LC/UV (not selective for finished products)
- Colorimetric/fluorescent kits (not selective; does not distinguish between types of proteins)

Raising of the Bar for Testing

- Agencies are increasing activity to enforce exactly what is on labels, which is requiring more accurate and precise testing
- Consumers are being influenced by recent events and becoming more knowledgeable
- Current industry practice for analysis of protein testing is vulnerable to exposure in the future

Alternative Approach

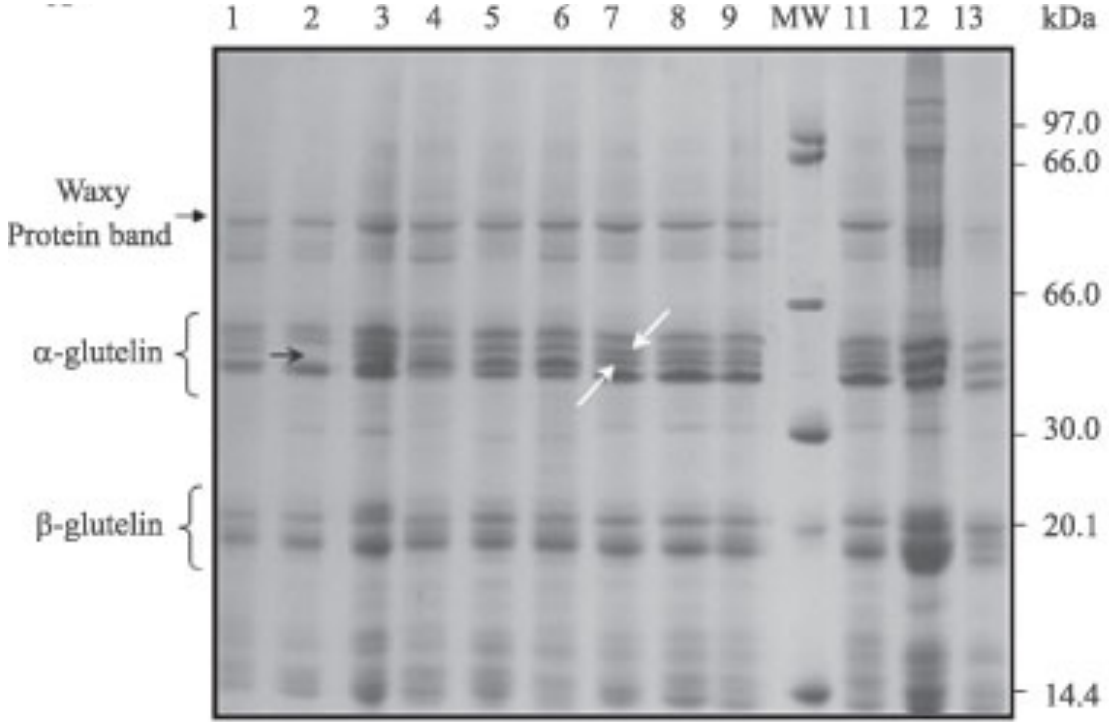
- Selection of Specific Protein
- Sample Preparation: break down protein into smaller peptide fragments
- Analysis: Liquid Chromatography with tandem mass spectrometry (LC/MS/MS)

Protein Selection

- Multiple proteins are present in samples
- We selected a specific protein to monitor based on literature data

Example:

SDS-Page of seed total protein in rice (glutelin chosen here)



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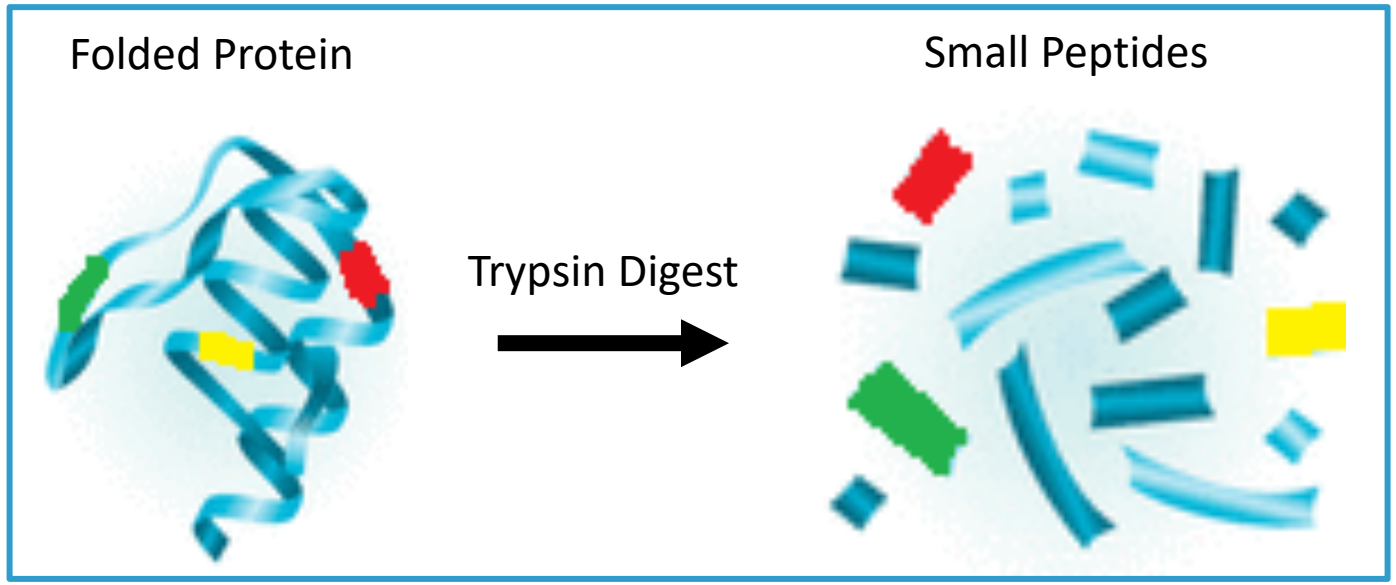
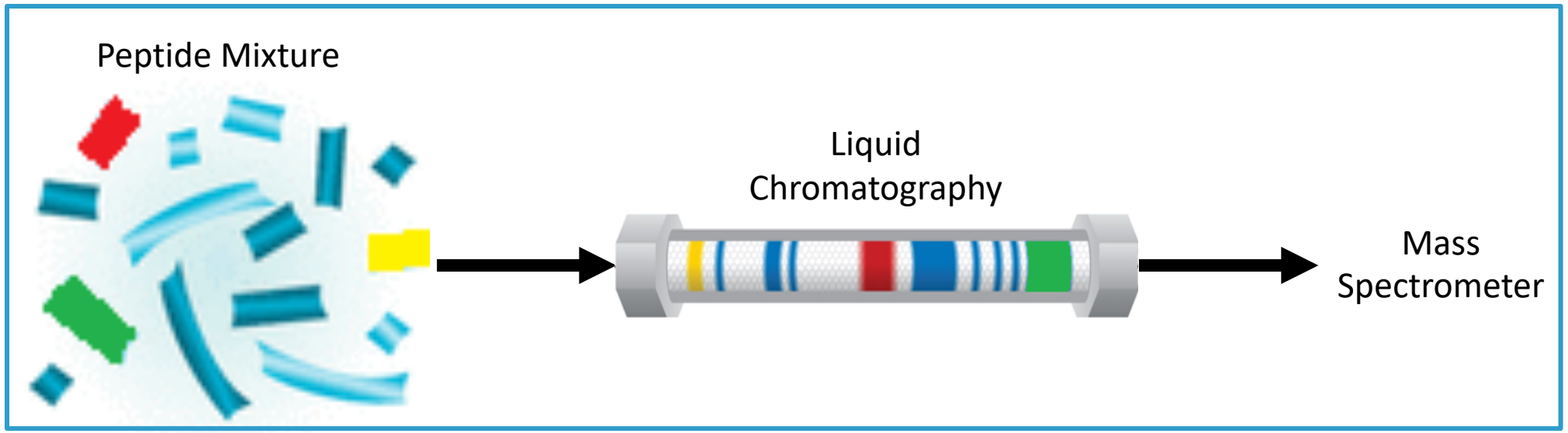
Sample Preparation

- Trypsin enzyme hydrolyzes proteins
- Cleavage occurs mainly at the carboxyl side of the amino acids Lysine (K) and Arginine (R)
- Smaller tryptic peptide fragments result from cleavage



Liquid Chromatography

- Peptides can be separated from each other and other components in the extract by liquid chromatography (Shimadzu Prominence)
- C18 column
- Acidic mobile phase



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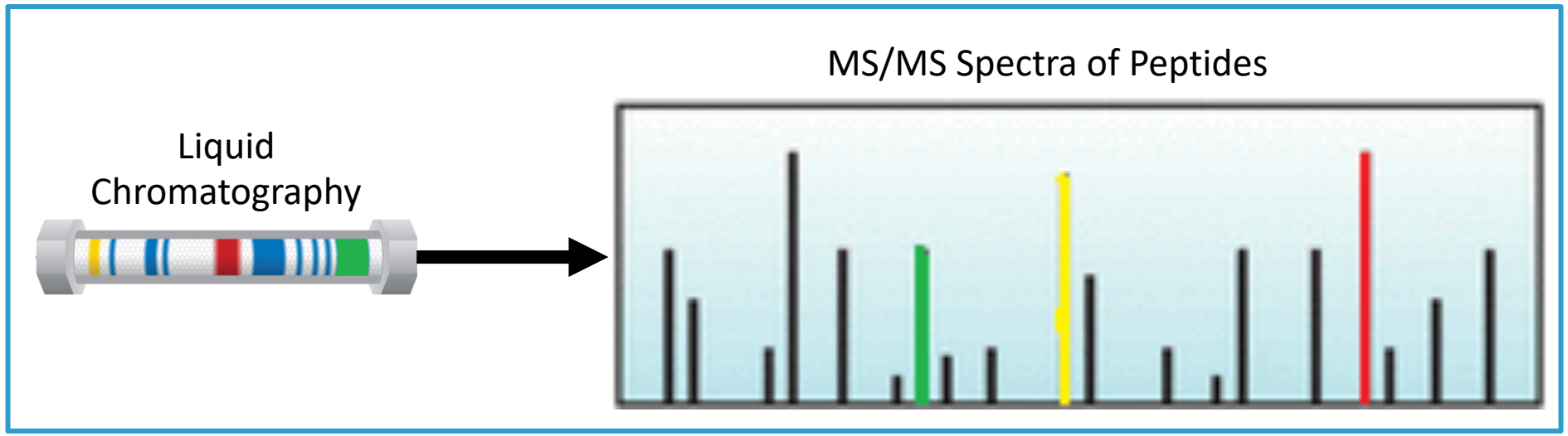
Tandem Mass Spectrometry

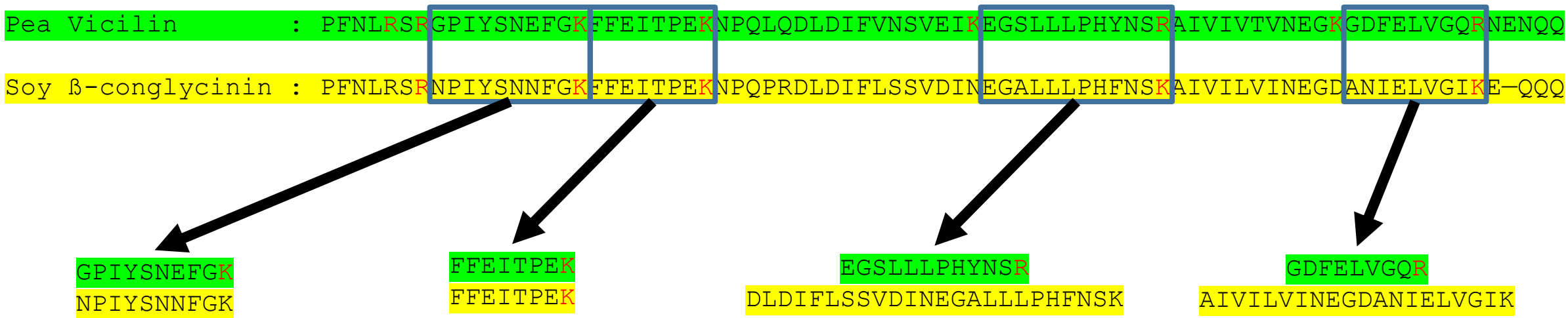
- Peptides can be detected in a mass spectrometer (Sciex API4000 QTrap) based on their mass-to-charge (m/z) ratio in positive-ion mode
- Peptides fragment in collision cell and product ions are monitored
- Typically, double-charge peptides provide highest signal



Peptide Selection

- Similar to DNA barcoding, only a specific region of the protein's amino acid sequence is chosen
- We selected unique tryptic peptides from the sequence
- Multiple peptide MS transitions can be monitored simultaneously (three for each protein)

Peptide Selection Example: Pea vs Soy



Same cleavage sites
Different sequences
Unique peptides

Same cleavage sites
Same sequences
Identical peptides

Different cleavage sites
Unique peptides

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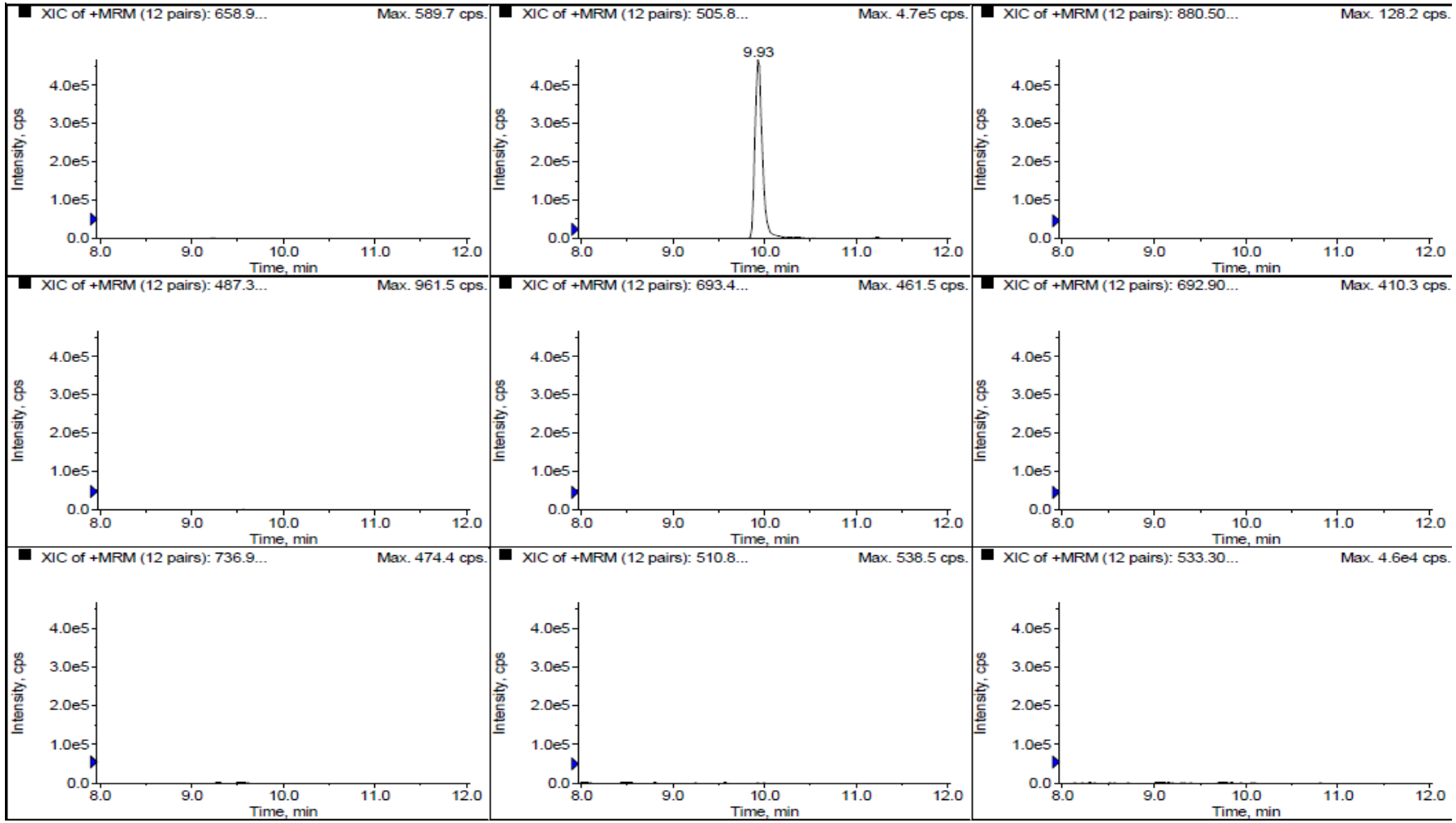
Example 1: Soy Raw Material Sample



Rice Pea Milk

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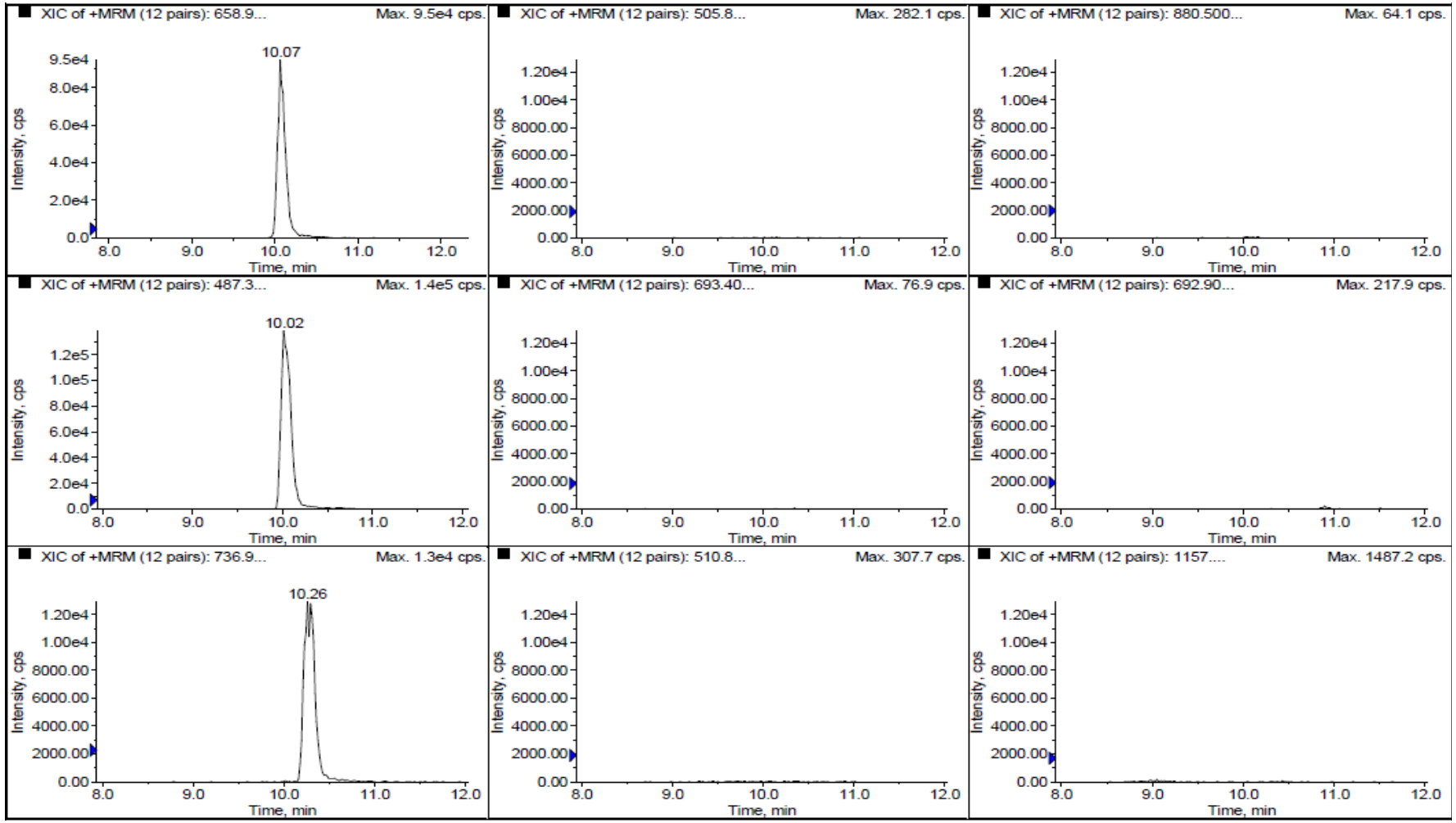
Example 2: Rice Raw Material Sample



Rice Pea Milk

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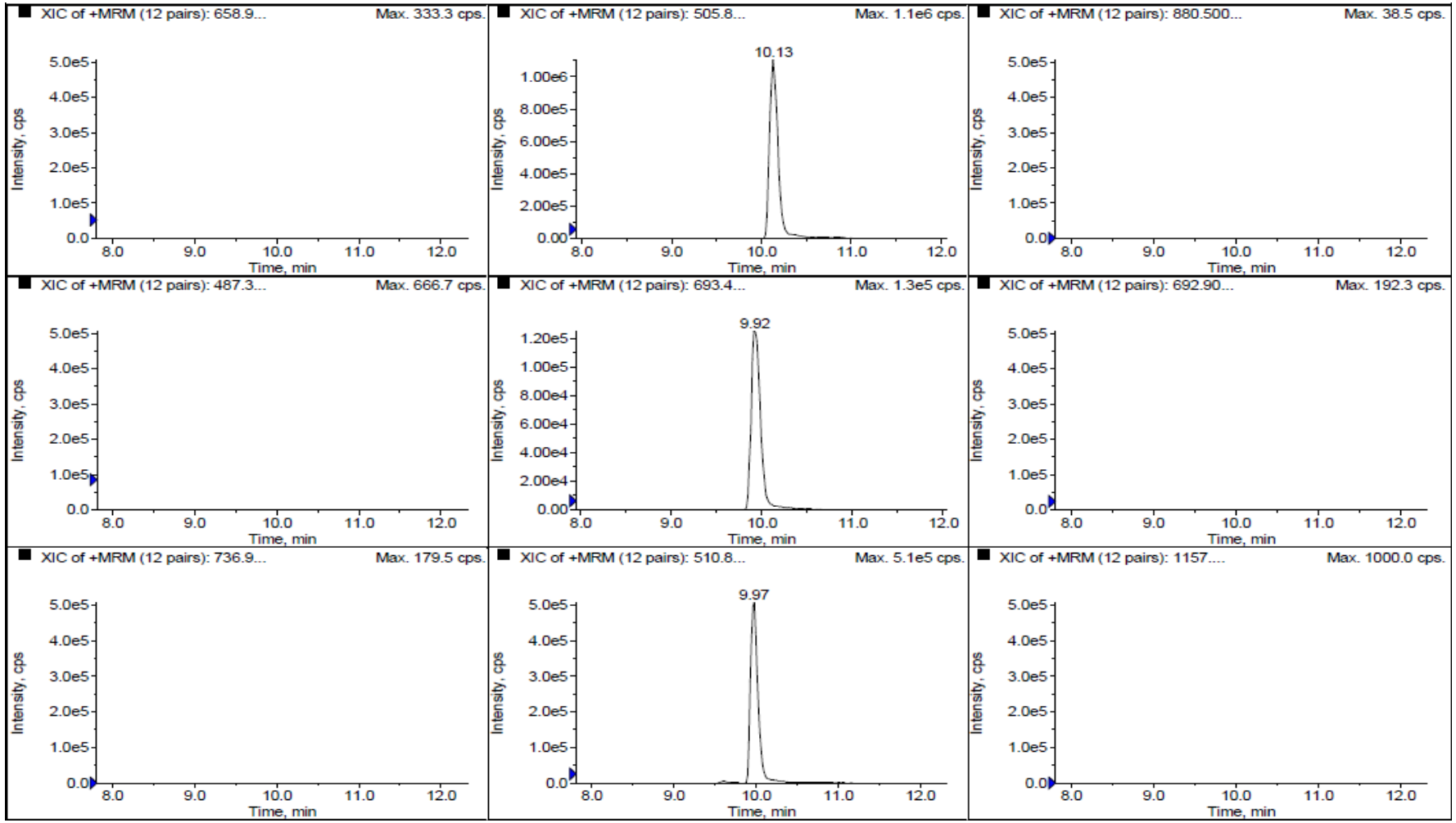
Example 3: Pea Raw Material Sample



Rice Pea Milk

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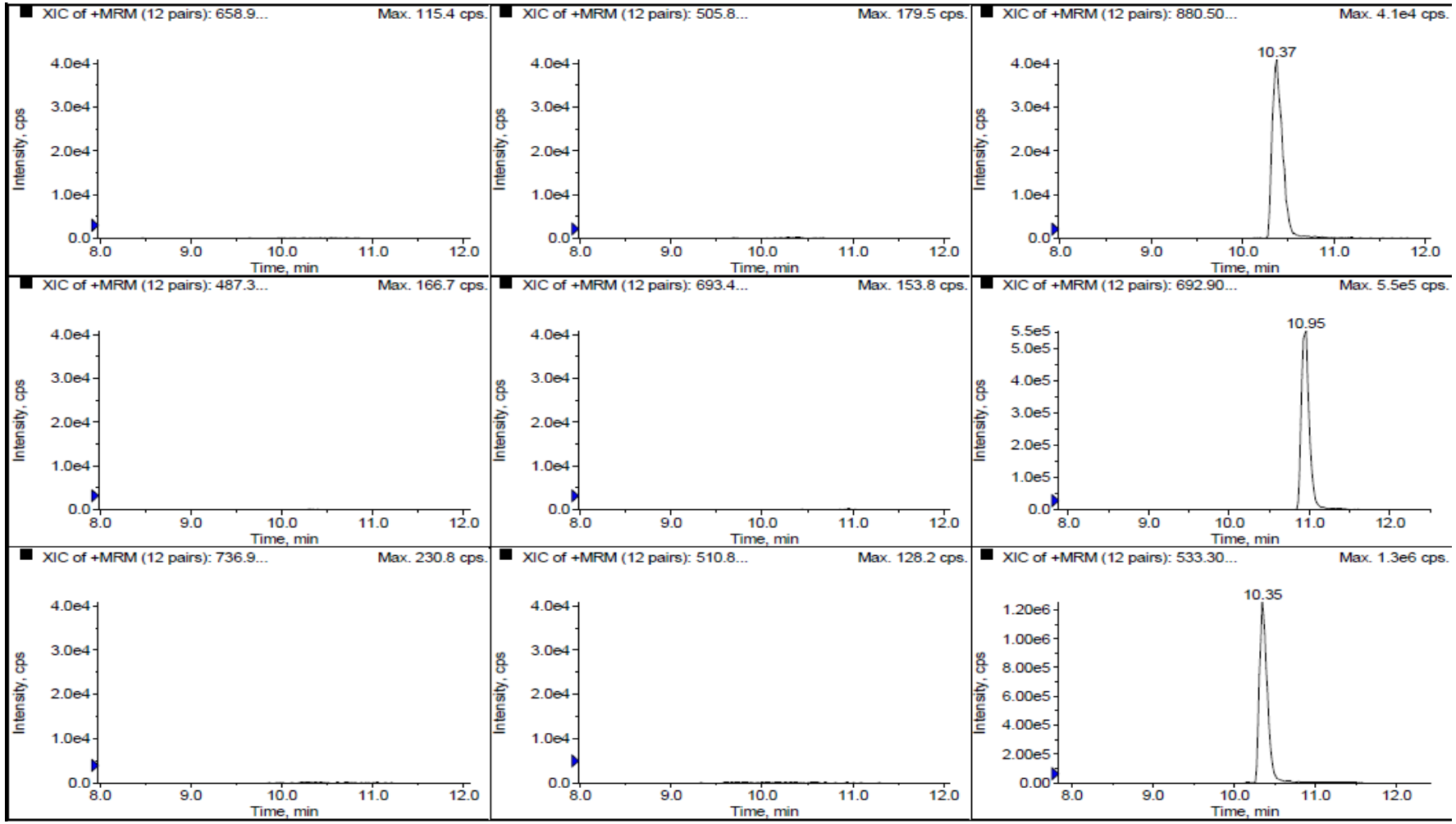
Example 4: Milk Finished Product Sample



Rice Pea Milk

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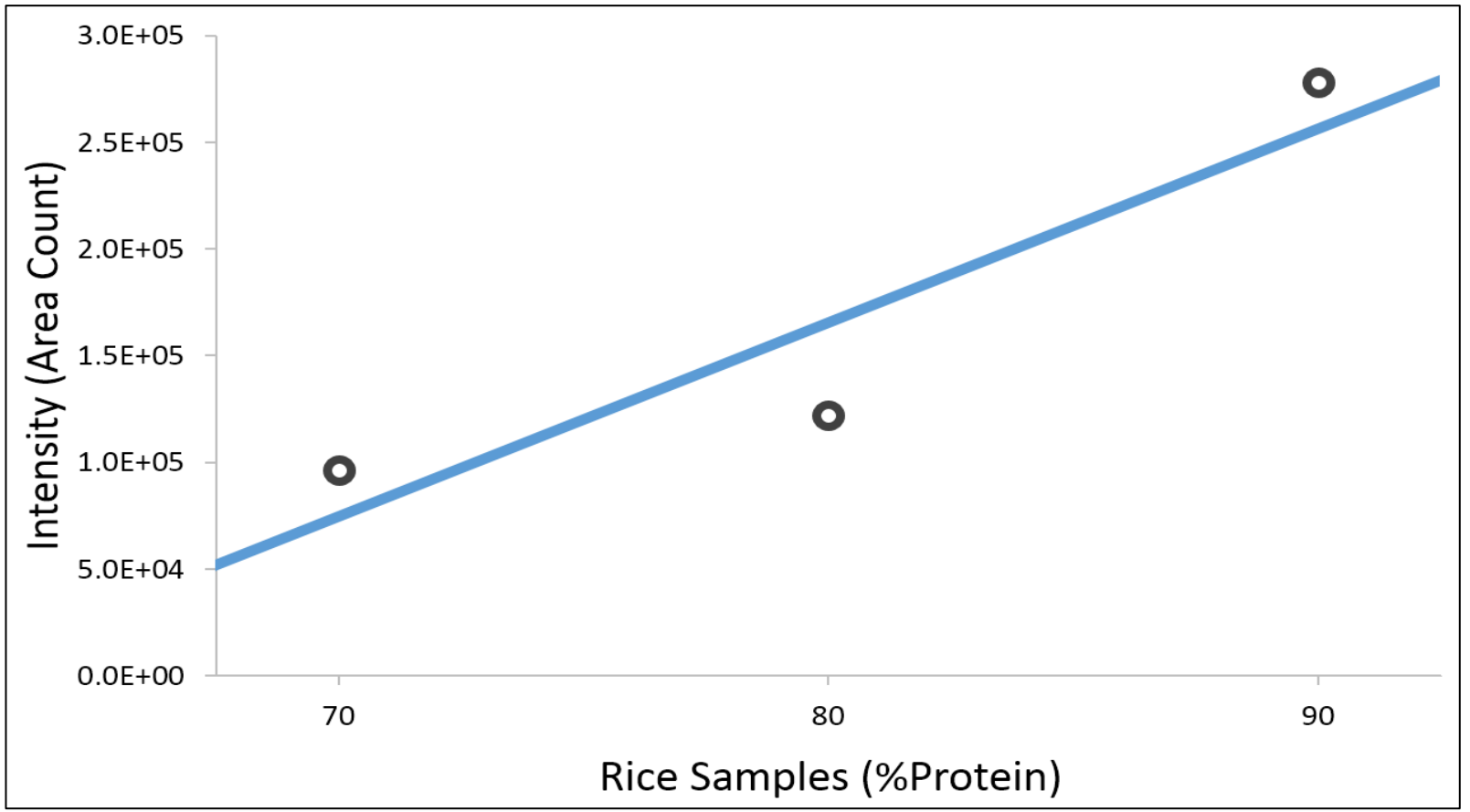
Example 5: Different Milk Products

- Peptide transitions for proteins in the for casein fraction (~80% of milk proteins) and whey fraction (~20% of milk proteins)

	Milk Product		Whey Product		Casein Product	
	Peak Area	% of Total	Peak Area	% of Total	Peak Area	% of Total
Sum of Casein MS Transitions	2.8E+07	**80**	2.8E+05	**1**	3.0E+07	**78**
Sum of Whey MS Transitions	7.0E+06	**20**	3.3E+07	**99**	8.7E+06	**22**

Example 6: Rice samples at 70, 80, 90% Protein Content



Conclusions

- Proteins are generally stable through processing, which allows analysis on finished products as well as raw materials
- Trypsin digest of samples followed by LC/MS/MS analysis provides conclusive protein identification
- Assay is selective:
  - Monitor unique and specific peptides from specific proteins
  - Chromatographic retention time of peptides is initial confirmation
  - Peaks using a mass transition of a fragmented peptide are monitored for additional confirmation
  - A peak in all three transitions is needed for confirmation
  - Positive (protein standards) and negative (blank samples) controls are additional confirmation

Future Work: Quantitation

- Obtain extracted pure standards for quantitation
- Examples of quantitation:
  - Differentiate between whey concentrate (~80% whey) and isolate (~90% whey) proteins
  - If sample is adulterated with less expensive proteins or amino acids, assay will differentiate

Future Work: Additional Proteins

- Current assay differentiates whey, casein, rice, pea, and soy products
- Additional products can be added by identifying unique peptides within the product of interest (example: hemp, seeds, etc)
- Differentiate sub-species (example: indica vs japonica subspecies of rice)