Research from value added analytical matter

Benjamin Katz

Pet Food Innovation Workshop

September 14th 2016

Acknowledgements

PI : Dr. John Tomich

Dr. Takeo Iwamoto
Nozomi M. Caton
Deane Lehman
Susan Whitaker

<u>Past Students</u>
Kelsey Pearson (Nursing)
Jammie Layman (Law School)
Kyle Steuber (Nutrisciences)
Jared Wilmoth (Grad School)
Marlena Birkel (Pharm School)
Denise Cobb (Grad School)
Macy Garcia

<u>Current Students</u>
Will Smith
Emily Wedeman
Kayla Wilkinson
Cassidy Frost

Dr. George Wang
Dr. Sunghun Park
Tzu-Yu Chen
Soyoung Lim
Jianteng Xu
Xiaoyu Su
Yanting Shen

Dr. Ying Fang
Rui Guo
Thomas Gallagher

<u>External</u>
Mike Molitor (U.Wisc. Madison)
Dan Abitz (GBA Engineering)

<u>Supported by</u>
Johnson Cancer Center
Kansas State University

Kansas State University Biotech Core

- Non-Profit initiative in the Vice President for Research Office.
- Work with >70 KSU investigators in a wide range of disciplines.
- Facilitate collaboration between academy and industry.
- Goal: Help promote new product design and entrepreneurship in the Midwest.

Kansas State University Biotech Core

- **Equipment**
  - Analytical and Preparative HPLC
  - MALDI-TOF and ESI-MS/MS
  - Proteomics and Metabolomics
  - Wet Lab, Roto-vap Distillation, Lyophilization
  - Microbiology culture, Fermentation Scale up
  - Super Critical Fluid Extraction and Spray Drying

- **Types of Projects**
  - Animal Nutrition and Disease
  - Food and Human Health
  - Agricultural Crops: Pest, Disease and Tolerance
  - Biosecurity BSL3ag support: Animal and Zoonosis
  - Fundamental Biology and Ecology
  - Classical Chemistry and Peptide Synthesis

Analyzing “Enriched” Protein Products

- Fingerprinting hydrolysates for peptides signature
  – Using MALDI-TOF
  – Like Bruker Biotyper for microbes

- Link nutrition information to product name
  – Enriched with…
    (Glutamine, BCAA, Sulfur AA…etc)
  – Blending example: Enriched Sunflower Meal

Outline of Talk

- By-product protein streams
- Negative image from consumers
- Rational design of hydrolysates
- Analytical service offered by KSU biotech core
- Controlling waste stream consistency
- Fingerprinting hydrolysates in blends
- Examples from industry

Grain Storage Proteins (prolamins)

- Great source of metabolic energy
- Essential source of nitrogen and sulfur rich amino acids
- Inherent poor digestibility
- Due to low/missed cleavage site by digestive enzymes
- Presences of trypsin inhibitors
- Negative image as cheap filler

Negative image from consumers

Consumer have been taught to AVOID:

- SOY FLOUR, WHOLE WHEAT FLOUR, CORN, CORN GLUTEN MEAL
- WHEAT BRAN, BREWER'S RICE, POTATO PRODUCT, MIDDLINGS/MIDS or MILL RUN of any kind.
- Unspecified grain sources like CEREAL FOOD FINES, CORN BRAN, OAT HULLS, RICE HULLS, PEANUT HULLS, DISTILLERS GRAIN FERMENTATION SOLUBLES, and CELLULOSE
- MEAT AND BONE MEAL, ANIMAL DIGEST from "4D" animals

Examples of protein by-products

- Corn Gluten Meal
  - Roquette America $520.00/ton FOB IOWA
  - 60% Protein @ 43c/lb protein
  - Zein prolamin proteins

- Wheat Midds, dried
  - ADM $88.00/ton FOB Kansas
  - 16% Protein @ 28c/lb protein
  - Gliadin prolamin proteins

Waste Streams updated list

Prolamin processing

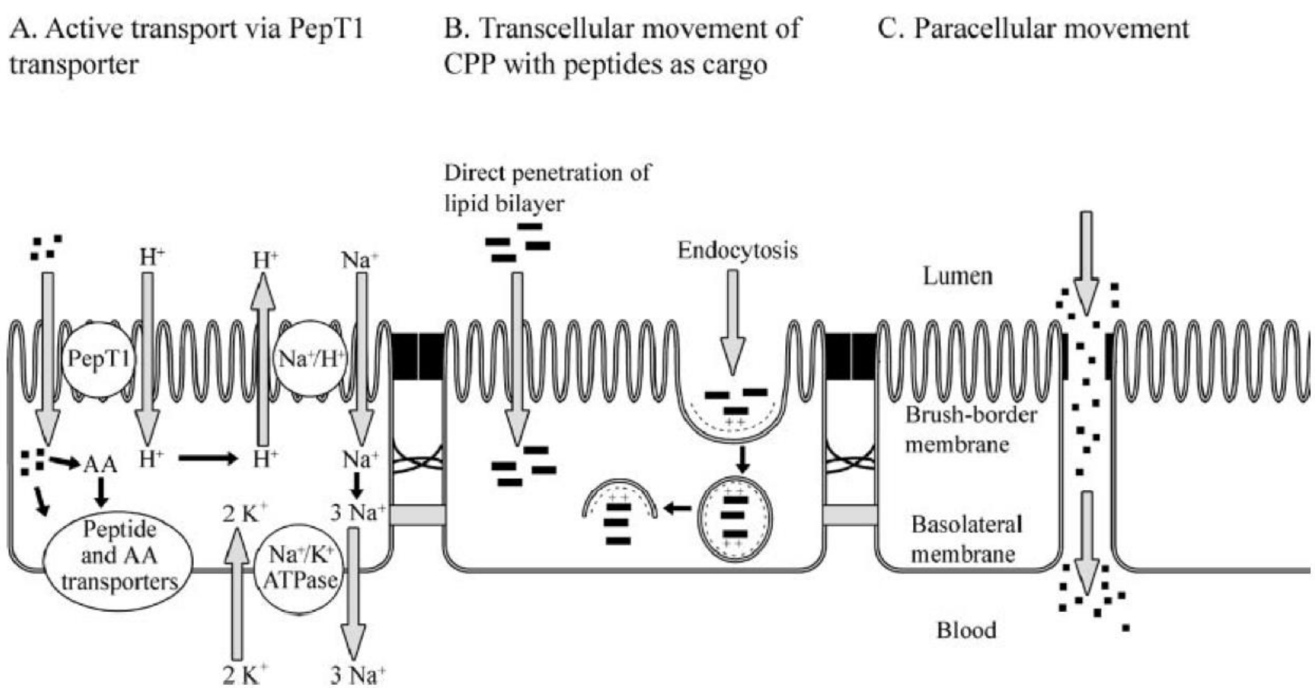
- Soluble in ~70% ethanol
- Difficult processing due to aggregation
- Insoluble aggregate floats and sink in water
  - Heavy and light fractions
- Difficult for enzymes to access cleavage sites
- Processing can cause bad flavors
- Denatured proteins cleave differently

Rational Prolamin Processing

- Utilize fungal/plant/bacterial enzymes
  - Able to cleave proteins at unique sites
  - Proteases
    - Help solubilized bulk protein
  - Endo & exo peptidases
    - Tailor flavor by eliminate low threshold taste modulators
    - Improve nutrition

Amino Acid Nutrition

Gilbert et al.



Group I: Hydrolysate > amino acid mix. > protein	
Essential amino acids	Thr, Lys
Nonessential amino acid	Tyr, His, Ser, Asx
Group II: Hydrolysate = amino acid mix. > protein	
Essential amino acids	Ile, Leu, Val, Met, Phe
Nonessential amino acid	Arg, Ala, Gly, Glx, Cys
Group III: Amino acid mix. > hydrolysate > protein	
Essential amino acids	–
Nonessential amino acid	Pro

Vol. 71, Nr. 7, 2006—JOURNAL OF FOOD SCIENCE S**521**

Rational Prolamin Processing

- Use analytical data to create multiple enzyme processes.
  - Eliminate known bad flavors and indigestible fragments

  - Completely digest into di/tri peptide supplements
    - Use PepT1 transporter for animal specific nutrition

  - Tailor peptide enrichment to specific markets
    - High glutamine & branch chain amino acids. (muscle energy)
    - High methionine & sulfur rich amino acids. (hair, skin and milk)

- Create peptide fingerprint of final product for QC
  - Check starting materials and in-line processes

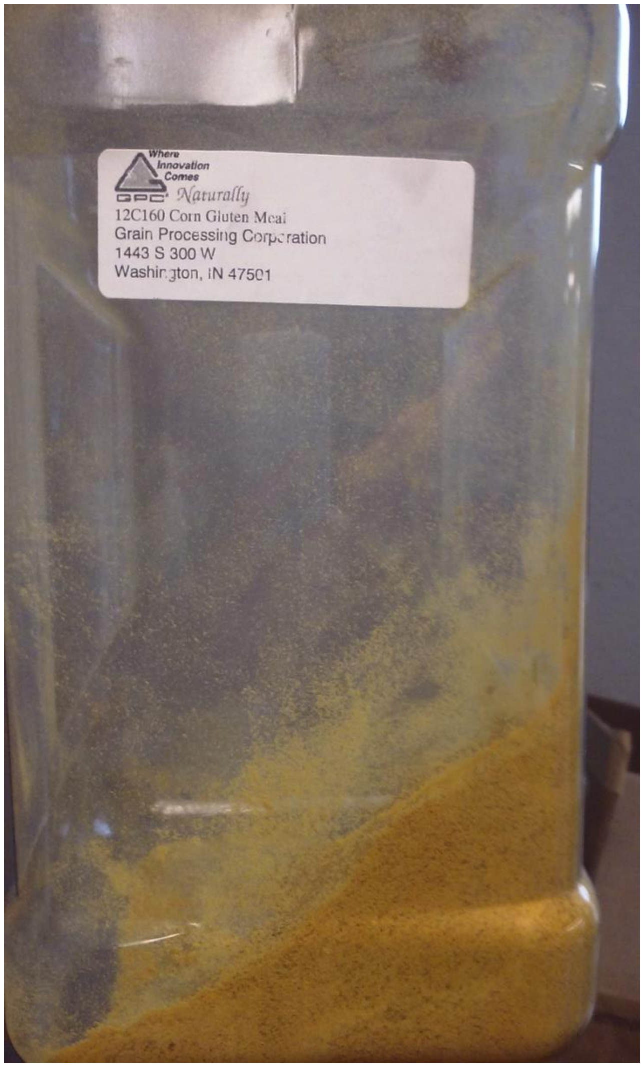
Analytical Service Provided

- ID proteins in by-products by MS1 mass
- Evaluate enzyme choices for desired product
- Fingerprint peptide signature of hydrolysates
- Analyze for AA length and content
- Link peptides to flavor and nutrition
- Track peptide fingerprints after blending

MALDI-TOF mass spectrometry

- Provides exact mass of proteins and peptides.
- Higher accuracy of intact large protein than 1D gels
- Better resolution of similar protein subunits
- Faster data acquisition with ability to automate

Corn Gluten Meal Example



- Corn Gluten Meal from corn syrup manufacturing
- Food grade 60% zein protein waste
- High in glutamine and proline
- Alpha and gamma Zein
  - High in BCAA
  - Leucine, Isoleucine & Valine
- Beta and delta Zein
  - High in sulfur containing AA
  - Methionine

Corn Gluten Meal Example

- Remove excess oil, color and and other hydrophobic impurities with 95% Ethanol.
- Can further purify with hexane/ethanol blends depending on quality.
- Ethanol can be distilled and reused

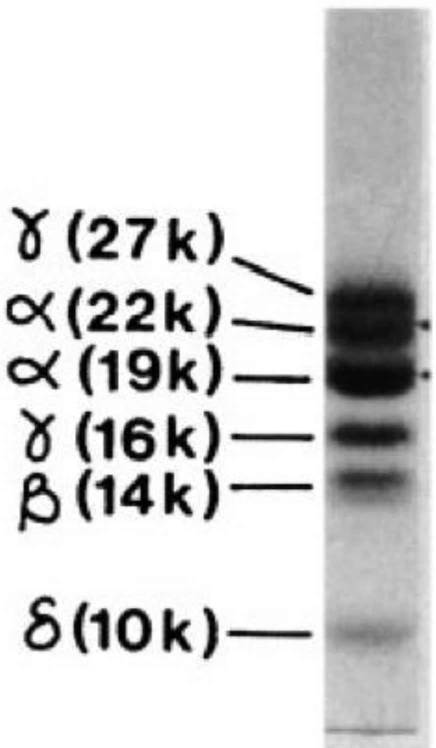
[Biotech Core Instagram](#)

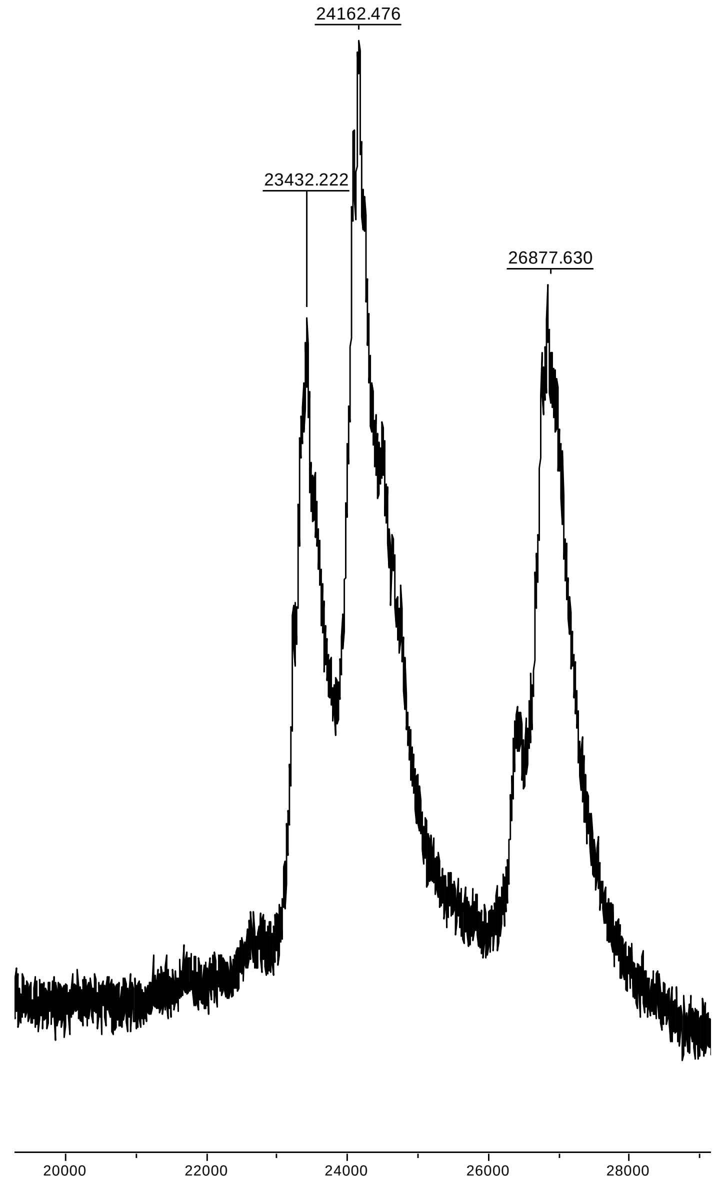
Corn Gluten Meal Example

- Repetitively extract prolamins in 70% ethanol.
- Can tailor ethanol % for desired protein composition.
- Purification and extraction may not be required for some products.

Biotech Core Instagram

Maize prolamins





Corn Gluten Meal Example

- Soluble Zein proteins aggregate and precipitate in water.
- Add to pH adjusted water with 0.1-1% enzyme protease.
- Keep final ethanol concentration at ~20%
  - Engineered food enzymes are still active.
  - Prevents microbial growth.
  - Helps solubilize peptides to improve accessibility.

Food Grade Enzyme Examples

- Promod™ 24L - Biocatalysts
  - Bacterial broad spectrum protease
- ENZECO® Dual - Enzyme Development Corp.
  - Bromelain and Papain from meat tenderizing
- Protamex® - Novozyme
  - Bacillus sp. bacterial protease from fish processing
- Flavourzyme – Novozyme
  - Protease and peptidase preparation from Aspergillus oryzae culture.
  - Complex mix of at least 8 enzymes

CGM Double Digest Example

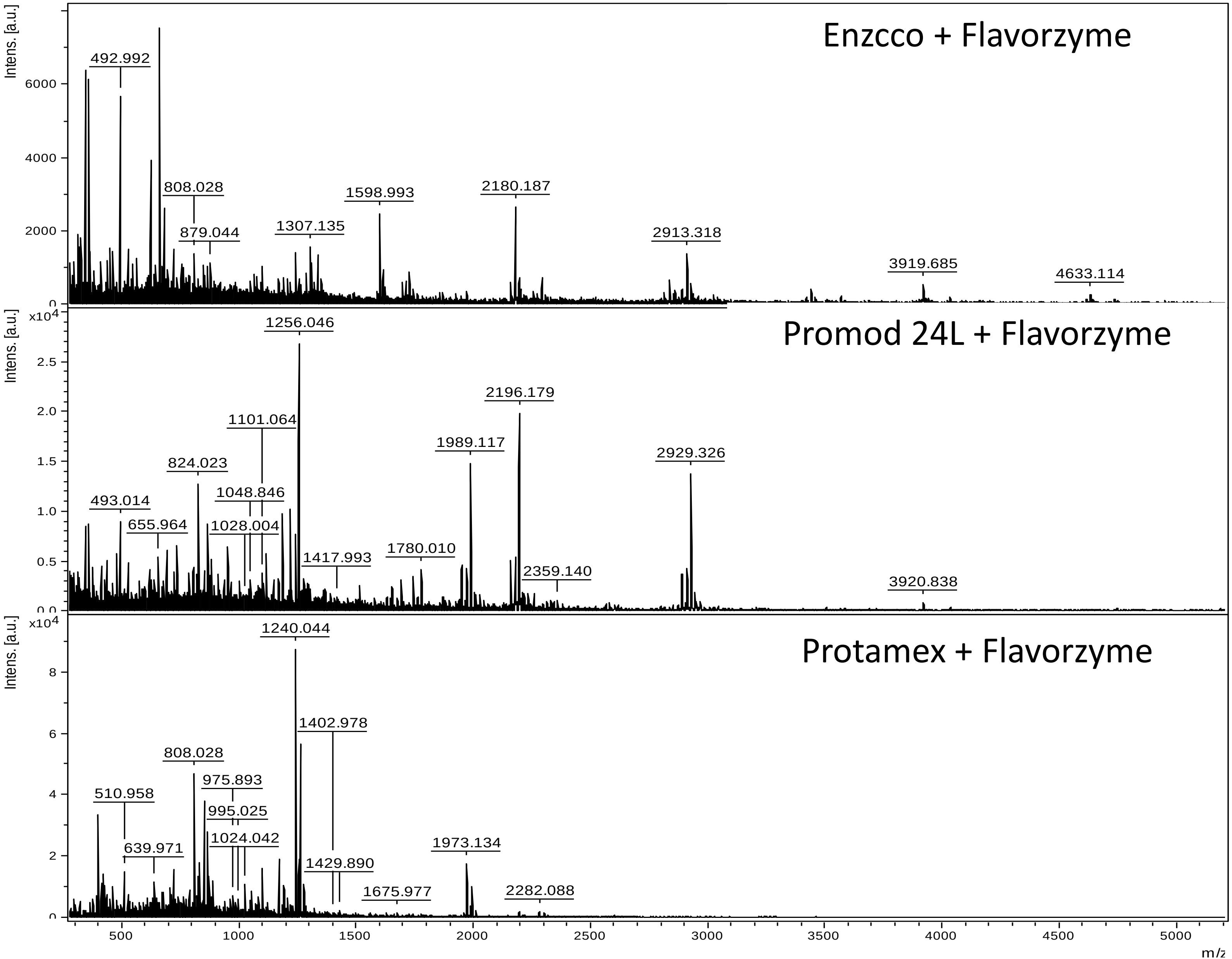
- Digest Zein with first protease until aggregate is broken.
- Digest with secondary enzyme, Flavourzyme, until entire solution is liquefied.
- Monitor hydrolysates process by MALDI-TOF fingerprinting.

Analytical Procedure

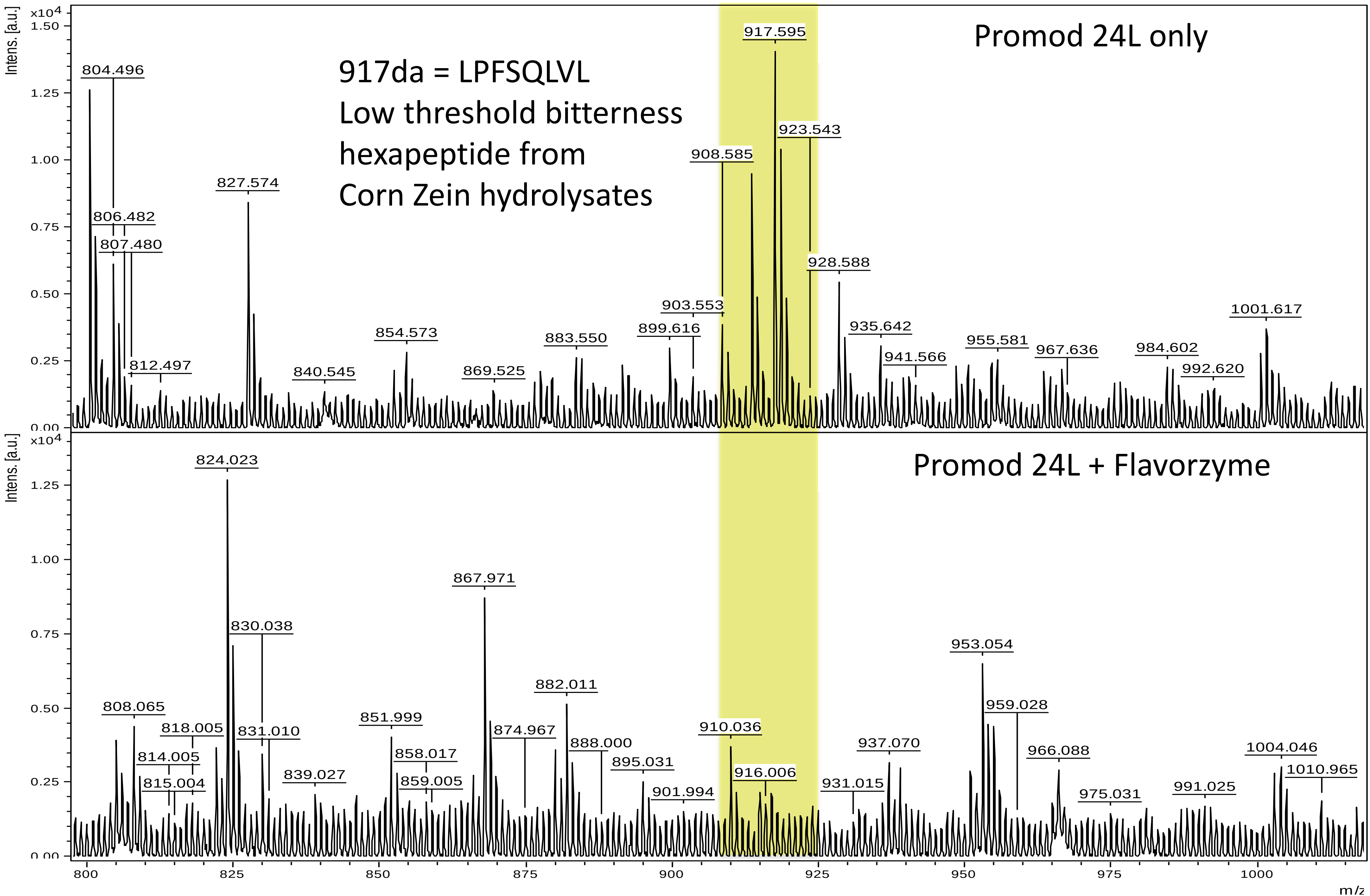
- Dilute 10uL of hydrolysate into:
  - 100uL of Acetonitrile and 90uL 1% TFA in water.
- Spot 1uL extract to MALDI-TOF
  - Use DHB matrix for peptide fingerprinting.

Biotech Core Instagram

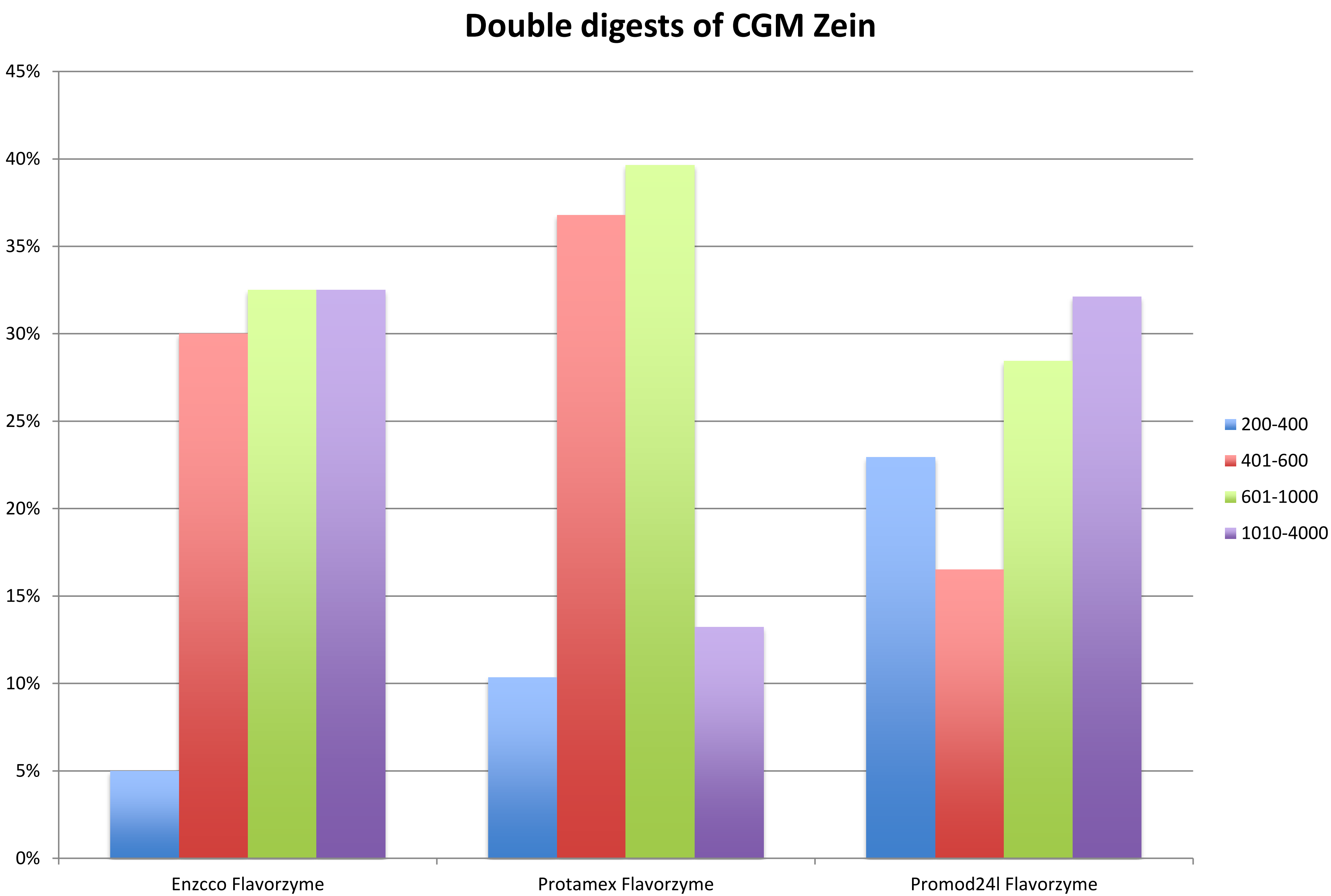
CGM Double Digests



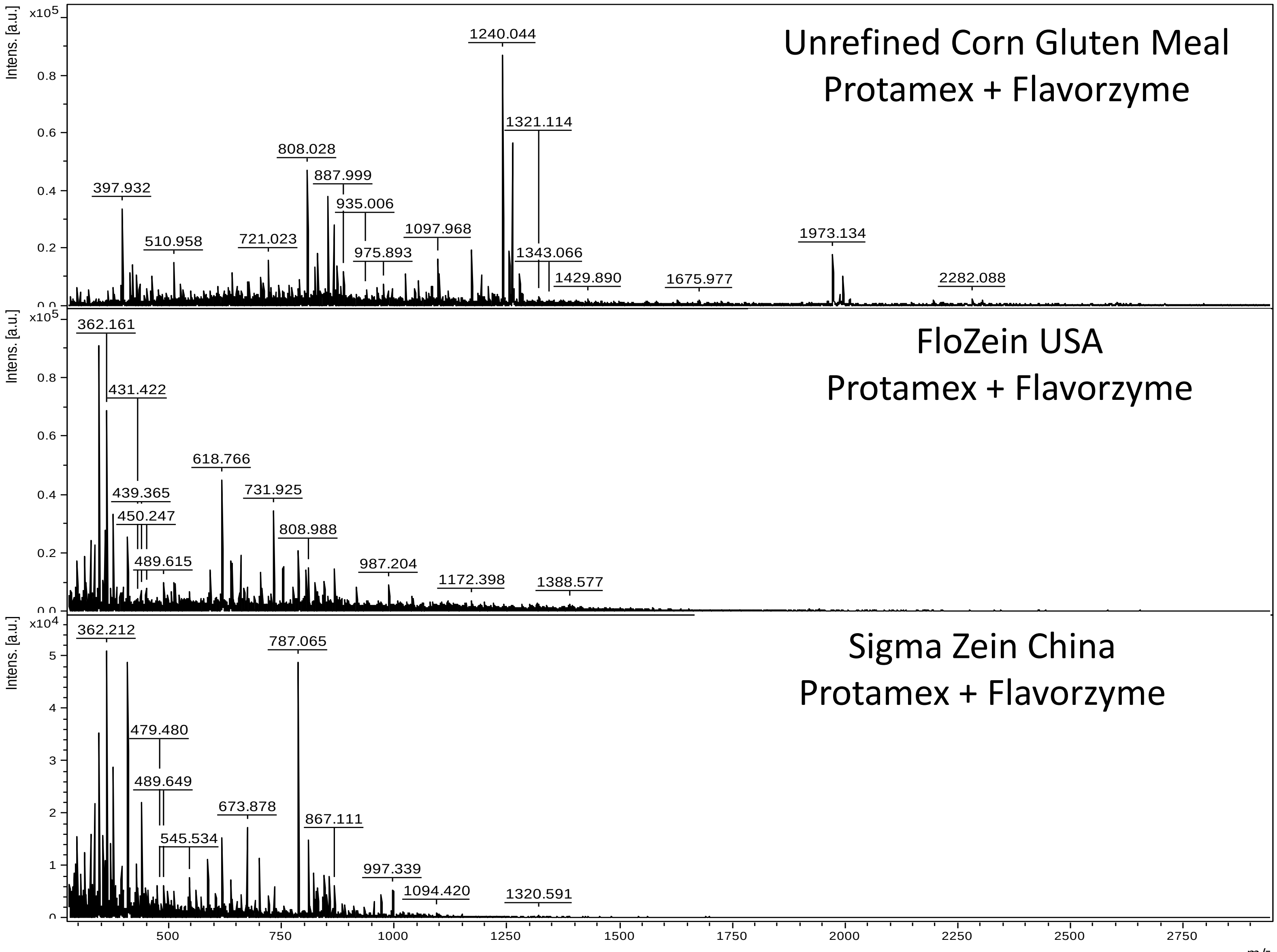
Why double digests?



Measuring improved digestibility



Comparing different Zein sources



Unique bulk filler sources

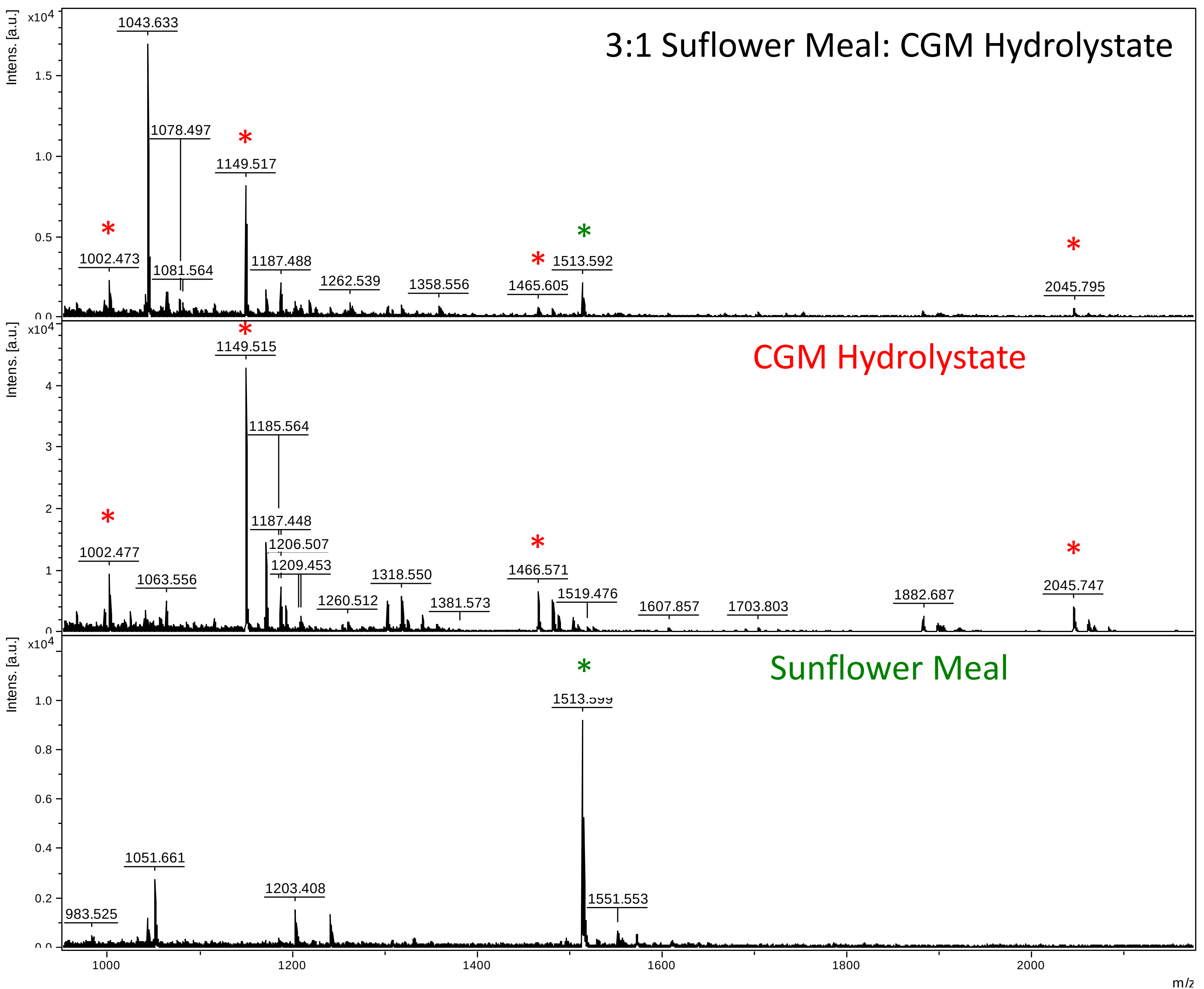
- Canola meal
  - $191/ton FOB from ADM
  - 36% protein
  - Accepted as a grain-free nutrient source.

- Sunflower meal
  - $280.00/ton FOB from CPE Feeds Inc – Texas
  - 30% protein
  - High in methionine, adds great flavor.

Blending with Filler

- New naming of product
  - Hydrolysate enrich by-products
  - “Protein enriched”
- Example product:
  - 3 parts Sunflower Meal : 1 part CGM hydrolysate
- Mix and heat extrude to kill enzymes
- Rename as “Enriched sunflower meal”
- GRAS due to all source ingredients being GRAS

Tracking peptides thru blending



CGM Enriched Products

- Turns high protein by-products into digestible nutrition without changing GRAS status.
- Masks the source of protein hydrolysates by blending into alternative sources.
- Analytical data used to normalize by-product streams.
- QC data can be used by plant manager to assign CAS#'s for new enrich products.

Acknowledgements

PI : Dr. John Tomich

Dr. Takeo Iwamoto
Nozomi M. Caton
Deane Lehman
Susan Whitaker

<u>Past Students</u>
Kelsey Pearson (Nursing)
Jammie Layman (Law School)
Kyle Steuber (Nutrisciences)
Jared Wilmoth (Grad School)
Marlena Birkel (Pharm School)
Denise Cobb (Grad School)
Macy Garcia

<u>Current Students</u>
Will Smith
Emily Wedeman
Kayla Wilkinson
Cassidy Frost

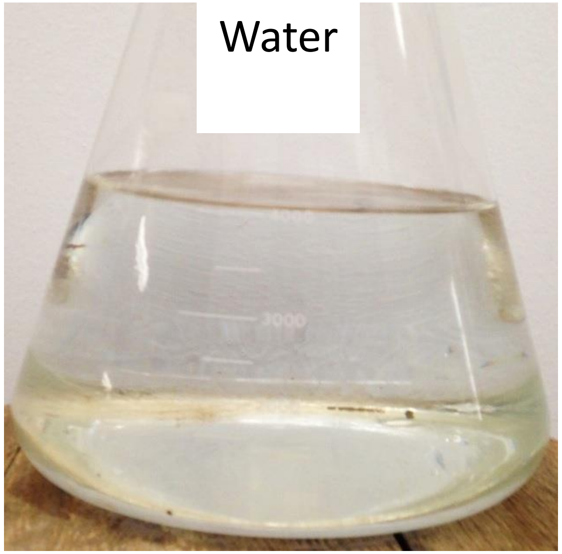
Dr. George Wang
Dr. Sunghun Park
Tzu-Yu Chen
Soyoung Lim
Jianteng Xu
Xiaoyu Su
Yanting Shen

Dr. Ying Fang
Rui Guo
Thomas Gallagher

<u>External</u>
Mike Molitor (U.Wisc., Madison)
Dan Abitz (GBA Engineering)

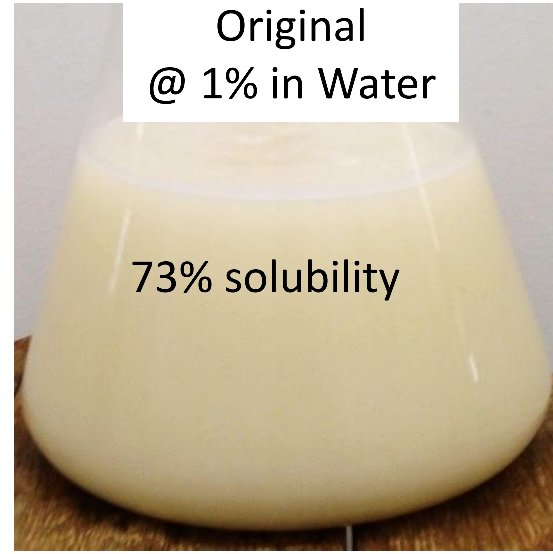
<u>Supported by</u>
Johnson Cancer Center
Kansas State University

Example 2: Gluten hydrolysate project



New Product

?



- Goal: Increase solubility in water
- Shorten peptide length for absorption
- Cleave known peptide epitopes and reduce bitterness

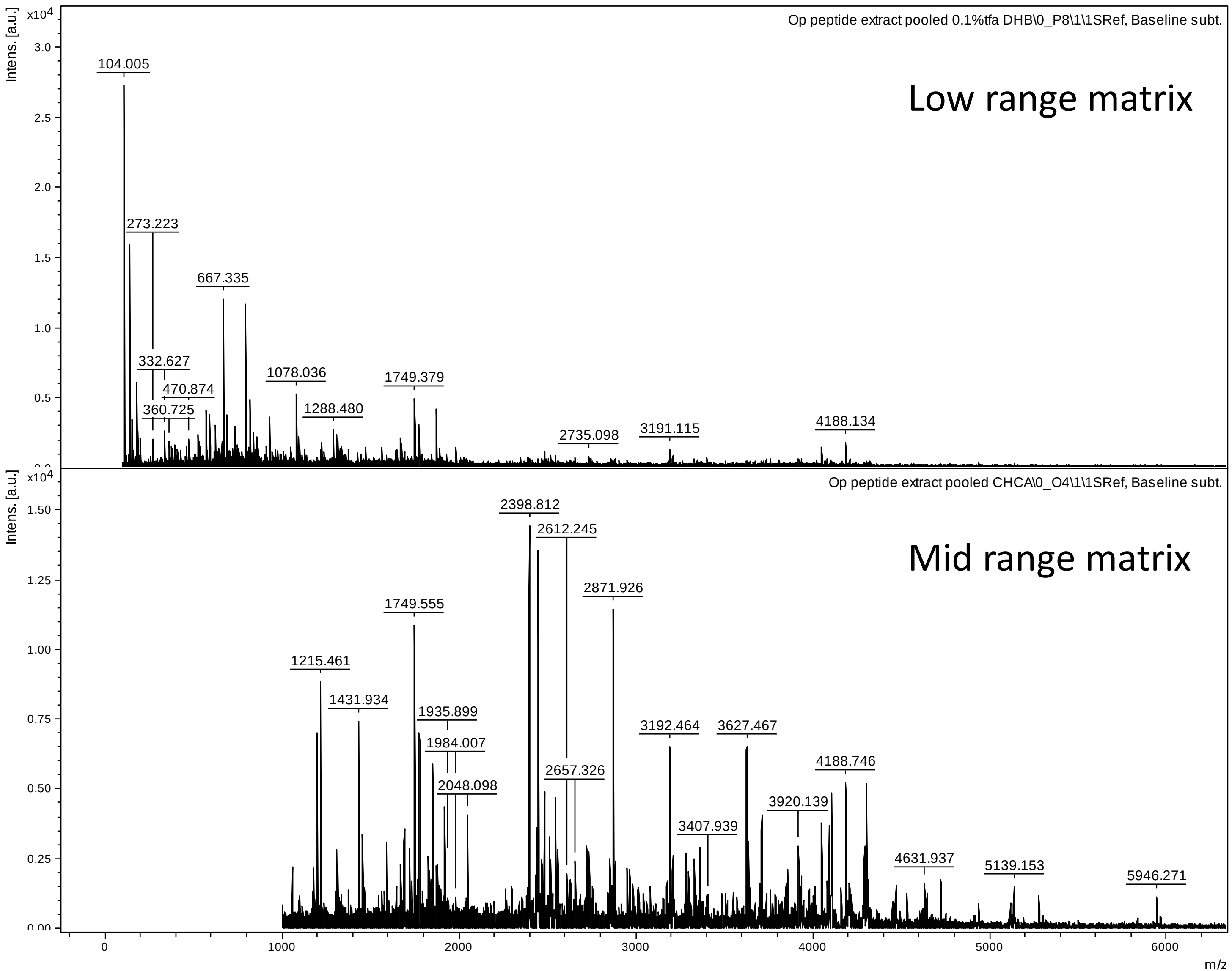
Full Analytical Extraction Protocol

- 50mg of spray dried hydrolysate was defatted in $CHCl_3$/MeOH/Water and freeze dried.

- Freeze dried samples were split into 4 fractions and resolubilized.
  - Fraction 1: w/ 0.1%TFA
  - Fraction 2: w/ 25% Acetonitrile : 75% 0.1% TFA
  - Fraction 3: w/ 50% Acetonitrile : 50% 0.1%TFA
  - Fraction 4: w/ 75% Acetonitrile : 25% 0.1%TFA

- Fractions were centrifuged and supernatant was collected and pooled.

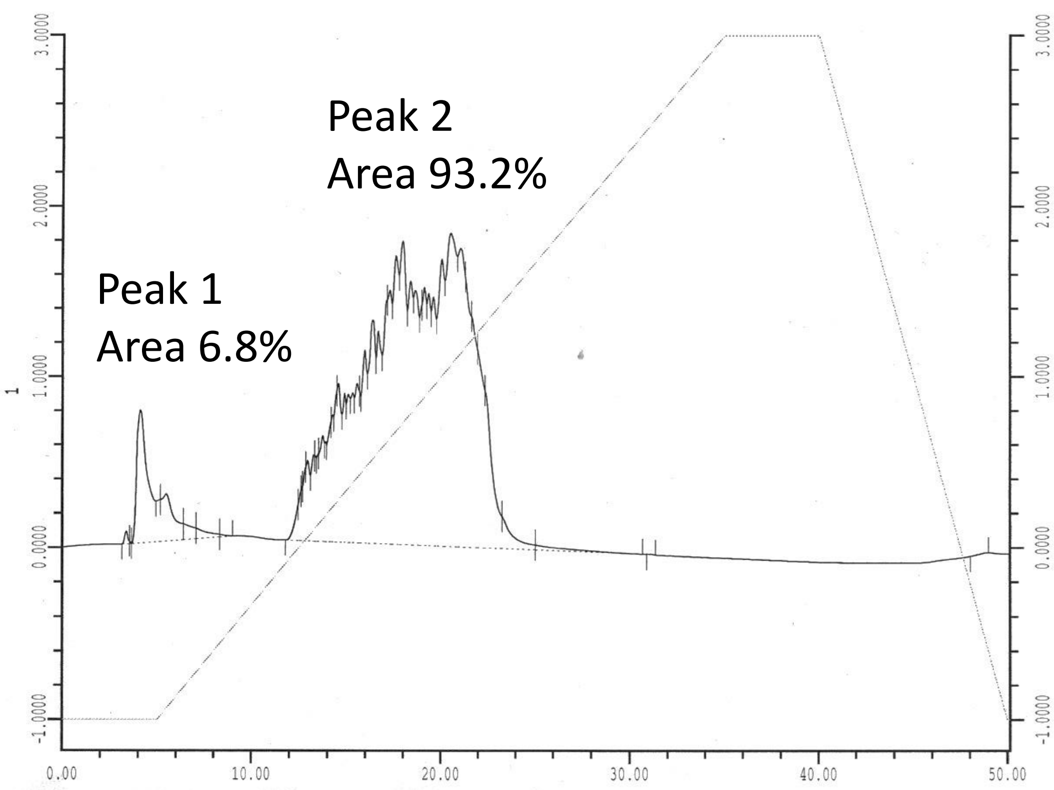
Raw MALDI-MS data of Pooled Sample



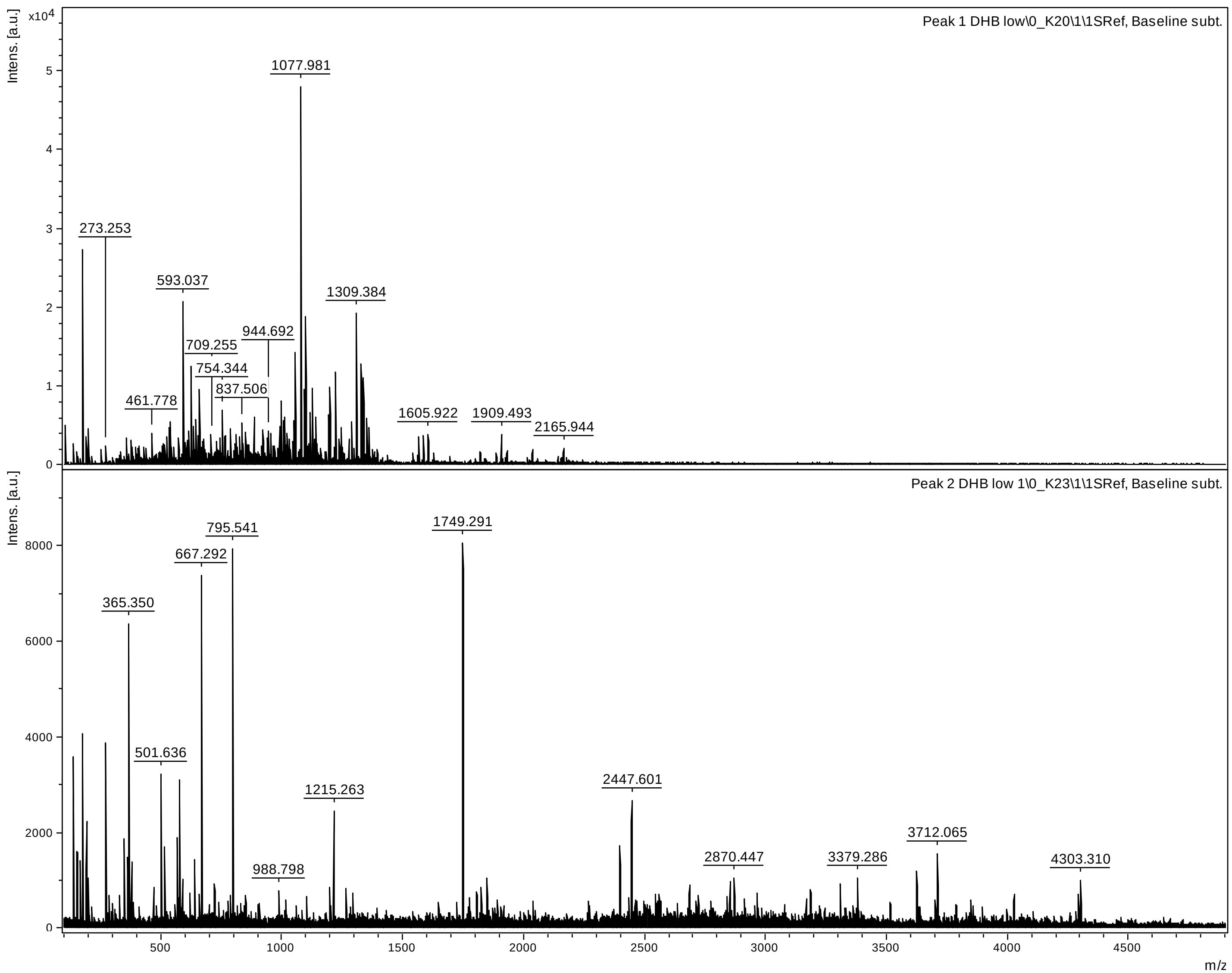
Peptide fractionation and distribution

- Pooled extracts were freeze dried and solubilized in 0.1% TFA for HPLC and Mass Spec (MS) analysis.
- HPLC was run on a 250 mm x 4.6 mm C4 column with Acetonitrile as the mobile phase.
- Two distinct broad peaks were collected and analyzed by MALDI-TOF MS.

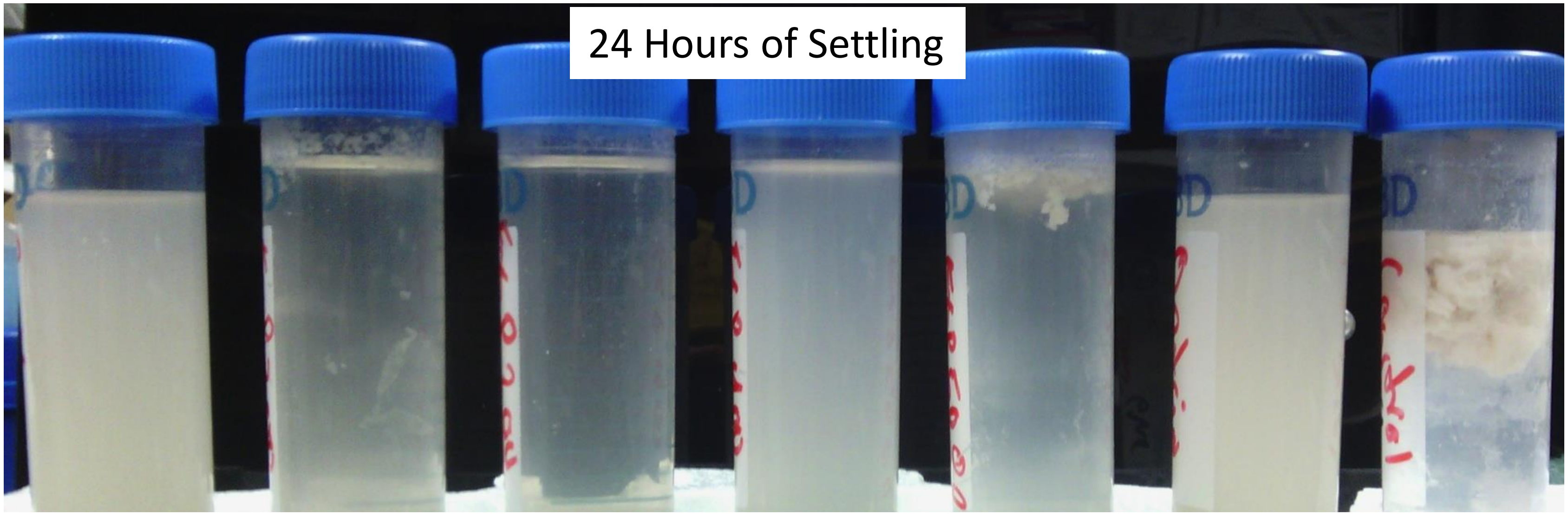
C4 HPLC of pooled extract



HPLC MALDI-MS data of Peak 1 & 2



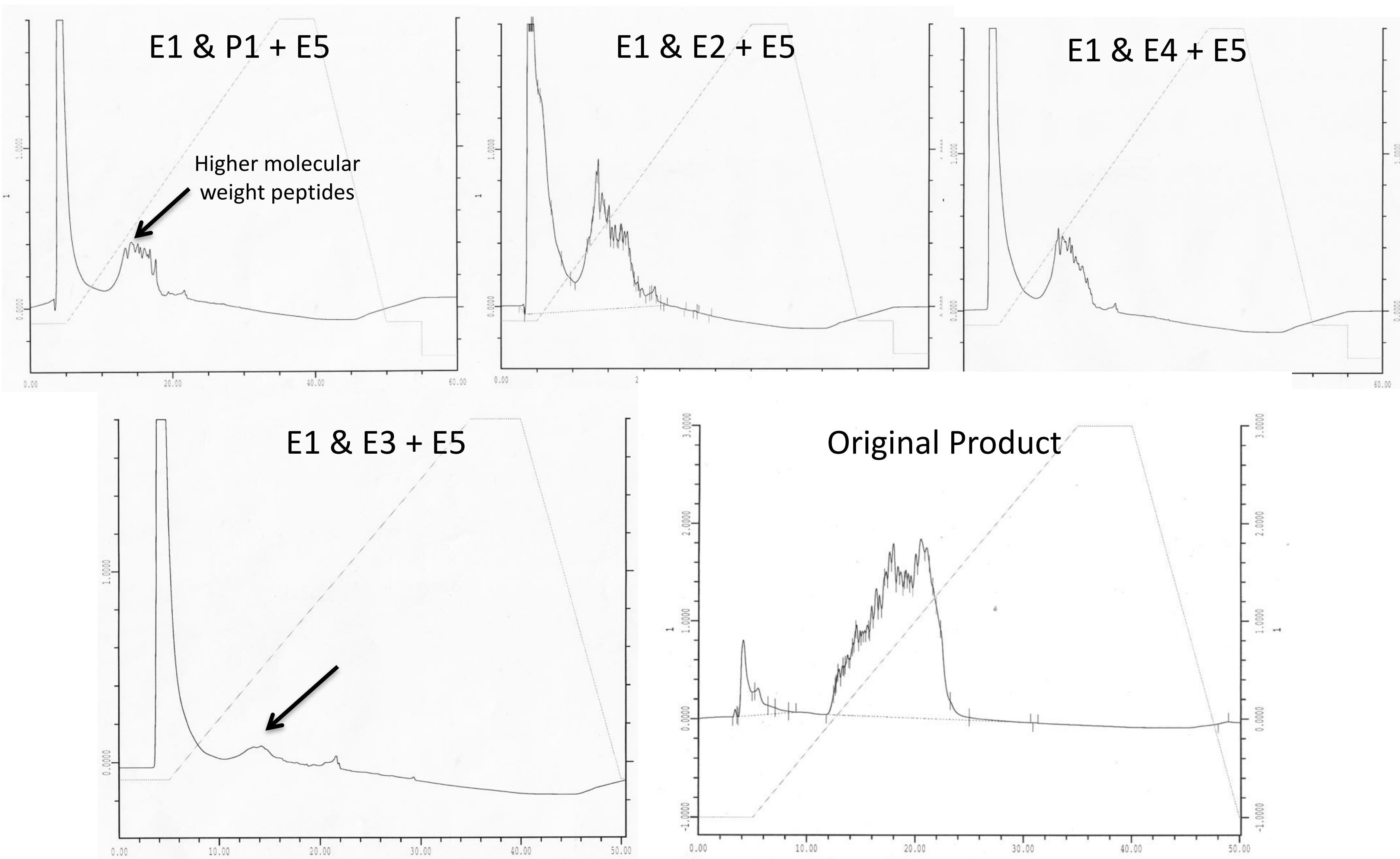
Single enzyme Solubilization and Dispersion of Gluten



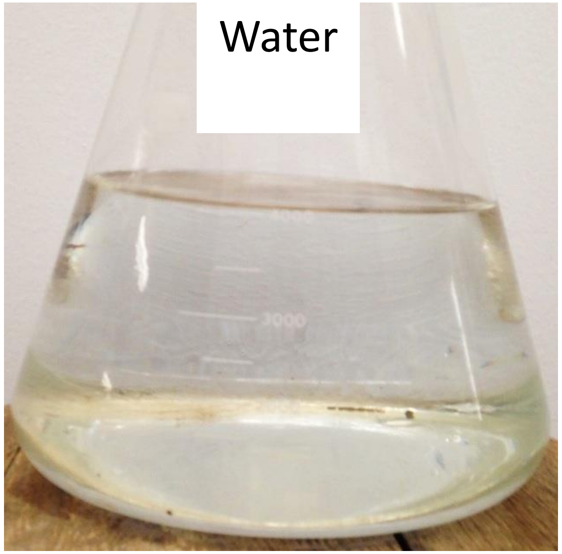
E1 E2 E3 E4 E5 P1 Gluten only

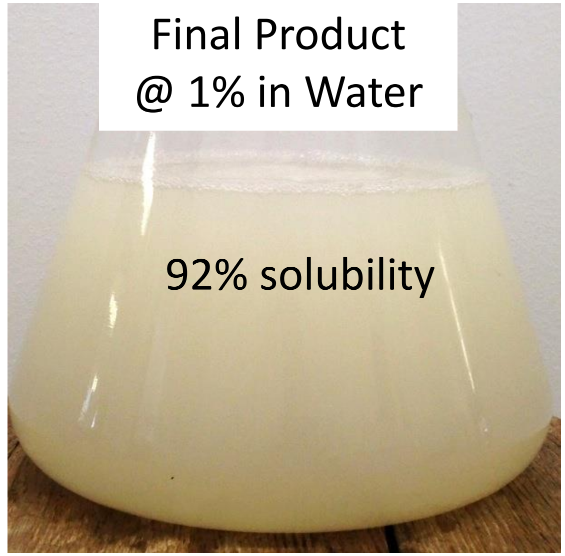
- All enzymes are at 1% w/w of gluten.
- E1, E4 and P1 were best at dispersing the gluten peptides after 24hrs of settling.
- E2 & E3 produced peptide rich soluble fractions but were unable to disperse large amount of gluten peptides.
- An enzyme combination strategy is necessary to both disperse and solubilize gluten peptides.

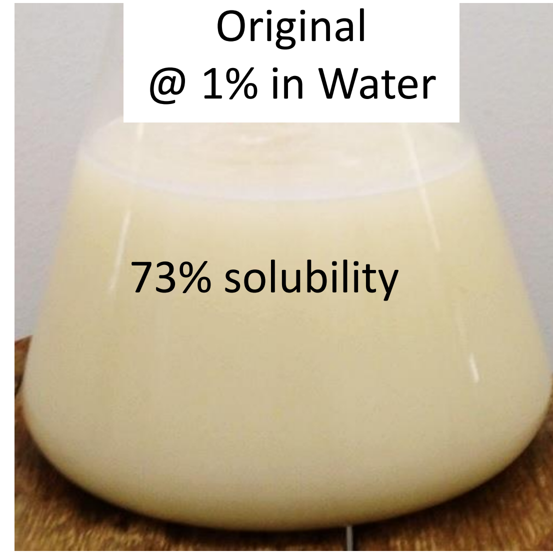
HPLC comparison of Enzyme Combos



Example 2: Gluten hydrolysate project







- Decreased insoluble precipitates by >300%
  - 27% w/v to 8% w/v
- Shorten peptide length for absorption
- Cleaved known peptide epitopes
- Reduced Bitterness

Acknowledgements

PI : Dr. John Tomich

Dr. Takeo Iwamoto
Nozomi M. Caton
Deane Lehman
Susan Whitaker

<u>Past Students</u>
Kelsey Pearson (Nursing)
Jammie Layman (Law School)
Kyle Steuber (Nutrisciences)
Jared Wilmoth (Grad School)
Marlena Birkel (Pharm School)
Denise Cobb (Grad School)
Macy Garcia

<u>Current Students</u>
Will Smith
Emily Wedeman
Kayla Wilkinson
Cassidy Frost

Dr. George Wang
Dr. Sunghun Park
Tzu-Yu Chen
Soyoung Lim
Jianteng Xu
Xiaoyu Su
Yanting Shen

Dr. Ying Fang
Rui Guo
Thomas Gallagher

<u>External</u>
Mike Molitor (U.Wisc., Madison)
Dan Abitz (GBA Engineering)

<u>Supported by</u>
Johnson Cancer Center
Kansas State University

Other projects and services

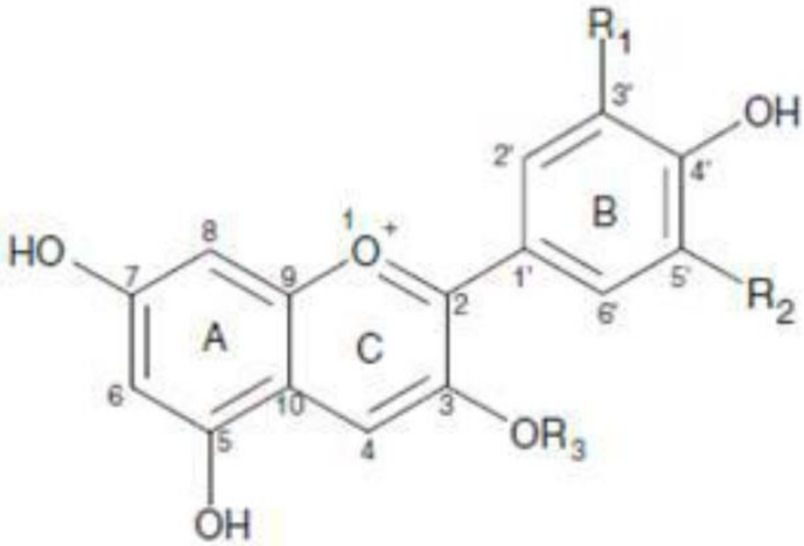
- Purple tomato and sweet potato anthocyanins
  - Discovering new small molecule with ESI-MS/MS

- PRRS virus using nanotubes for intercellular spread
  - Protein ID and Mapping

Anthocyanin Rich Sweet Potatoes and Tomatoes

Good dietary antioxidants for cancer prevention specifically colorectal cancer.

Structures of common anthocyanidins and anthocyanins



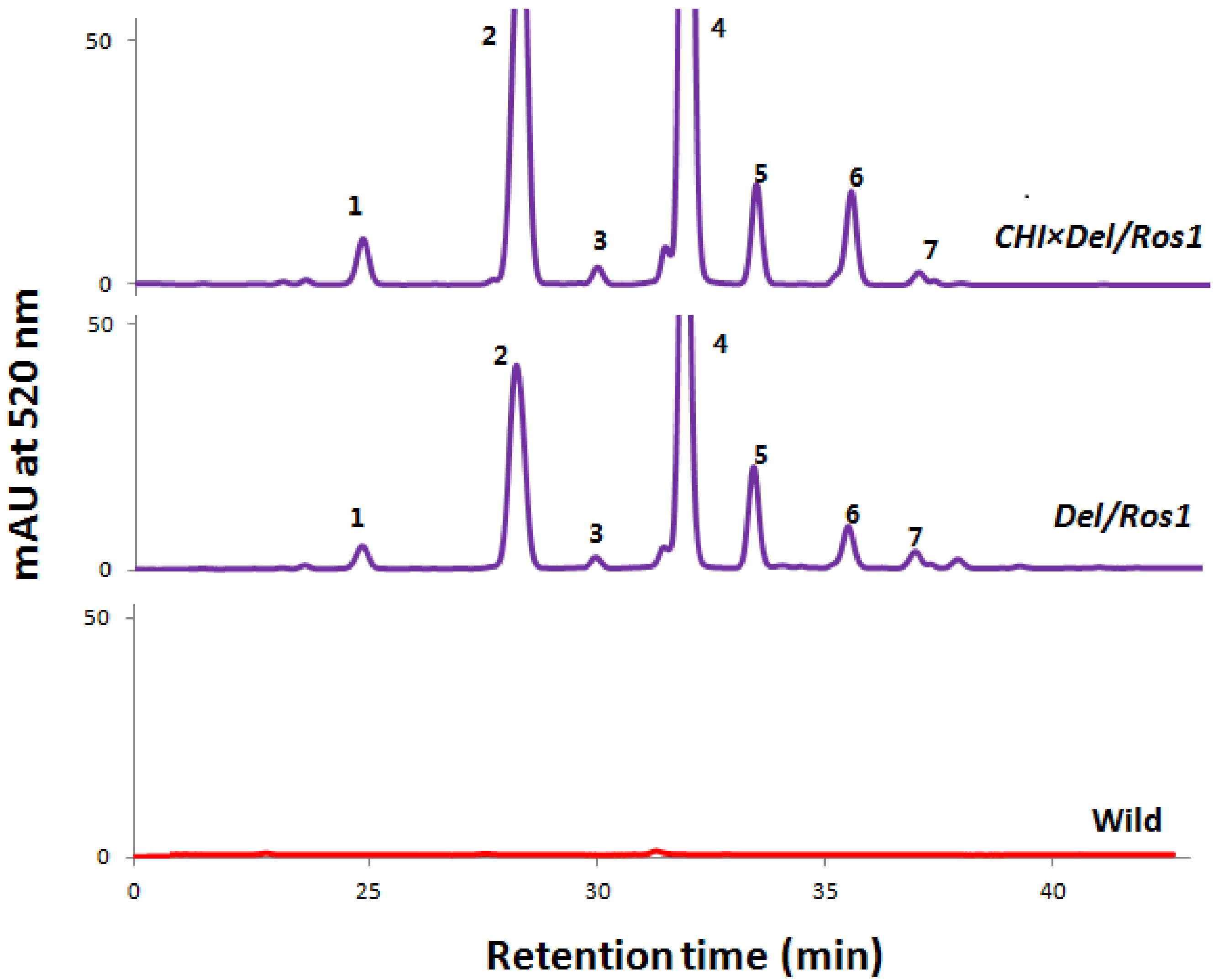
	R_1	R_2
Cyanidin	OH	H
Pelargonidin	H	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

R_3 = Glucose, galactose, rhamnose, xylose, or arabinose

Cyanidin = 287.2 g/mol
Malvindin = 331.3 g/mol

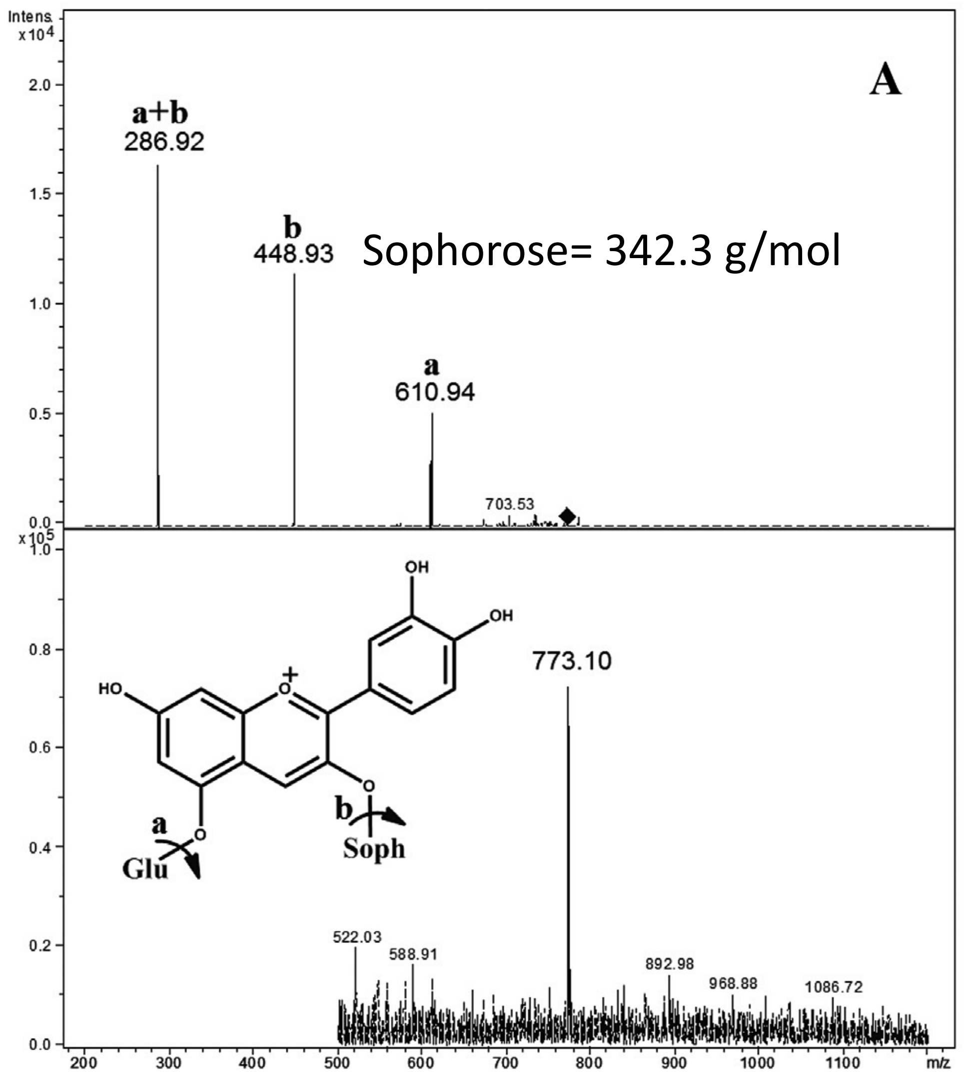
PhD Thesis. Xiaoyu Su. Kansas State University 2014

C18 Reverse phase HPLC separation of a methanolic extract



PhD Thesis. Xiaoyu Su. Kansas State University 2014

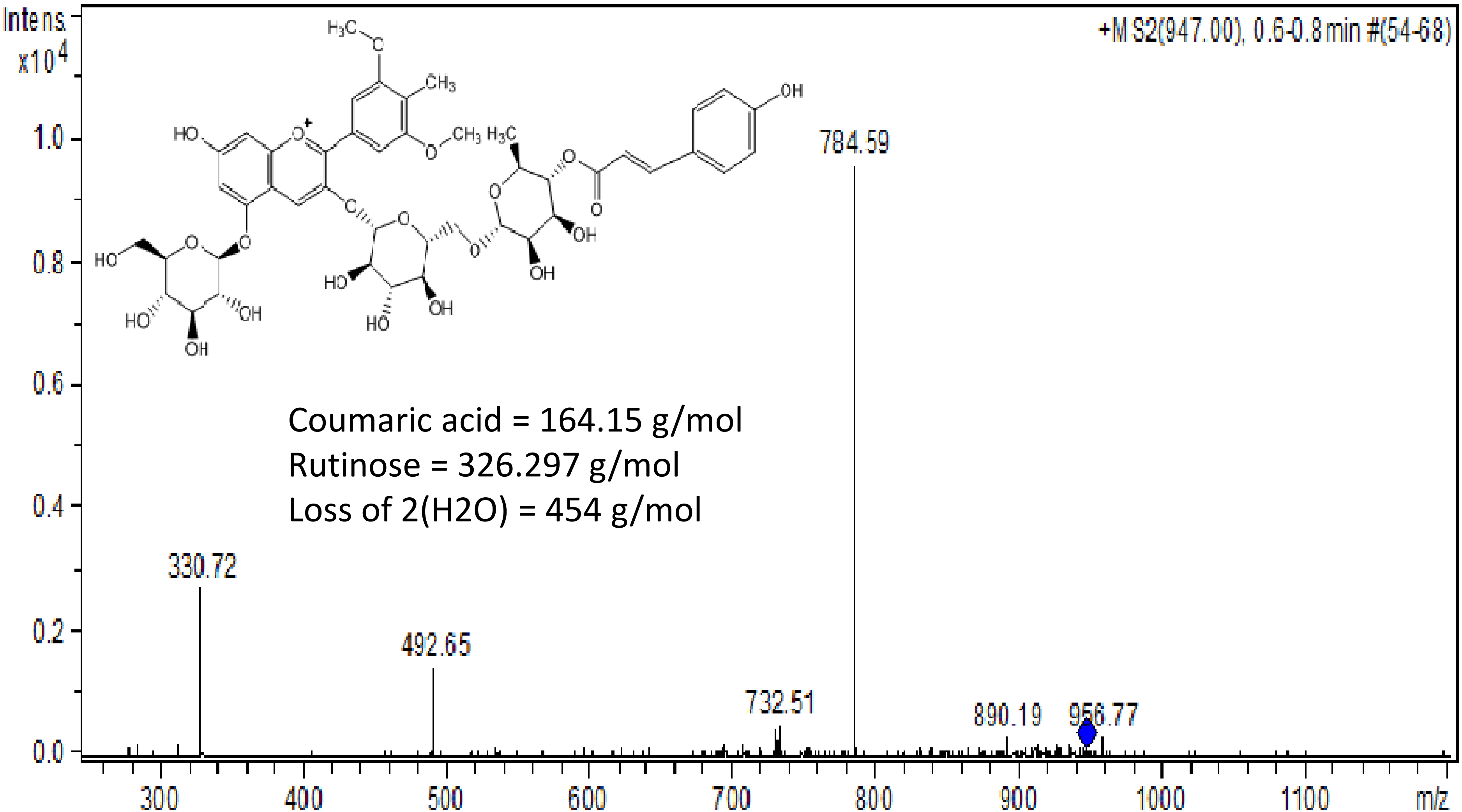
Dissecting the ESI-MS/MS daughter ions



Peak 2 : Cyanidin 3-sophoroside-5-glucoside

Characterization and stability of anthocyanins in purple fleshed sweetpotato P40. J Xu, B.Katz et al. Food Chemistry, 2014 Elsevier

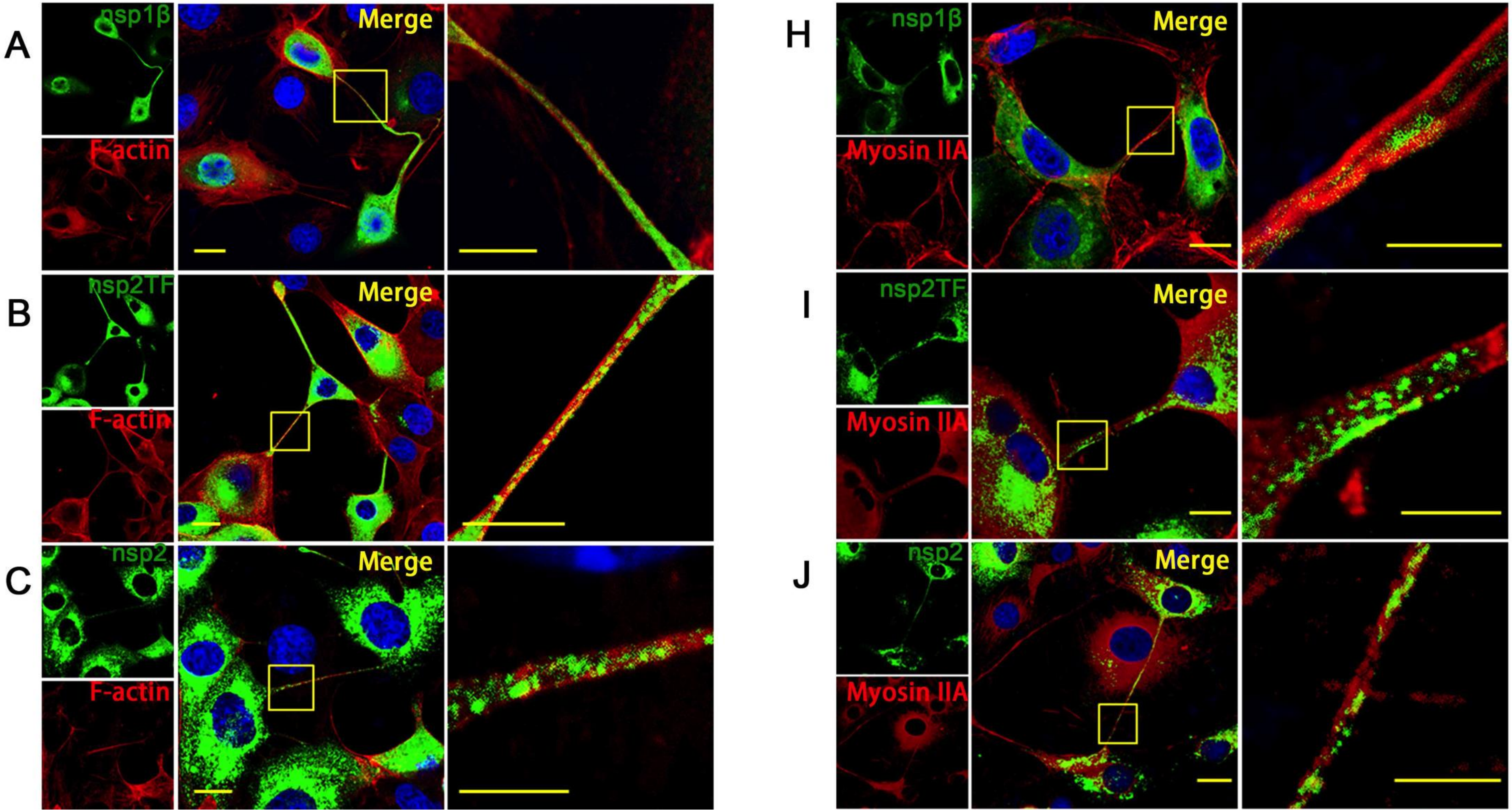
Discovering unknowns by fragmentation pattern



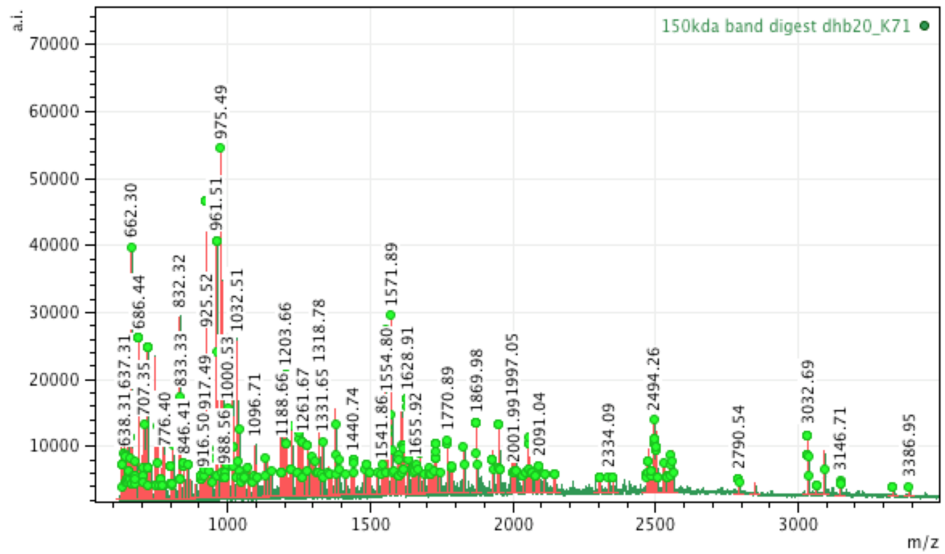
Peak 6: malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside

Porcine reproductive and respiratory syndrome virus utilizes nanotubes for intercellular spread.

Rui Guo, Benjamin B. Katz, et al. In Press Journal of Virology. 2016



Myosin II Identification using In-gel Trypsin Digestion with MALDI-TOF



Sequence - >gi|635102017|ref|XP_007973837.1| PREDICTED: myosin-9 [Chlorocebus sabaeus]

Accession	Length	Mo. Mass	Av. Mass	Coverage	Matched Int.
	1960	228713.77	228853.49	88.2 %	59.3 %

```
MAQQAADKYL YVDKNFINNP LAQADWAAKK LVWVPSDKSG FEPASLKEEV GEEAIVELVE NGKKVKVNKD DIQKMNPPKF SKVEDMAELT CLNEASVLHN LKERYYSGLI YTYSGLFCVV INPYKNLPIY
SEEIVEMYKG KKRHEMPPHI YAITDTAYRS MMQDREDQSI LCTGESGAGK TENTKKVIQY LAYVASSHKS KKDQGELERQ LLQANPILEA FGNAKTVKND NSSRFGKFIR INFDVNGYIV GANIETYLLE
KSRAIRQAKE ERTFHIFYYL LSGAGEHLKT DLLLEPYNKY RFLSNGHVTI PGQQDKDMFQ ETMEAMRIMG IPEEEQMGLL RVISGVLQLG NIVFKKERNT DQASMPDNTA AQKVSHLLGI NVTDFTRGIL
TPRIKVGRDY VQKAQTKEQA DFAIEALAKA TYERMFRWLV LRINKALDKT KRQGASFIGI LDIAGFEIFD LNSFEQLCIN YTNEKLQQLF NHTMFILEQE EYQREGIEWN FIDFGLDLQP CIDLIEKPAG
PPGILALLDE ECWFPKATDK SFVEKVMQEQ GTHPKFQKPK QLKDKADFCI IHYAGKVDYK ADEWLMKNMD PLNDNIATLL HQSSDKFVSE LWKDVDRIIG LDQVAGMSET ALPGAFKTRK GMFRTVGQLY
KEQLAKLMAT LRNTNPNFVR CIIPNHEKKA GKLDPHLVLD QLRCNGVLEG IRICRQGFPN RVVFQEFRQR YEILTPNSIP KGFMDGKQAC VLMIKALELD SNLYRIGQSK VFFRAGVLAH LEEERDLKIT
DVIIGFQACC RGYLARKAFA KRQQQLTAMK VLQRNCAAYL KLRNWQWWRL FTKVKPLLQV SRQEEEMMAK EEELVKVREK QLAAENRLTE METLQSQLMA EKLQLQEQLQ AETELCAEAE ELRARLTAKK
QELEEICHDL EARVEEEEER CQHLQTEKKK MQQNIQELEE QLEEEESARQ KLQLEKVTTE AKLKKLEEEQ IILEDQNCKL AKEKKLLEDR IAEFTTNLTE EEEKSKSLAK LKNKHEAMIT DLEERLRREE
KQRQELEKTR RKLEGDSTDL SDQIAELQAQ IAELKMQLAK KEEELQAALA RVEEEAAQKN MALKKIRELE SQISELQEDL ESERASRNKA EKQKRDLGEE LEALKTELED TLDTTAAQQE LRSKREQEVN
ILKKTLEEEA KTHEAQIQEM RQKHSQAVEE LAEQLEQTKR VKANLEKAKQ TLENERGELA NEVKVLLQGK GDSEHKRKKV EAQLQELQVK FNEGERVRTE LADKVTKLQV ELDNVTGLLS QSDSKSSKLT
KDFSALESQL QDTQELLQEE NRQKLSLSTK LKQVEDEKNS FREQLEEEEE AKHNLEKQIA TLHAQVADMK KKMEDSVGCL ETAEEVKRKL QKDLEGLSQR HEEKVAAYDK LEKTKTRLQQ ELDDLLVDLD
HQRQSACNLE KKQKKFDQLL AEEKTISAKY AEERDRAEAE AREKETKALS LARALEEAME QKAELERLNK QFRTEMEDLM SSKDDVGKSV HELEKSKRAL EQQVEEMKTQ LEELEDELQA TEDAKLRLEV
NLQAMKAQFE RDLQGKDEQS EEKKKQLVRQ VREMEAELED ERKQRSMAVA ARKKLEMDLK DLEAHIDSAN KNREEAIKQL RKLQAQMKDC MRELDDTRTS REEILAQAKE NEKKLKSMEA EMIQLQEELA
AAERAKRQAQ QERDELADEI ANSSGKGALA LEEKRRLEAR IAQLEEELEE EQGNTELIND RLKKANLQID QINTDLNLER SHAQKNENAR QQLERQNKEL KVKLQEMEGT VKSKYKASIT ALEAKIAQLE
EQLDNETKER QAACKQVRRT EKKLKDVLLQ VDDERRNAEQ YKDQADKAST RLKQLKRQLE EAEEEAQRAN ASRRKLQREL EDATETADAM NREVSSLKNK LRRGDLPFVM PRRMARKGAG DGSDEEVDGK
ADGAEAKPAE
```

Acknowledgements

PI : Dr. John Tomich

Dr. Takeo Iwamoto
Nozomi M. Caton
Deane Lehman
Susan Whitaker

<u>Past Students</u>
Kelsey Pearson (Nursing)
Jammie Layman (Law School)
Kyle Steuber (Nutrisciences)
Jared Wilmoth (Grad School)
Marlena Birkel (Pharm School)
Denise Cobb (Grad School)
Macy Garcia

<u>Current Students</u>
Will Smith
Emily Wedeman
Kayla Wilkinson
Cassidy Frost

Dr. George Wang
Dr. Sunghun Park
Tzu-Yu Chen
Soyoung Lim
Jianteng Xu
Xiaoyu Su
Yanting Shen

Dr. Ying Fang
Rui Guo
Thomas Gallagher

<u>External</u>
Mike Molitor (U.Wisc., Madison)
Dan Abitz (GBA Engineering)

<u>Supported by</u>
Johnson Cancer Center
Kansas State University