Novel Approaches for the Discovery of Allergenic Components in Gluten

Robert D. Voyksner and Jennifer Sealey-Voyksner
LCMS Limited
Durham, NC, USA
www.lcmslimited.com
Overview of this presentation:

• Introduction to gluten and gluten allergies and intolerance.

• Summarize the process that led to the identification of gluten marker immunogenic peptides.

• Discuss practical applications of the LC/MS/MS assay for the quantitative determination of the presence of immunogenic gluten peptides in foods.

• Compare the LC/MS/MS approach to current approached to measure gluten.
The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) has identified eight foods as the most common food allergens. The FDA regulates the labeling of foods, such that these eight foods and any ingredient that contains protein derived from one or more of them, has to be clearly identified. Wheat is one of these eight foods.

“Gluten” is a generic term that refers to the water-insoluble fraction of a large family of seed storage proteins found in mature cereal grain seeds of the Triticeae tribe (wheat, rye and barley) of the grass (Gramineae) family.

Dietary gluten detection and quantification is important because some gluten proteins are implicated in a variety of immune diseases, food allergies and intolerances.
Gluten allergies or intolerance

- Gluten allergy: IGE and histamine response: skin and eyes irritation, hives, coughing, stomach pains

- Gluten intolerance: celiac disease
  strong antibodies reacting to the proteins in gluten (gliadins and glutenins). These overreacting antibodies lash out at your intestinal track.

People need to know their food is free of gluten. Food labeling, manufacturing practices and QC procedures would benefit from an analytical procedure which can selectively and sensitively identify and detect gluten
What is Celiac Disease?

- chronic autoimmune disease affecting ~1% world’s Western population
- symptoms manifest themselves in the small intestine
- multi-factorial disease:
  - (1) exposure to gluten
  - (2) genetic predisposition to the disease
  - (3) other environmental factors
- gluten proteins are not completely metabolized making them “toxic” (immunogenic) peptides
  - bind to HLA-DQ2 with a specific confirmation (P content)
  - selective deamidation from TG2 (Q content)
  - T cells are targeted … malabsorption of food … death
  - The goal is to identify these immunogenic peptides
Effects of gluten in Celiac Disease

villi
Adaptive and Innate autoimmune responses to gluten peptides

- Intestinal villous atrophy
- Small peptides resistant to digestive enzymes
- Dietary gluten
- Intestinal digestive enzymes

- TG2
- APC
- DQ2/8
- CD4+ T cell
- Activated T cell
- Anti-gluten antibodies
- Anti-TG2 antibodies
- Villous atrophy

- Adaptive and Innate autoimmune responses to gluten peptides
**In-vitro** enzymatic digestion of wheat flour

Which peptides trigger the immune response?
In-vitro procedure that mimics the in-vivo TG2 targeting reaction: specific glutamine residues in physiologically relevant gluten peptides are deamidated then fluorescent tagging of that site occurs.
Sample prep:
Native cereal grains were proteolytically digested using conditions and enzymes that model the human digestion process and then treated with human tissue transglutaminase 2 (TG2) and directly analyzed by LC/MS/MS

LC separation:
column: Poroshell 120 SB C18 2.1x50mm
0-40% B in 60 or 90 min then to 70% B in 15min
A= 95/5 water/acetonitrile 0.025% TFA; B= 95/5 water/acetonitrile 0.025% TFA
flowrate 0.25 ml/min; 10-40 ul injected

Fluorescence detection (detection of tagged peptides):
Agilent 1260 with 4 ul cell, 1:1 split post column between fluorescence detector and MS
MDC ab= 335 nm  em= 526 nm; RhC  ab= 557 nm  em= 581 nm

LC/Q-TOF (peptide discovery and identification):
Agilent 6530 High Definition Q-TOF with 1290 Infinity LC
reference ions at m/z  322, 1222 and 2422 added via a post column tee
operated in +ESI in auto MS/MS mode

Ion Trap (peptide discovery and identification):
Agilent Ion trap MS (Classic) with 1100 series LC system operated in +ESI in auto MS mode

Triple Quadruple (Target peptide quantitation):
Agilent 6410 Triple with 1200 series LC system operated in +ESI in MRM mode observing the 2 most sensitive transitions
Data analysis process for the identification of immunogenic gluten peptides

Grain sample (digested with proteolytic enzymes) → MS spectra → TG2 + chemical tag reaction with MDC or RhC → MS spectra

TG2 → LC/fluorescence chromatograms → verify m/z of untagged peptide

MS spectra → data base search and interpretation of MS/MS spectra → potential target peptide sequence

postulated MW of tagged peptide → back calculate peptide MW with no tags → determine the presence/absence of this peptide m/z in other grains

** this peptide could have several tags! **

verify target peptide sequence mass and its measured mass agree to within 5 mmu

define # of tags
AF Chromatogram for RhC tagged peptides identified in Wheat

• note that the labeled peaks are the unique peptides that were identified; there are other large peaks present that were found to be in the control
#       MW                        Sequence        Mass Error  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th>(mmu)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1957.9857</td>
<td>LQPQNPSQQQPPSEQVPL</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2148.0388</td>
<td>TQQPQQPFPQPQQPQPFPQ</td>
<td>1.2</td>
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<tr>
<td>3</td>
<td>2478.2867</td>
<td>VPVPQLQPQNPSQQQPQEVL</td>
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<tr>
<td>4</td>
<td>1625.7950</td>
<td>RPQQPYQPQPQPQY</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2148.0388</td>
<td>QPQQPFPQTPQQPQPFPQ</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>702.3336</td>
<td>PQQSPF</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1531.7783</td>
<td>QPQQQLPQPQPQPF</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

Immunogenic Gluten Peptides in Wheat
Peptides 1-7 represent all classes of wheat gluten proteins

wheat gluten proteins

- gliadins (monomers)
  - ω-gliadins
    - peptide 1
    - peptide 4
  - γ-gliadins
    - peptide 2
    - peptide 5
    - peptide 6
  - α/β-gliadins
    - peptide 1
    - peptide 2
    - peptide 3
    - peptide 4

- glutenins (polymers)
  - LMW subunits
    - peptide 3
    - peptide 6
    - peptide 7
  - HMW subunits
    - peptide 3
AF Chromatogram for RhC tagged peptides identified in barley and rye

- note that the labeled peaks are the unique peptides that were identified; there are other large peaks present that were found to be in the control
# Immunogenic Gluten Peptides in Barley

<table>
<thead>
<tr>
<th>#</th>
<th>MW</th>
<th>Sequence</th>
<th>Mass Error (mmu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>856.4443</td>
<td>QPQQPFL</td>
<td>0.9</td>
</tr>
<tr>
<td>9</td>
<td>616.3089</td>
<td>QPQPF</td>
<td>0.6</td>
</tr>
<tr>
<td>10</td>
<td>727.3405</td>
<td>AHQQQPF</td>
<td>0.8</td>
</tr>
<tr>
<td>11</td>
<td>1246.5982</td>
<td>LQQPYPQNPY</td>
<td>0.6</td>
</tr>
<tr>
<td>12</td>
<td>899.4376</td>
<td>QPQPWQP</td>
<td>0.4</td>
</tr>
<tr>
<td>13</td>
<td>1194.6397</td>
<td>IIPQQPQQPF</td>
<td>0.6</td>
</tr>
<tr>
<td>14</td>
<td>1531.7783</td>
<td>QPQQPLPQPQQQPF</td>
<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>1548.7685</td>
<td>QQVPQPQPPQQQPF</td>
<td>2.1</td>
</tr>
<tr>
<td>16</td>
<td>830.4664</td>
<td>VQVQIPF</td>
<td>0.5</td>
</tr>
<tr>
<td>17</td>
<td>1758.8108</td>
<td>QVNMQQQQQHYSQQL</td>
<td>3.4</td>
</tr>
</tbody>
</table>
## Immunogenic Gluten Peptides in Rye

<table>
<thead>
<tr>
<th>#</th>
<th>MW</th>
<th>Sequence</th>
<th>Mass Error (mmu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>727.3405</td>
<td>AHQQQPF</td>
<td>0.2</td>
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<tr>
<td>19</td>
<td>954.5032</td>
<td>LQPPQQML</td>
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<tr>
<td>20</td>
<td>1531.7783</td>
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<td>1.4</td>
</tr>
<tr>
<td>21</td>
<td>1581.7729</td>
<td>APPAPHWPPPPQQPY</td>
<td>4.5</td>
</tr>
<tr>
<td>22</td>
<td>1148.6554</td>
<td>VLVQPQQQQL</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Verifying MW of peptide #8 using MDC tag

MDC-peptide MW is 1175.5501
untagged peptide MW is 856.4441

EIC for 1 MDC

EIC for 2 MDC

EIC for 3 MDC

EIC for Corn at same mass as 1MDC
(signal is unique to Barley)
Looking for postulated peptide #8 (MW 856.4441) in barley

Barley showing [M+H]+ ion at 857.4519 for the target peptide

The target peptide is absent in corn indicating this is a gluten peptide
RhC to determine MW of tagged #8 peptide

**AF for RhC**

**EIC**

m/z 677

[M+2H]^{+2} based on isotope spacing

MW = 1353.6758

Untagged peptide MW is 856.4441 (1 RhC attached)
MS/MS Sequencing and database search results for the peptide #8 (MW 856.4441)

Hordein C – barley

1 MKTFLTFVLL AMAMSIIVTTA RQLNPSHQL E QSPQQPFLKQ QSYLQQPY P Q
51 QPYLPQQPFP TPQFFPYLP QQTFFPSQQP NPLQPQQPFP LQPQPPQQPQFP
101 PQPQQPNPQQ PQPPFPPRPQP QIVPQQPQPQ FPQQPQPPFP QPQQPFSWQP
151 QQQFPFLQQLXL QPLQAQQQPFP LQPQPLFPQFP QQPIGQQPKQ PLLQQPQQTi
201 PQPQPPQQFPL QPPQQPPQPP QQLPQPQQQQ IISSQQPQQPQ PLQPQPPQPFP
251 PQPFPQEQPQ QAFPLQPPQQ FPFESEQIIT QQPFPFLQPQQL LFPQQQQPQPL
301 PQPQQPFPRQL PKYIPQPQQQ QPPLLQPQHPQ QQPYAQQDIW SDIALLG
## HPLC-MS/MS analysis of food and consumer products

### Native flours:
- * corn flour
- * soy flour
- wheat flour
- wheat gluten
- rye flour
- barley flour
- * quinoa flour
- * oat flour
- * rice flour

### Processed foods:
- bread
- crackers
- goldfish crackers
- cheerios
- pasta
- orzo
- * rice crackers
- * powdered ice tea mix

### Gluten-free labeled products:
- * gluten free pasta (1)
- * gluten free pasta (2)
- * gluten free bread
- * gluten free pretzels
- * gluten free rice seasoning mix
- * gluten free crackers
- * gluten free beer
- * gluten free pad thai seasoning
- * quinoa pasta
- * soy pasta
- * protein bar
- * quinoa cereal

### Beverages and sauces:
- beer
- gin
- * potato vodka
- hot, hot, hot sauce
- vinegar
- * rum
- * red/white wine

### Consumer products:
- body wash
- * toothpaste
- * body lotion
## LC/MS/MS Experimental Conditions

Agilent 6410 triple quad ESI + operated in MRM mode

<table>
<thead>
<tr>
<th>Peptide #</th>
<th>Sequence</th>
<th>Monoisotopic MW (Da)</th>
<th>Time segment</th>
<th>MRM transition*</th>
<th>Product ion type</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LQPQNPSQQPQEQQVPL</td>
<td>1958.0</td>
<td>4</td>
<td>980.7(+2)–866.6(+2) 980.7(+2)–1150.7</td>
<td>b15 b10</td>
<td>20 30</td>
</tr>
<tr>
<td>2</td>
<td>TQPQPQPFPQQPQPFPQ</td>
<td>2148.0</td>
<td>5</td>
<td>1075.9(+2)–1195.6 1075.9(+2)–956.6 1075.9(+2)–244.4</td>
<td>y10 b8 y2</td>
<td>30 25 17.5</td>
</tr>
<tr>
<td>3</td>
<td>VPVPLQPQNPSQQPQEQQVPL</td>
<td>2478.3</td>
<td>6</td>
<td>1240.9(+2)–1126.7(+2) 1240.9(+2)–762.4</td>
<td>b20 b7</td>
<td>25 30</td>
</tr>
<tr>
<td>4</td>
<td>RPQQPYPQPQPY</td>
<td>1627.8</td>
<td>3</td>
<td>814.6(+2)–1221.8 814.6(+2)–407.3</td>
<td>b10 y3</td>
<td>25 30</td>
</tr>
<tr>
<td>5</td>
<td>QPQPFPQPTQQPQPFPQ</td>
<td>2148.0</td>
<td>5</td>
<td>1075.9(+2)–726.3 717.6(+3)–244.1 1075.9(+2)–1308.8</td>
<td>b6 y2 b11</td>
<td>27.5 17.5 25</td>
</tr>
<tr>
<td>6</td>
<td>PQQSF</td>
<td>702.3</td>
<td>2</td>
<td>703.4–441.4 703.4–263.3</td>
<td>b4 y2</td>
<td>25 35</td>
</tr>
</tbody>
</table>

Target peptide sequences, their respective monoisotopic molecular weights, time program segments, parent and product MRM ion transitions and respective collision energies used for all analyses (*charge state other than 1 is listed in parentheses: MRM transitions in bold were used for quantification; MRM transitions in italics were used for confirmation).
Confirming Peptide 5 is present in Wheat

- Standard
  - Quantifying ion 827 → 1126.7
  - Confirming 1240.9 – 1126.7
  - Confirming 1240.9 – 762.4

- Corn

- Wheat
LC/MS/MS determination of peptide 7 in different grains - First target peptide marker reported common to wheat, rye and barley.

- Standard
- Peptide 7
- Gluten
- Wheat
- Rye
- Barley
Calibration Curves

Calibration range from 0.01 to 100 ng/mg (0.2 – 2000 pg injected on column)
Accuracy of the recovery of the six target peptides spiked at 0.06 ng/mg and 30 ng/mg into wheat

<table>
<thead>
<tr>
<th>#</th>
<th>Sequence</th>
<th>% Accuracy (0.06ng/mg)</th>
<th>% Accuracy (30 ng/mg)</th>
<th>LOQ ng/ml</th>
<th>Linear corr</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>LQPQNPSQQQPQEQVPL</td>
<td>96.2</td>
<td>92.8</td>
<td>0.02</td>
<td>0.9976</td>
</tr>
<tr>
<td>2</td>
<td>TQQPQQPFPQQPQQPFPQ</td>
<td>85.6</td>
<td>95.8</td>
<td>0.01</td>
<td>0.9960</td>
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<tr>
<td>3</td>
<td>VPVPQLQPQNPSQQQPQEQVPL</td>
<td>103.5</td>
<td>97.1</td>
<td>0.05</td>
<td>0.9992</td>
</tr>
<tr>
<td>4</td>
<td>RPQQPYQPQPQPYQY</td>
<td>90.4</td>
<td>92.3</td>
<td>0.02</td>
<td>0.9916</td>
</tr>
<tr>
<td>5</td>
<td>QPQQPFPQTQPPQQPFPQ</td>
<td>90.1</td>
<td>99.1</td>
<td>0.01</td>
<td>0.9963</td>
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<tr>
<td>6</td>
<td>PQQSPF</td>
<td>68.7</td>
<td>97.6</td>
<td>0.01</td>
<td>0.9977</td>
</tr>
<tr>
<td>7</td>
<td>QPQQPLPQPPQQPF</td>
<td>92.2</td>
<td>98.6</td>
<td>0.01</td>
<td>0.9969</td>
</tr>
</tbody>
</table>

Relative standard deviation (%) 11.7 5.9 0.9967
Quantitative results from the analysis of native and processed products by LC-MS/MS.

<table>
<thead>
<tr>
<th>Product description</th>
<th>Product</th>
<th>Measured amount of peptide (ng/mg)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Peptide 1</td>
</tr>
<tr>
<td>Native flours</td>
<td>Corn flour*</td>
<td>HD</td>
</tr>
<tr>
<td></td>
<td>Soy flour*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Wheat flour</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>Wheat gluten</td>
<td>510</td>
</tr>
<tr>
<td></td>
<td>Rye flour</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Barley flour</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Quinoa flour*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Oat flour*</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>Rice flour*</td>
<td>ND</td>
</tr>
<tr>
<td>Processed foods</td>
<td>Bread</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Sun chips</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Goldfish crackers</td>
<td>190</td>
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<tr>
<td></td>
<td>Cheerios</td>
<td>3.3</td>
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<tr>
<td></td>
<td>Wheat pasta</td>
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</tr>
<tr>
<td></td>
<td>Orzo</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Rice crackers*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Powdered ice tea mix*</td>
<td>0.046</td>
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<tr>
<td>Gluten-free products</td>
<td>Gluten-free pasta (1)*</td>
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</tr>
<tr>
<td></td>
<td>Gluten-free pasta (2)*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gluten-free bread*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gluten-free pretzels*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gluten-free rice seasoning mix*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gluten-free cracker*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gluten-free beer*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Gluten-free pad thai seasoning*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Quina pasta*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Soy pasta*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Protein bar*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Quinoa cereal*</td>
<td>ND</td>
</tr>
<tr>
<td>Beverages and sauces</td>
<td>Beer (light)</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>Gin</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Potato vodka*</td>
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<td></td>
<td>Hot sauce</td>
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<td></td>
<td>Vinegar</td>
<td>D</td>
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<tr>
<td></td>
<td>Rum*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Red wine*</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>White wine*</td>
<td>ND</td>
</tr>
</tbody>
</table>
Gluten peptides 1-7 detected in wheat pasta and gluten free pasta

B  Made gluten free in a facility that processes wheat products
C  Made in a gluten free facility
Gluten peptides detected in ice tea mix

Gluten is not listed in as an ingredient on the product label of this product.
Gluten peptides detected in body wash
HPLC-MS/MS assay for the quantitative determination of peptides 1-7

• capability for trace level gluten (both prolamines (gliadins) and glutelins) detection:
  
  10 pg/mg to 100 ng/mg (10 ppb – 100 ppm in food)

• ability to detect gluten in native, processed and cooked foods
  
  * manufacturing processes
  * package labeling
  * food safety

Quantitative determination of trace levels of gluten in commercially available food and consumer products.
# ELISA Method Comparison

<table>
<thead>
<tr>
<th></th>
<th>ω-gliadin ELISA</th>
<th>R5 ELISA</th>
<th>LC/MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>What it measures</strong></td>
<td>Monoclonal antibody to ω-gliadin fraction of wheat</td>
<td>Monoclonal antibody to QQPFP present in wheat barley and rye</td>
<td>Many physiological relevant peptides representing wheat barley and rye gluten</td>
</tr>
<tr>
<td><strong>LOQ</strong></td>
<td>150 ppm</td>
<td>5 ppm</td>
<td>10 ppb</td>
</tr>
<tr>
<td><strong>Intended Use</strong></td>
<td>Measures gluten in cooked and uncooked foods</td>
<td>Measures gluten in cooked and uncooked foods</td>
<td>Measures gluten in cooked and uncooked foods</td>
</tr>
<tr>
<td><strong>Strengths</strong></td>
<td>ω -gliadin not denatured when heated — address cooked and uncooked foods</td>
<td>QQPFP heat resistant; can measure gluten in cooked and uncooked foods; works for wheat gliadin, barley hordein, and rye secalin</td>
<td>Sensitive! Measures gluten peptide markers in cooked and uncooked foods and liquids from wheat, barley and rye; Numerous markers cover many classes of prolamins and glutelins resulting in less false neg or pos</td>
</tr>
<tr>
<td><strong>Drawbacks</strong></td>
<td>Underestimates barley prolamin; Does not work for hydrolyzed gluten; Measurement varies depending on gliadin standard used</td>
<td>Can overestimate barley prolamin; Does not work for hydrolyzed gluten</td>
<td>Instrumentation costs; Requires synthetic peptide standards</td>
</tr>
</tbody>
</table>

ELISA summary from: Thompson T, Mendez E, J Am Diet Assoc 2008 108
Conclusions from this research project:

- The fluorescence tagging of the TG2 activated sites together with LC/q-TOF and Ion trap has enabled the discovery of 22 peptides that can serve as markers for the presence of wheat, rye and barley in foods. These peptides were not found in foods considered gluten free such as corn, rice or soy.

- LC/MS/MS methods were developed capability to screen commercially available food and consumer products for the quantitative detection of these peptides with at least 100x better sensitivity than current ELISA methods.

Significance of the work:

- These efforts will pave the way for eventual commercial application, as a service to both the Celiac community and others who are gluten-sensitive, thus enabling scientists to address important issues faced worldwide in disease management, food safety, quality control and food labeling.
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