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Excellence in Science

Overcoming Challenges of Protein Sample Preparation for Food Allergen Analysis

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Overcoming Challenges of Protein Sample Preparation for Food Allergen Analysis

1.Overview

An on-line sample preparation workstation that automates proteolysis, de-salting and reversed phase chromatography for proteomic workflows has been described.
Two major benefits of the system as they relate to food analysis are reproducbility and speed. By configuring the system to enable multiplexing, we have successfully shown the ability to digest and analyze 200 samples per day.

This sample preparation method has been applied to analyze Ara h 1, a common food allergen associated with peanuts. Complete digestion of the Ara h 1 protein was demonstrated reproducibly, with identification of the common tryptic peptides found in Ara h 1 protein. One common peptide, SFNLDEGHALR (*m/z* 629.8) was identified by MS/MS with abundant representation of b and y ions.

NOVEL ASPECT: The ability to quickly and efficiently digest proteins found in foods allows rapid identification and quantitation of food allergens.

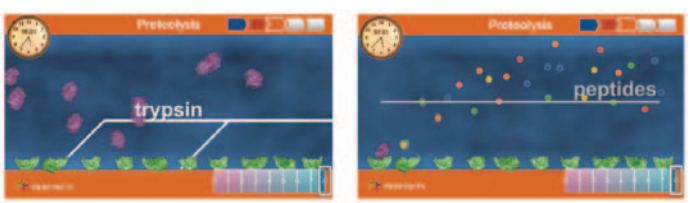
2. Introduction

Food allergens are prevalent in today's society and can result in reactions which range in severity from skin irritations to anaphylaxis. Milk, eggs, peanuts, tree nuts, fish, shellfish, soy and wheat contribute to 90% of food allergies. Regulations from the Food and Drug Administration (FDA) require proper labeling of foods to ensure safety. Currently, the only solution available for food allergy is avoidance, and as these allergens can appear as trace contaminants in unexpected food products, it is important to develop sensitive methods to detect low levels of the allergens that result in immune responses. This presentation will investigate the ability to implement an automated digestion platform prior to mass spectrometric analysis to analyze proteins associated with food allergies.

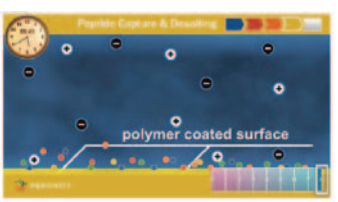
3. Methods - Perfinity iDP

The Perfinity iDP is an online protein digestion system incorporating trypsin digestion, HPLC separation and MS detection.

- Automates and integrates key proteomic workflow steps:
  - Trypsin digestion
  - Online Desalting
  - Reversed phase LC
- Reduces sample preparation times from 24 hours to less than 1 hour
- Acheives exceptional reproducibility (CV less than 10%)

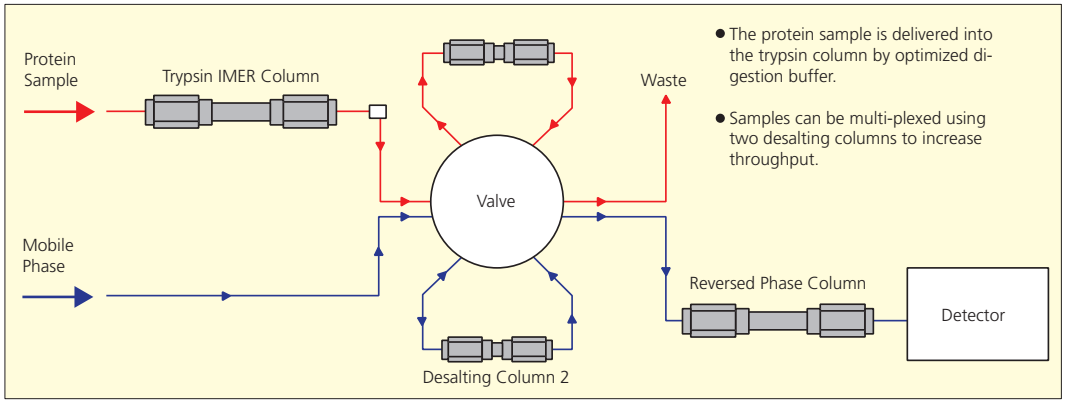


Schematic of IMER column with immobilized trypsin

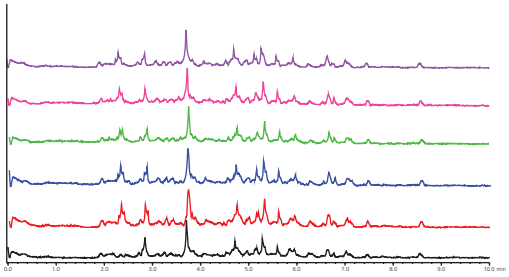


Peptide trap and desalting before RPC

4.Multiplexing with Perfinity iDP



A crude extraction with 80% ethanol was performed prior to Perfinity iDP-LCMS analysis.



Glaidin from Wheat: No red/alk. 4 minute digestion with 15 minute linear gradient using NoRA trypsin IMER. Six consecutive injections using multiplexing.

5. Methods

Sample Pretreatment: A 50 µL aliquot of 1.2 mg/mL stock of Ara h1 protein (INDOOR Biotechnologies, Charlotesville, VA) were reduced with dithiothreitol (DTT) and incubated at 60°C for 1 hr. Once the solution cooled to room temperature, the sample was alkylated with iodoacetamide and allowed to incubate in the dark at room temperature for 1 hour. After reduction and alkylation the sample was quenched with 50 mM TRIS buffer to yield a final Ara h 1 concentration of 220 µg/mL (ppm). 10 µL of Ara h 1 was injected onto an immobilized enzyme trypsin (IMER) column for digestion using flow-through incubations with variable times.

Matrix Samples: Three different concentrations of red/alk Ara h1 standard (1, 5, 10 ug/mL) were added to four different baby food matricies (beef, juice, bananas, sweet potatoes). Approximately 1.5 g of baby food was weighed and suspended in 6 mL of TRIS buffer. Then varying amounts of Ara h1 standard were added. Samples were vortexed and centrifuged and the supernatant was directly injected into the Perfinity iDP.

Perfinity Integrated Digestion Platform (iDP): An immobilized enzyme reactor (IMER) trypsin column was used for digestion using flow-through incubations with variable times and the resulting peptides were collected onto a peptide C18 guard column. Digestion times varied from 2-8 minutes at two temperatures (40°C and 70°C).

LCMS: The peptides were eluted and separated using a Phenomenex Aeris PEPTIDE (3.6 µm × 2.1 × 100 mm) (Torrance, CA) column with a flow rate of 500 µL/min. Mobile phase A consisted of 2% acetonitrile, 98% water and 0.1% formic acid. Mobile phase B consisted of 90% acetonitrile, 10% water and 0.1% formic acid with a 15 minute linear gradient from 5-50% B. The reversed phase column was directly connected to a Shimadzu ion trap-time of flight (IT-TOF) instrument for identification of tryptic peptides.

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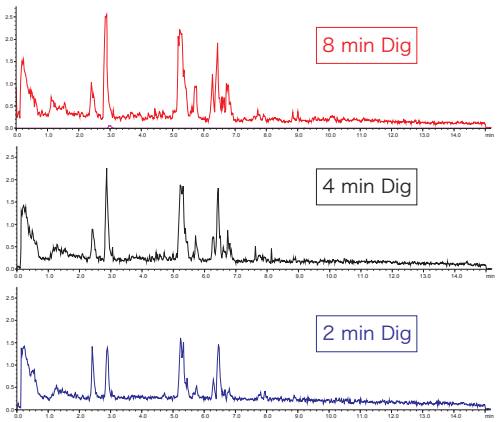
6. Optimization of Digestion Conditions



*Perfinity Optimization of Digestion Conditions:
- Temperature (40, 50, 60 degrees C)
- Digestion time (2, 4, 6, 8 minutes)

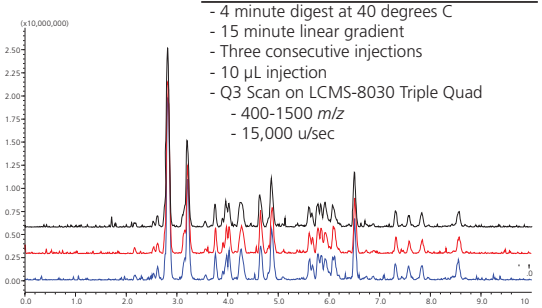
*Look for differences in peak intensity, peak area and resolution between peaks.

*Determine which condition provides the best digestion efficiency for the protein of interest and then design experiment from the chosen conditions.

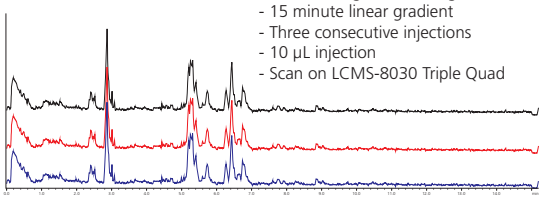
Sample : 2 ug Red/Alk Ara h1
Column 1: Perfinity Optimized Trypsin Column
Column 2: Phenomenex Kinetex 2.1mm × 100mm

Mobile Phase A: 2% ACN, 98% Water, 0.1% FA
Mobile Phase B: 90% ACN, 10% Water, 0.1% FA
MS Detection - LCMS-IT-TOF

7. Reproducibility



2 µg/mL Hemoglobin (Reduced/Alkylated)
- 4 minute digest at 40 degrees C
- 15 minute linear gradient
- Three consecutive injections
- 10 µL injection
- Q3 Scan on LCMS-8030 Triple Quad
  - 400-1500 *m/z*
  - 15,000 u/sec

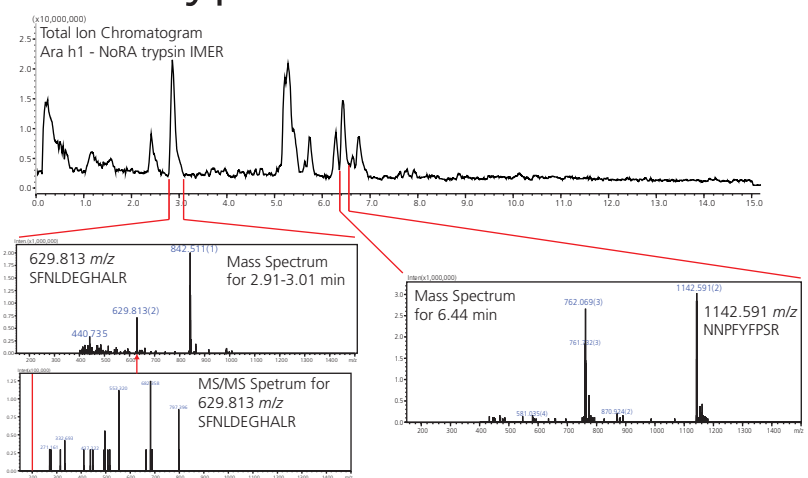


2 µg/mL Ara h1 (Reduced/Alkylated)
- 8 minute digest at 70 degrees C
- 15 minute linear gradient
- Three consecutive injections
- 10 µL injection
- Scan on LCMS-8030 Triple Quad

Representative digest chromatograms generated from Perfinity iDP with process CV's less than 10% (shown in table to the right)

Retention Time	*m/z*	Run 1	Run 2	Run 3	Average	St. Dev	%RSD
1.168	542.7506	3225850	2881846	3348254	3151983	241819	8%
1.532	1046.552	2077744	1959670	2057771	2031728	63198	3%
2.419	583.3228	7349359	5984942	6342068	6558790	707555	11%
2.867	842.5095	18527438	17430051	18429873	18129121	607374	3%
5.291	1106.554	18164616	18248820	18614069	18342502	238923	1%
5.735	970.9722	5994627	5531490	6513029	6013049	491029	8%
6.275	788.2987	7772178	6338726	7438893	7183266	750137	10%
6.44	1142.595	11134605	12293313	12824852	12084257	864299	7%
6.639	1143.078	6514803	6675553	6911765	6700707	199673	3%

8. NoRA Trypsin IMER



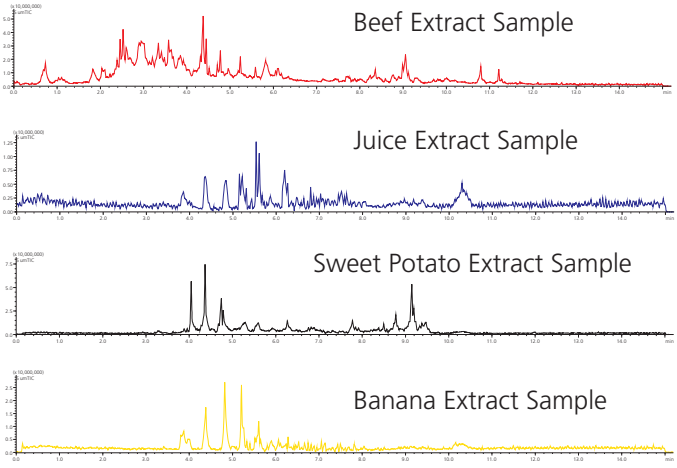
No Reduction and Alkylation (NoRA) Trypsin IMER

Trypsin digestion at high temperature (70°C)
Protein denatured at high temperature
Faster sample prep by eliminating reduction and alkylation steps

Sample: 2 ug Red/Alk Ara h1
8 minute digestion at 70°C
15 minute gradient 5-50%B

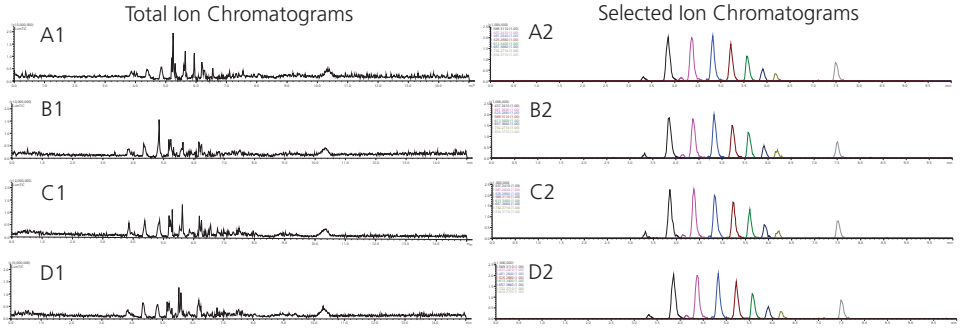
9. Results - LCMS-IT-TOF

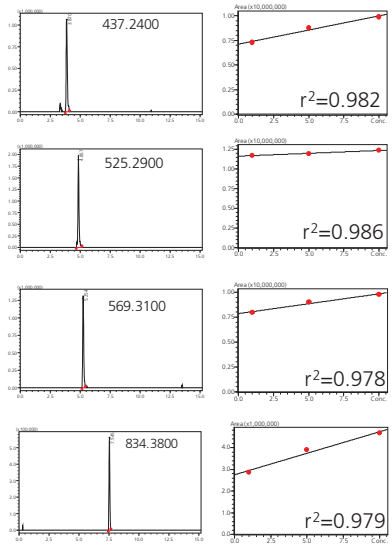
Analysis of Ara h1 (peanut allergen) in baby food matricies using LCMS-IT-TOF



*Total Ion Chromatograms (TIC) for four extract samples
- Beef, juice, sweet potato, banana
- 8 minute digestion at 40°C
*Each matrix has different peptide profiles
*Beef extract is most complex because of fat content found in the material.
*Quantitation experiments were performed using the juice extract samples.

Quantitation of Ara h1 in Juice extract sample





Juice extract samples with (A) 10 ug/mL (B) 5 ug/mL (C) 1 ug/mL (D) 0 ug/mL spiked Ara h1 standard. Chromatograms on the left (A1-D1) display the TIC from the LCMS-IT-TOF. Chromatograms on the right (A2-D2) show the specific ions used for quantitation. The table lists the respective retention times and exact masses for each ion. Calibration curves for four of the ions are shown at the far right. Each ion shows a linear response with spiked extract samples.

Ret. Time	*m/z*
3.86	437.2410
4.25	481.2640
4.68	525.2880
5.13	569.3110
5.49	613.3400
5.83	657.3660
6.13	732.2710
7.45	834.3770

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10. Conclusions

The Perfinity iDP automates and integrates protein digestion, desalting and LC separation while adding speed, quality and value to MS-based assay development. Complete, rapid digestion of the Ara h1 protein was demonstrated reproducibly and common tryptic peptides of Ara h1 protein were successfully identified on the Perfinity iDP. The Perfinity iDP coupled to LCMS-IT-TOF was able to generate linear calibration curves for spiked food samples indicating quantitative recovery of trypsic peptides. These factors suggest that this new platform is ideal for food safety applications.

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