Research Article:

Isolation and detection of gluten using Liquid Chromatography Mass Spectrometry (LC-MS/MS)

ABSTRACT:

Gluten intolerance has been a serious problem to a certain group of population. This research article aims to determine the presence of gluten on Hearty Bread products with wheat flour, rye flour and gluten free flour used as comparison samples. Method of analysis involve LC-MS/MS which is more sensitive than ELISA detection. In conclusion wheat and rye contains gluten in both beta-amylase and secalin marker peptides while gluten free flour (obtained from local market) and Hearty Bread products (n=10) are absent of gluten.

INTRODUCTION

Definition:

Gluten is a complex mixture of hundreds of related but distinct proteins, mainly gliadin and glutenin. Collectively, the gliadin and glutenin proteins are referred to as prolamins. Which represent seed proteins which are insoluble in water, but extractable in aqueous ethanol and are characterized by high levels of glutamine (38%) and proline residues (20%). (1)

Gluten may be classified to different subgroups dependent on key differences including sulfur content and molecular weight and then further classified according to their different primary structures into alpha, beta, gamma and omega (α, β, γ, and ω) gliadins. Strong covalent and non-covalent bonds connect gluten molecules which contributes to the shape and unique properties of gluten (2)

Demographics:

"gluten-related disorders" encompasses all conditions related to gluten intake; it includes autoimmune, allergic, and non-autoimmune and non-allergic diseases [3,4]. Celiac disease is an autoimmune-like gluten-related disorder, which is triggered by gluten from wheat, rye and barley. This condition affects between 0.5% and 1% of the general population [5]. In Argentina,

gluten sensitivity is commonly reported and physicians/gastroenterologists are aware of celiac disease diagnosis. [6]

Significance of the study:

Celiac disease is characterized as an inflammatory disorder of the small intestine that affects genetically predisposed individuals upon ingestion of gluten. Genes encoding HLA-DQ2 and HLA-DQ8 predispose to CD by causing the preferential presentation of Pro-rich gluten peptides that have undergone deamidation by tissue transglutaminase (tTG) to mucosal $CD4^+$ T cells. (7) Gliadin-reactive $CD4^+$ T cells have a key role in the damage of the intestinal mucosa that culminates with villus atrophy and crypt hyperplasia . (9) The prevalence of CD worldwide is increasing; it is estimated to be 0.5 to 2.0% in most of the European countries and the United States. (8) Clinical trial evidence supports an important role of diet in the generation of irritable bowel syndrome that may be in the form of gastrointestinal bleeding and flatulence. Certain diet plan alleviates such symptom. Among which include diets that are low in Fats; Carbohydrates; Gluten; or Fermented Oligosaccharides, Disaccharides, Monosaccharides and Polyols (FODMAPs). (10) The main therapeutic intervention for Celiac Disease is a gluten free diet.

FDA Guidelines on "Gluten-free" labelling:

In general, foods may be labeled "gluten-free" if they meet the definition and otherwise comply with the final rule's requirements. More specifically, the final rule defines "gluten-free" as meaning that the food either is inherently gluten-free, or does not contain an ingredient that is: 1) a gluten-containing grain (e.g., spelt wheat); 2) derived from a gluten-containing grain that has not been processed to remove gluten (e.g., wheat flour); or 3) derived from a gluten-containing grain that has been processed to remove gluten (e.g., wheat starch), if the use of that ingredient results in the presence of 20 parts per million (ppm) or more gluten in the food. Also, any unavoidable presence of gluten in the food must be less than 20 ppm. (11)

Effect of Lactobacillus on gluten:

Lactobacillus have different species namely *L. alimentarius, L. brevis, L. sanfranciscensis,* and *L. hilgardii*. Lactobacillus demonstrates the capacity to hydrolyze and degrade immunogenic gluten peptides thru variable peptidase activity. (12)

"Presently, the only effective treatment for celiac disease is a life-long gluten-free diet. In this work, we used a new mixture of selected sourdough lactobacilli and fungal proteases to eliminate the toxicity of wheat flour during long time fermentation. Immunological (R5 antibody-based sandwich and competitive enzyme-linked immunosorbent assay [ELISA] and R5 antibody-based Western blot), two-dimensional electrophoresis, and mass spectrometry (matrix-assisted laser desorption ionization-time of flight, strong-cation-exchange-liquid chromatography/capillary liquid chromatography-electrospray ionization-quadrupole-time of flight [SCX-LC/CapLC-ESI-Q-TOF], and high-pressure liquid chromatography-electrospray ionization-ion trap mass spectrometry) analyses were used to determine the gluten concentration. Assays based on the proliferation of peripheral blood mononuclear cells (PBMCs) and gamma interferon production by PBMCs and intestinal T-cell lines (iTCLs) from 12 celiac disease patients were used to determine the protein toxicity of the pepsin-trypsin digests from fermented wheat dough (sourdough). As determined by R5-based sandwich and competitive ELISAs, the residual concentration of gluten in sourdough was 12 ppm. Albumins, globulins, and gliadins were completely hydrolyzed, while ca. 20% of glutenins persisted. Low-molecular-weight epitopes were not detectable by SCX-LC/CapLC-ESI-Q-TOF mass spectrometry and R5-based Western blot analyses. The kinetics of the hydrolysis of the 33-mer by lactobacilli were highly efficient. All proteins extracted from sourdough activated PBMCs and induced gamma interferon production at levels comparable to the negative control. None of the iTCLs demonstrated immunoreactivity towards pepsin-trypsin digests. Bread making was standardized to show the suitability of the detoxified wheat flour. Food processing by selected sourdough lactobacilli and fungal proteases may be considered an efficient approach to eliminate gluten toxicity." (13)

Lactic acid bacteria (LAB) was evaluated using SDS-PAGE for its proteolytic activity. Lactobacillus was evaluated as able to utilize gluten as nitrogen source and basic amino acids increased (due to proteolysis) mainly due to release of ornithine. (14)

Method of analysis of gluten using High Performance Liquid Chromatography Mass Spectroscopy (HPLC-MS):

Over 20 years, liquid chromatography coupled with mass spectrometry (HPLC-MS) has been used in proteomics research. LC-MS has the ability to detect species based on multiply markers with multiple points of confirmation which makes it less susceptible to producing false negatives and positives. This is in comparison with the case of R5 assay, the peptide sequence glutamine-glutamine-proline-phenylalanin-proline (QQPFP) are susceptible to false positive or negative results. (15-19).

Principles of LC-MS/MS:

Mass spectrometers involve converting the analyte molecule to an ionized state which is then subjected to analysis on the basis of their mass to charge ratio (m/z). There are different types of technologies used for ionization and ion analysis. Electrospray ionization (ESI) is one of the most widely used technology. ESI works well with moderately polar molecules and is thus well suited for analysis of xenobiotics and peptides. Liquid samples are pumped through a metal capillary maintained at 3 to 5 kV and nebulized at the tip of the capillary to be sprayed into fine charged droplets. The droplets are then evaporated by the application of heat and dry nitrogen as such the residual electrical charges are transferred to the analytes. Ionized analytes are transferred to high vacuum mass spectrometer. ESI is considered as "soft ionization" which means little energy is imparted to the analyte and hence little fragmentation occurs. (This is in contrast with GC-MS for which the electron impact causes extensive fragmentation). (20)

Quadruple Analysers

There is a combination of constant and varying (radio frequency) voltages allows the transmission of a narrow band of m/z values along the axis of the rods. By varying the voltages with time, it's possible to scan across a range of m/z values resulting a mass spectrum. Majority of quadruple analysers operate at <4000 m/z and scan speeds up to 1000 m/z per sec or more are common. (A>0.1m/z). The first and third quadrupoles can also be simultaneously stepped to different m/z values, and panels of precursors or product ions pairs can be created to specifically detect a large number of targeted analytes. This process is also known as multiple reaction monitoring (MRM), commonly used in LC-MS assay run. (21)

MATERIALS & METHODOLOGY:

The method involves classical proteomic approach as adapted from previous journals. The process starts with extraction of protein from the matrix. After extraction, proteins were reduced, alkylated and digested. Extracts were diluted and analyzed with LC-MS/MS (20).

Preparation of Tryptic Digests

Extraction of Protein

0.5 g of bread (Hearty Bread) were powdered and placed into the test tube (15 mL) with extraction buffer (5 mL of 50:50 mixture of ethanol containing 2M urea and 50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)). This is shaken and heated in water bath (40°C, 60 minutes).

Reduction and Alkylation of Proteins

Once extracted the samples were centrifuged (2500 rpm, 5 min, 20°C). The supernatant (0.5 mL) was then reduced by the addition of TCEP [tris(2-carboxyethyl)phosphine, 0.2 M, 50 µL, 60 °C, 60 min in a thermal mixer] and cooled to room temperature. MMTS (methyl methanethiosulfonate, 0.2 M, 100 µL) was added and the sample left in the dark (30 min) to alkylate the free cysteine residues. (20)

Tryptic Digestion of Proteins

Once the proteins had been alkylated the sample were diluted with buffer (1.35 mL, 0.1 M ammonium bicarbonate solution) and trypsin was added. The proteins were then digested for one hour. The digestion was quenched by taking the digest extract (100 µL) and adding 0.1% formic acid (300 µL). The sample was centrifuged (13,000 rpm, 5 min) and then the supernatant was injected into the LC-MS/MS system (20).

LC-MS/MS Analysis of Tryptic Digests

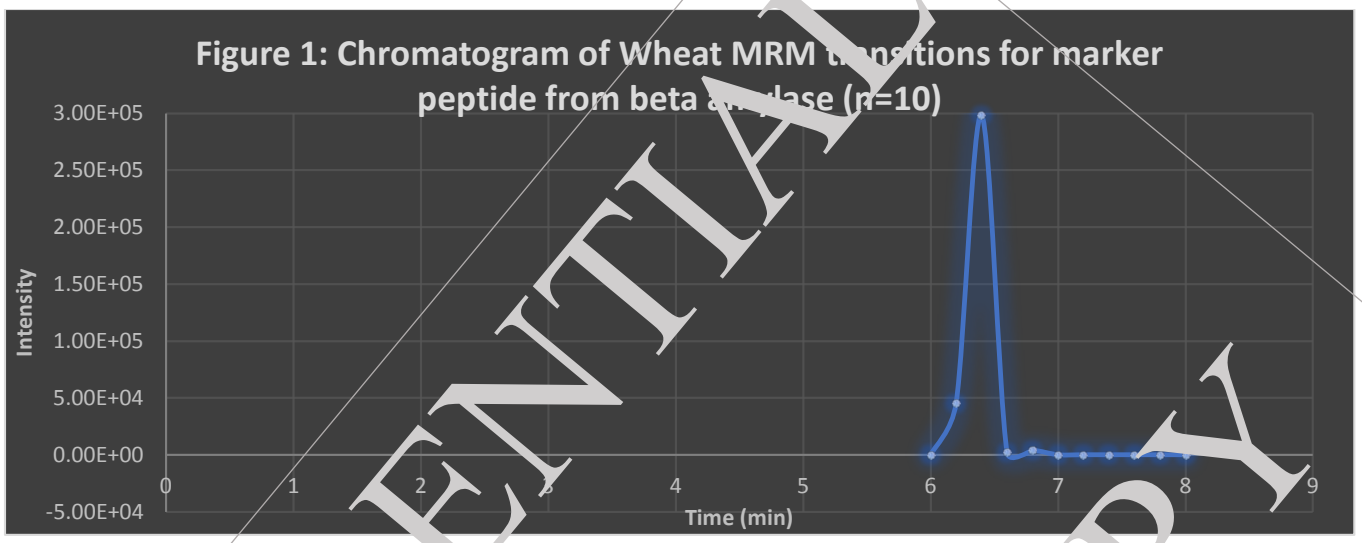
The extracts (10 µL injection) were separated on a reversed-phase column at a temperature 40C using the gradient conditions shown in Table 1 where A was water, B was acetonitrile with both phases containing 0.1% formic acid. (20)

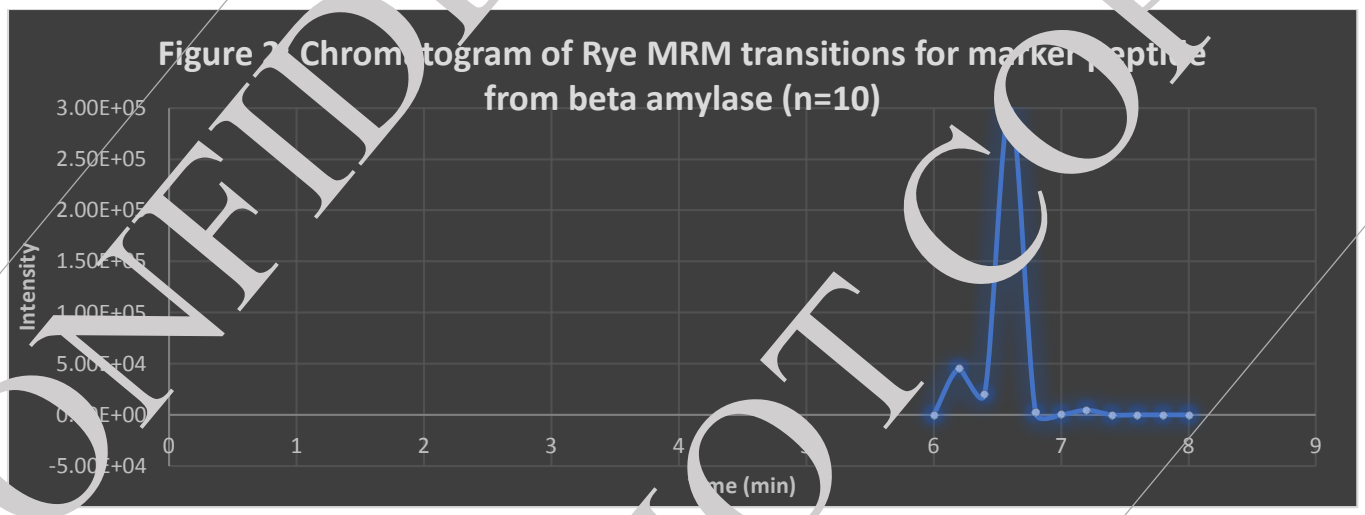
Table 1: Gradient elution used for analysis of extracts.

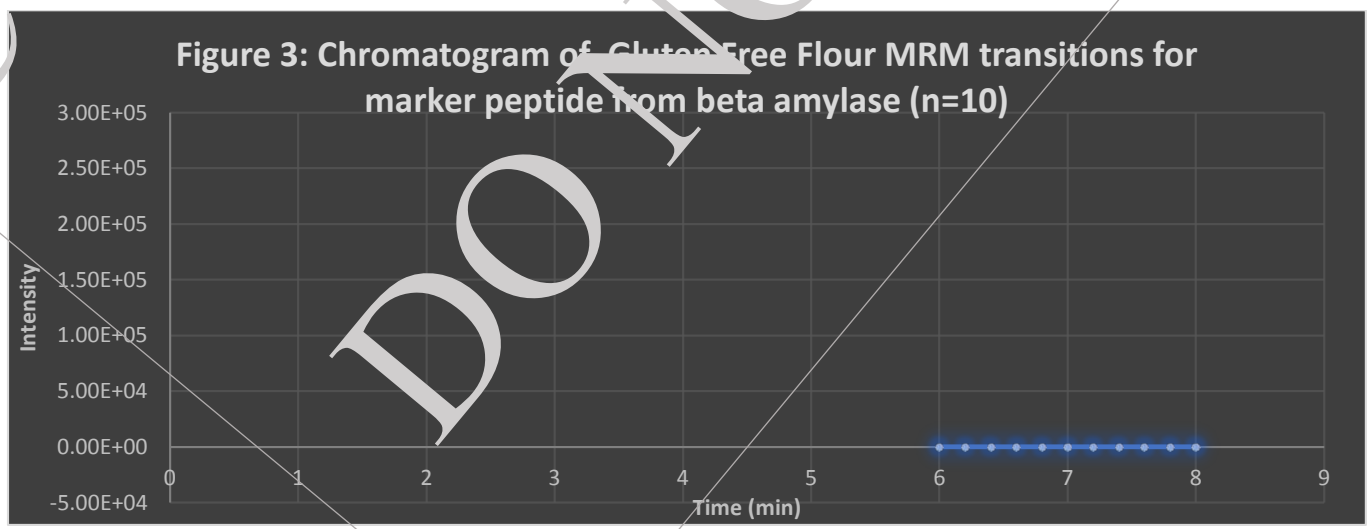
Step	Time (mins)	Flow rate (µL/min)	%A	%B
1	1	25	[illegible]	5
2	6	25	75	25
3	8	25	5	95
4	9	25	[illegible]	95
5	9.2	25	95	5
6	12	25	95	5

Table 2: Marker peptides and sequence information used for gluten species markers (the peptide information was taken from Swiss-Prot database (14,20). [This information shall be used as reference]

Species	Peptide	Protein	Entry number	Peptide sequence
Hordeum vulgare (barley)	1	B1-hordein	P06470	TLPMMCSVNVPLYR
	2	B1-hordein	P06470	GVGPSVGV
	3	B3-hordein	P06471	TLPTMCSVNVPLYR
	4	B3-hordein	P06471	IVPLAIDTR
	5	B3-hordein	P06471	SQMLQQSSCHVLQQ QCCQQLPQIPEQLR
Avena sativa (oats)	1	Avenin-3	P80356	QFLVQQCSPVAVVPFLR
	2	Avenin-3	P80356	SQILQQSSCQVMR
	3	Avenin-3	P80356	QLEQIPEQLR
	4	Avenin-3	P80356	QQCCR
Secale cereal (rye)	1	75k gamma secalin	E5KZQ3	NVLLQQCSPVALVPSLR
	2	75k gamma secalin	E5KZQ4	EGVQILLQQSHQQHVGQGAL AQVQCIIQQQLSQLEVVR
	3	75k gamma secalin	E5KZQ5	SLVLQNLPTMCNVYVPR
	4	75k gamma secalin	E5KZQ5	QCSIQAPEASIVTGIVGH
Triticum aestivum (wheat)	1	Glutenin, subunit DY10	P10387	QVVDQQLAGR
	2	Glutenin subunit PW212	P08489	IPVGIPALLK
	3	Glutenin, subunit DY10	P10387	SVAVSQVAR
	4	Glutenin Subunit DY10	P10387	LPWSTGLQMR
	5	Beta-amylase	P93594	YDPTAYNTILR
	6	Alpha-amylase inhibitor 0.19	P01085	EHGAQEGQAGTGAFPR







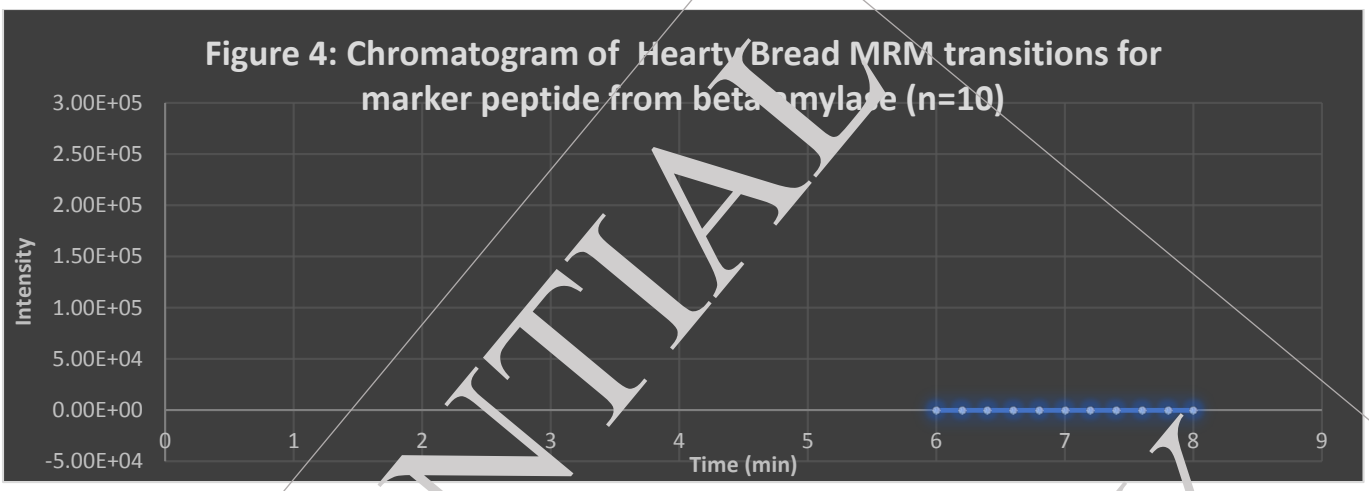
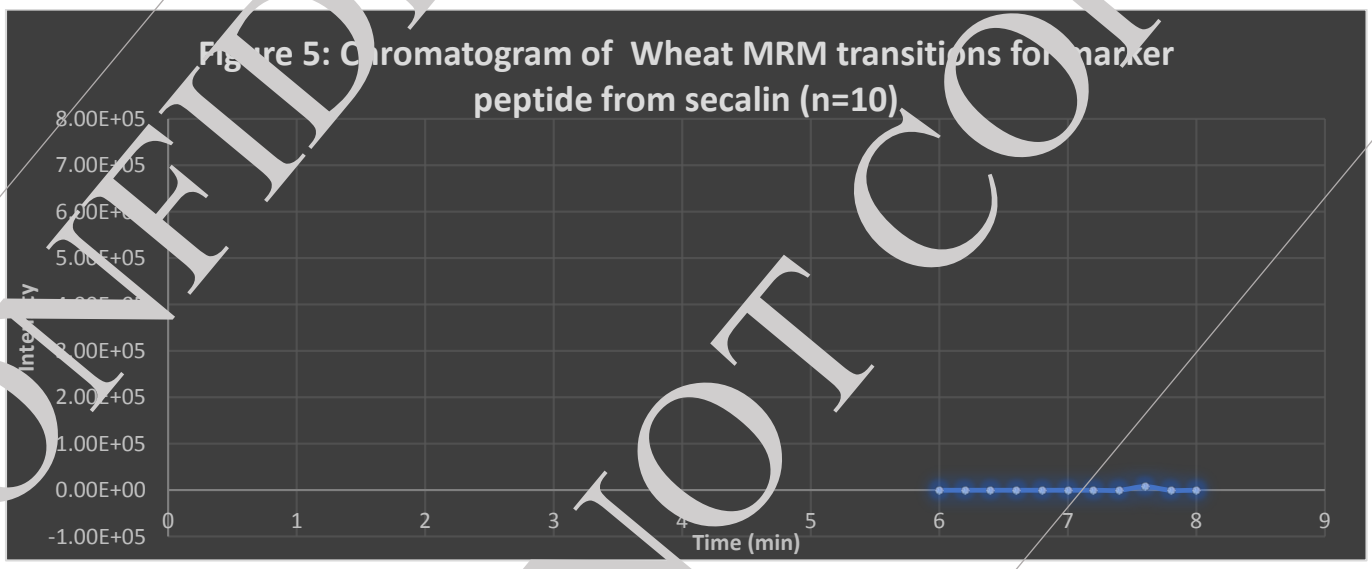


Figure 1-4 indicates the results obtained from MRM analysis of wheat flour, rye flour, gluten free flour (bought from local markets), and Hearty Bread. Beta amylase is used as the marker peptide for Figure 1-4.



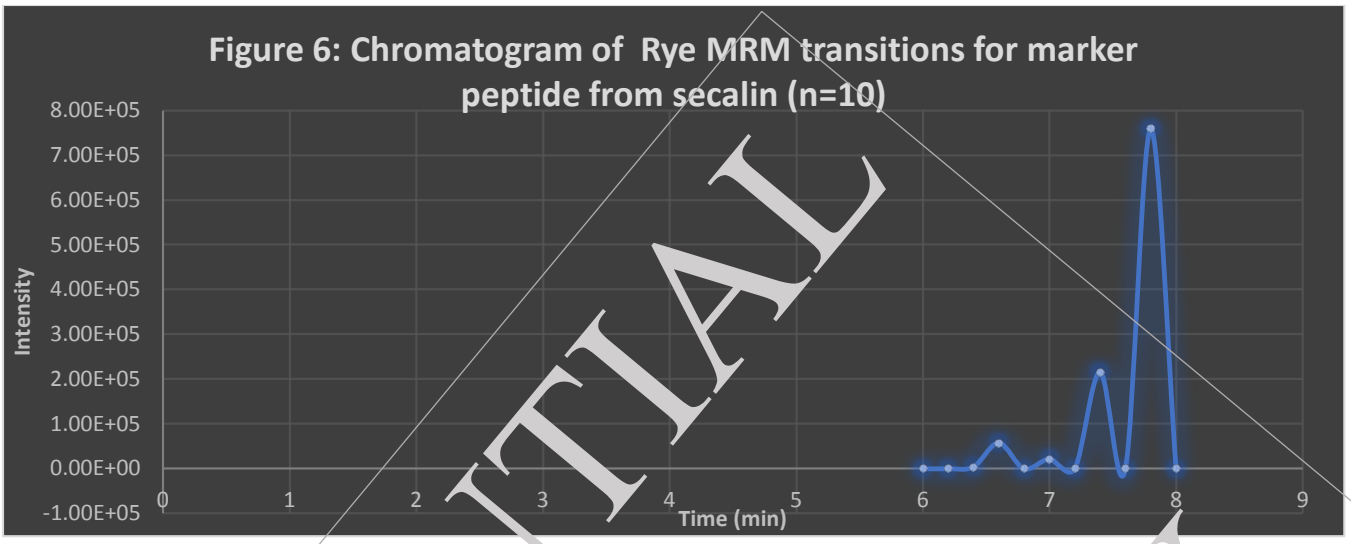


Figure 6: Chromatogram of Rye MRM transitions for marker peptide from secalin (n=10)

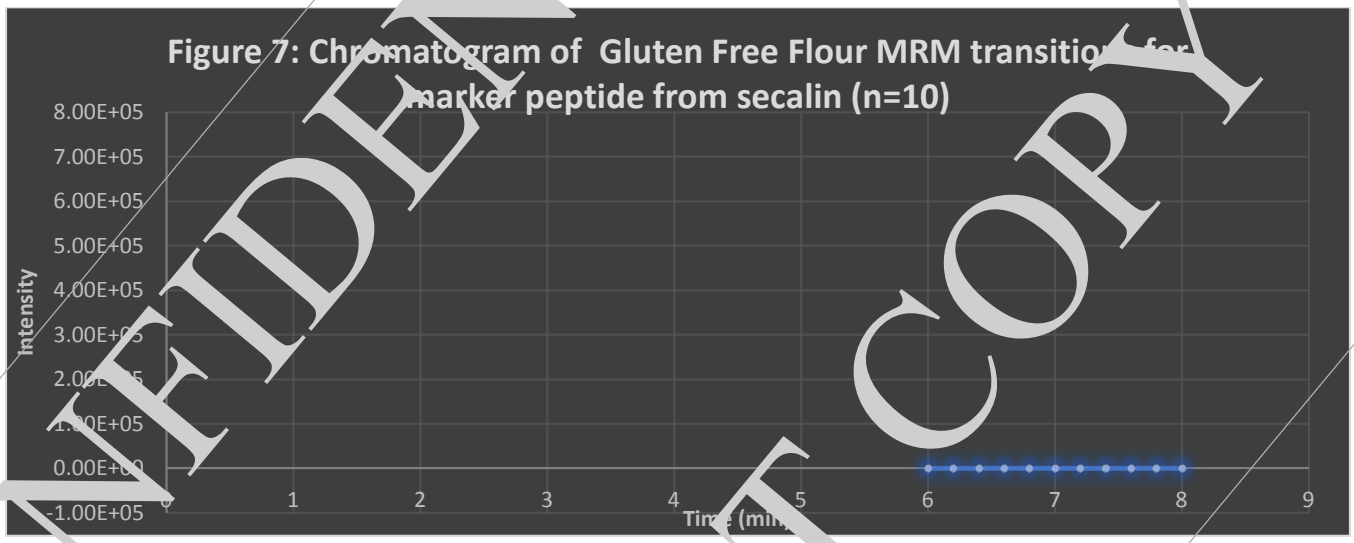


Figure 7: Chromatogram of Gluten Free Flour MRM transitions for marker peptide from secalin (n=10)

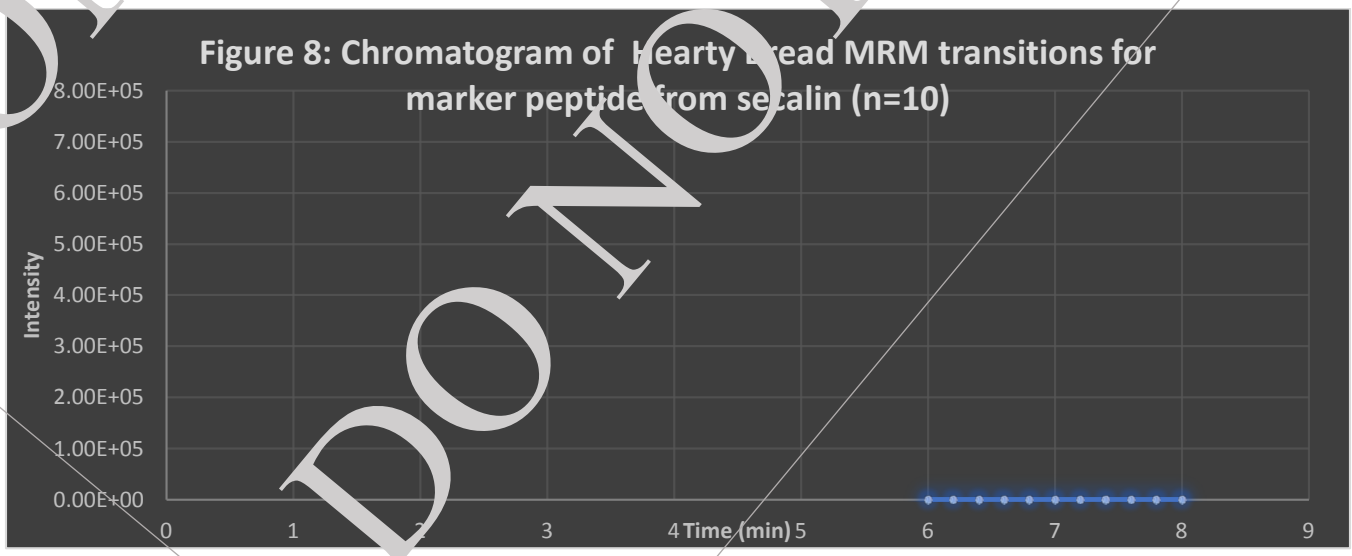


Figure 8: Chromatogram of Hearty Bread MRM transitions for marker peptide from secalin (n=10)

Figure 5-8 indicates MRM analysis results of Wheat flour, Rye flour, Gluten free flour (from local market) and Hearty Bread.

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