**LC-MS/MS quantitation of bioactive proteins and peptides
during sourdough breadmaking**

by

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Abstract

Sourdough involves the fermentation of flour with yeast and lactic acid bacteria and is increasingly capturing the interests of industrial and artisanal bakers. It modifies wheat proteins through pH-dependent proteolysis and accumulation of low molecular weight thiols, such as glutathione (GSH). By altering protein structure, sourdough may reduce the bioactivity of wheat allergens, such as α-amylase/trypsin inhibitors (ATI). The purpose of this investigation was to develop LC-MS/MS approaches to quantify the ATI protein CM3 and GSH in sourdough during breadmaking to understand how fermentation affects their abundances. GSH and CM3 were extracted from wheat sourdough, MRM-based methods were designed, and the analytes were quantified using LC-QTRAP MS. Doughs were prepared using two wheat cultivars, Red Fife and Brennan, and fermented with *Fructilactobacillus sanfranciscensis*, *F. sanfranciscensis ΔgshR* and *Latilactobacillus sakei*; chemically acidified dough and straight dough served as controls. The abundance of GSH in wheat flour was 35-55 nmol/g flour. Across all treatments, GSH decreased after mixing and after proofing, but increased after baking ($P<0.001$). CM3 relative abundance remained similar after mixing and after proofing but decreased after baking ($P<0.001$), regardless of fermentation. This trend was supported by SDS-PAGE analysis of the extracts ($P<0.001$). Overall, the general baking process exerted a greater effect on GSH and CM3 abundance than individual fermentation conditions. Application of LC-MS/MS for analysis of CM3 and GSH will be valuable in elucidating the ability of sourdough to modify wheat allergens for increased tolerability by wheat sensitive consumers.

Preface

This is an original work by Savanna Won.

This thesis contains a manuscript submitted to the Journal of Applied Microbiology for publication. The title is "LC-MS/MS quantitation of α-amylase/trypsin inhibitor CM3 and glutathione during wheat sourdough breadmaking" and the authors are Savanna Won, Michael Gänzle and Jonathan Curtis. SW performed the experiments and data analysis, as well as wrote the manuscript. MG and JC conceptualized the experiment and revised the manuscript.

Chapter 1 provides an introductory literature review of the topics related to the thesis, as well as the hypothesis and objectives. Chapter 2 presents the materials and methods. Chapter 3 details the results of the experiment, including the method development process and quantitative data. Chapter 4 discusses the experimental results in the context of current literature, compares them to other studies in the field and recommends directions for future research.

Dedication

To those who believed in and encouraged me ~

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AA	Ascorbic acid
AAO	Ascorbic acid oxidase
ACN	Acetonitrile
AOAC	Association of Official Agricultural Chemists
ATI	α-amylase/trypsin inhibitors
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CA	Chemically acidified dough
CE	Collision energy
CFU	Colony forming units
COV	Coefficient of variance
DHAA	Dehydroascrobic acid
DTT	Dithiolthreitol
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunoassay
EtOH	Ethanol
FODMAPs	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FT-ICR	Fourier-transform ion cyclotron resonance
GC	Gas chromatography
GSH	Glutathione
GSH-DH	Glutathione dehydrogenase
GshR	Glutathione reductase
GSSG	Dimeric form of glutathione
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
IAM	Iodoacetamide
IBS	Irritable bowel syndrome
LC	Liquid chromatography

LIT	Linear ion trap
LOD	Limit of detection
LOQ	Limit of quantification
LSAK	*Latilactobacillus sakei*
MeOH	Methanol
mMRS	Modified de Man, Rogosa and Sharpe
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
NADH/NAD+	Nicotinamide adenine dinucleotide (reduced/oxidized)
NADPH/NADP+	Nicotinamide adenine dinucleotide phosphate (reduced/oxidized)
NCWS	Non-celiacs wheat sensitivity
NTR system	TRX, NADPH and NADP-TRX reductase
PKP	Phosphoketolase pathway
QQQ	Triple quadrupole mass spectrometer
QTOF	Quadrupole – Time of flight
S/N	Signal to noise ratio
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SF	*Fructilactobacillus sanfranciscensis*
SFM	*Fructilactobacillus sanfranciscensis ΔgshR*
SMPR	Standard method performance requirements
STR	Straight dough
TLR4	Toll-like receptor 4
TOF	Time of flight
TRX	Thioredoxin
WDEIA	Wheat-dependent exercise-induced anaphylaxis
WGA	Wheat germ agglutinin

CHAPTER 1   INTRODUCTION

1.1   Sourdough breadmaking

Sourdough is increasingly used by industrial and artisanal bakers to produce breads with desirable properties and refers to the fermentation of flour with yeasts and lactic acid bacteria (Gänzle and Zheng, 2019). Over the course of fermentation, the dough is acidified and favorably altered by microbial metabolic processes. As many consumers are familiar with sourdough, it is a promising "clean label" method by which foods can be modified without the use of additives or chemicals that are unfamiliar to the general public. Sourdough is currently used in the food industry as a baking improver and for flavor enhancement, though research has moved towards exploring how specific lactic acid bacteria traits can be harnessed to create foods with specific nutrient profiles. The major organisms used in sourdough include *Fructilactobacillus sanfranciscensis*, *Levilactobacillus brevis* and *Latilactobacillus plantarum* (Arora et al., 2021; de Vuyst et al., 2014; Gänzle and Zheng, 2019). During fermentation, lactobacilli grow to densities of around log 8 CFU/g and the pH of the dough decreases to around pH 4 (Arora et al., 2021; Corsetti and Settanni, 2007). While initial interest in sourdough was limited to rheology and taste, it has been shown to modulate specific nutritional properties via bacterial metabolism, such as carbohydrates, glycemic index, mineral bioavailability, and gluten content (Corsetti and Settanni, 2007; Gänzle, 2014; Gobbetti et al., 2019; Loponen and Gänzle, 2018). These metabolic traits are often strain-specific, making sourdough a flexible and promising tool for food innovation.

1.2   Wheat-related health conditions limit consumer choice

Wheat is a major dietary staple in many cultures across the globe. However, certain health conditions can prevent consumers from enjoying wheat-based foods. These include gluten-mediated conditions like wheat anaphylaxis, wheat-dependent exercise-induced anaphylaxis (WDEIA) and Celiac's disease, as well as non-gluten-mediated conditions, such as baker's asthma and non-celiacs wheat sensitivity (NCWS) (Leonard et al., 2017). This thesis is relevant to the latter, non-gluten-mediated health conditions. Baker's asthma is an asthmatic reaction to wheat flour inhalation and is considered an occupational hazard in the baking industry. It has been linked to non-gluten wheat proteins, including α-amylase trypsin inhibitors (ATI). (Tatham and Shewry, 2008). NCWS, by contrast, is triggered by the consumption of wheat and is characterized by a variety symptoms including abdominal discomfort, bloating and irritable bowel syndrome (IBS)-like symptoms, fatigue, headaches, and depression. This condition is estimated to affect up to ten percent of people, based on a self-reported study across multiple continents (Aziz, 2018). NCWS is generally diagnosed by a process of exclusion and the means to address it are limited by lack of research and established biomarkers. (Mansueto et al., 2014; Schuppan et al., 2015). It has been speculated that NCWS is transient, and some patients are recommended to reintroduce wheat into their diet after a period of exclusion (Leonard et al., 2017). The current solution for wheat-related health conditions, including NCWS, is elimination of wheat from the diet. However, wheat-free diets are challenging due to risk of nutrient deficiencies and wheat-free alternatives are often more expensive and less readily available (Vici et al., 2016). Innovative food processing technologies to remove allergens from wheat-based foods may be valuable for consumers experiencing wheat sensitivity.

1.3 Triggers of non-celiacs wheat sensitivity

The pathology of NCWS is poorly understood, but recent studies have linked multiple wheat components to adverse reactions in humans. The first group is fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) (Leonard et al., 2017; Schuppan et al., 2015). FODMAPs are undigestible carbohydrates that pass through the stomach and small intestine intact to be fermented by microbiota in the large intestine. (Priyanka et al., 2018). Due to accumulation of gas during fermentation and osmotic activity, FODMAPs are linked to symptoms of abdominal discomfort, such as gas and bloating (Murray et al., 2014; Shepherd et al., 2014). Certain symptoms of NCWS can be alleviated by following a low FODMAP diet. In addition to undigestible carbohydrates, it is suspected that non-gluten wheat proteins may play a role in NCWS by inciting pro-inflammatory immune responses (Matsuo et al., 2015). The main non-gluten wheat proteins linked to immune responses in humans are lipid transfer proteins, wheat germ agglutinin (WGA), and α-amylase/trypsin inhibitors (ATI) (Tatham and Shewry, 2008). As the focus of this thesis is on ATI, the following section will describe them and their relation to NCWS in more detail.

1.4 ATI structure & bioactivity

Found in wheat (*Triticum aestivum*), α -amylase/trypsin inhibitor (ATI) proteins are involved in nutrient mobilization during seed germination and defend the plant against predation (Geisslitz et al., 2021). They belong to the prolamin superfamily, a class of allergens comprising of the major seed storage proteins in cereals and grasses (Radauer and Breiteneder, 2007). This superfamily includes gluten and non-specific lipid transfer proteins and is characterized by structural features: high proline and glutamine content, alpha helices and a conserved cysteine backbone that forms

multiple disulfide bonds (Mills et al., 2010; Radauer and Breiteneder, 2007). Due to such structural features, these proteins are stabilized against thermolytic and proteolytic degradation and may persist in food post-processing and -digestion (Breiteneder and Mills, 2005; Radauer and Breiteneder, 2007).

Structurally, ATI are bifunctional inhibitors of α-amylase and trypsin that contain separate inhibition sites: one protein can inhibit trypsin and amylase simultaneously (Cuccioloni et al., 2016). It is suggested that they discourage predation by inactivating insect digestive enzymes. ATI have monomeric, dimeric and tetrameric isoforms that are often classified based on their electrophoretic mobility. The monomers range in size from 12-15 kDa, the dimers are around 24-30 kDa, and the tetramers are 60 kDa (Bose et al., 2020). In analyzing the wheat proteome, Dupont et al. suggests that wheat flour consists of roughly 53% tetramers, 31% dimers and 16% monomers (Dupont et al., 2011). A subset of ATI are named for their extractability in chloroform-methanol solutions and are referred to as "CM" proteins. The most commonly reported ATI proteins are described in Table 1.1 (Geisslitz et al., 2021; Matsuo et al., 2015; Tatham and Shewry, 2008). Further information on ATI structure and function has recently been provided by Geisslitz et al. (Geisslitz et al., 2021).

Table 1.1 Description of α-amylase/trypsin inhibitors in wheat. Notes: *The ATI homodimer comprises of two 0.19 subunits. **The ATI tetramer is 60 kDa and comprises of one CM1 or CM2 subunit, one CM16 or CM17 subunit, and two CM3 subunits.

Structure	Name	IUIS name	Molecular weight of the monomeric form (kDa)
Monomer	0.28	Tri a 15	12
Dimer*	0.19	Tri a 28	13
Tetramer**	CM1/ CM2	Tri a 29	13
	CM3	Tri a 30	16
	CM16/ CM17	n/a	17

ATI are implicated in the pathology of baker's asthma, and possibly NCWS. They are classified as IgE-mediated allergens and are suspected to engage the adaptive and innate immune systems (Geisslitz et al., 2021). In a study by Zevallos et al., it was observed that ATI provoke immune responses via the TLR4 pathway and Bellinghausen et al. reported that dietary ATI exacerbated birch pollen allergies in humanized mice. This suggests that, in addition to being pro-inflammatory themselves, they can act as adjuvants for pre-existing conditions (Bellinghausen et al., 2019; Zevallos et al., 2017). Modern wheat species were documented to provoke a greater cytokine release than older wheat variants, such as einkorn or emmer, though quantitative data does not suggest that older wheat variants have a considerably different ATI profile than modern ones (Geisslitz et al., 2018; Sielaff et al., 2021; Zevallos et al., 2017). CM3 and 0.19 isoforms were reported as the most potent activators of the immune response and, for this reason, CM3 (accession number P17314) was chosen as the focus of this thesis (Sander et al., 2011; Tundo et al., 2018). Due to their structural stability, it is possible that ATI may persist in food after

processing and interact with the human digestion system to trigger NCWS symptoms. Understanding how these proteins are affected by food processing is essential to assessing their pathophysiology. The following subsection describes how sourdough fermentation can modulate protein bioactivity, such as that of ATI.

1.5   Sourdough affects protein bioactivity

1.5.1   Role of Thiols in plants and breadmaking

Plants contain many naturally occurring thiol compounds, such as glutathione (GSH) and thioredoxin (TRX). These species play important roles in plant development, metabolism, cell signaling and stress responses (Foyer and Noctor, 2008).

GSH is a tripeptide of glycine-cysteine-glutamate that is ubiquitous in both plants and animals. In plants, it is involved in many functions such as defense, detoxification, metabolism and redox signaling (Foyer and Noctor, 2008). This compound readily participates in redox reactions to form disulfide bridges with itself (GSSG) or to glutathionylate another protein. In this manner, it acts as a redox buffer and reserve of cysteine. Many plant enzymes, such as glutaredoxins and glutathione S-transferases, are involved in their utilization (Noctor et al., 2012; Rouhier et al., 2008).

TRX is a 12 kDa dithiol that plays a significant role in seed growth and photosynthetic regulation. Indeed, removal of genes related to TRX and NADPH-dependent TRX reductase (NTR) severely reduced the growth rate and chloroplast function of *Arabidopsis thaliana* (Ojeda et al., 2017). TRX is also indicated to be involved in the mobilization of nutrients during seed germination by (1) inhibiting protease inhibitors, such as ATI, and (2) reducing storage proteins,

such as glutenins and gliadins, for enzymatic hydrolysis (Buchanan et al., 1997; Joudrier et al., 2005; Kobrehel et al., 1992; Lozano et al., 1996).

Low molecular weight thiols, such as GSH or TRX, participate in redox reactions beyond the life of the plant to affect the downstream properties of flour and bread in the food industry. For example, as the abundance of reduced GSH in wheat flour declined during storage, the quality of bread produced by it, as measured by loaf volume, increased (Chen and Schofield, 1996). Similarly, Reinbold and colleagues confirmed that the amount of sulfur in fertilizer significantly affects the free thiol content of wheat flour and, subsequently, its breadmaking properties. Sulfur-deficient wheat flour had lower levels of free thiols and produced tougher and less extensible breads, despite having the comparable total protein levels to sulfur-rich flours (Reinbold et al., 2008). In summary, wheat naturally contains reducing thiols that may affect its breadmaking properties during subsequent food processing.

1.5.2 Sourdough affects protein structure

Sourdough has a proteolytic effect on wheat proteins, such as gluten, due to two factors: (1) acid-dependent proteolysis and (2) accumulation of reducing power in the form of low molecular weight thiols (Gänzle, 2014). This process alters protein structure, thereby modulating its function and immunogenicity (Gänzle et al., 2008; Gobbetti et al., 2019; Poutanen et al., 2009). The following section outlines the mechanism by which sourdough alters protein structure.

1.5.2.1 Acidification of the food matrix

During fermentation, lactic acid bacteria generate lactic and acetic acid as a by-product of their central carbon metabolism (Arora et al., 2021). The pH of the dough drops towards the pH optimum of wheat proteolytic enzymes, facilitating increased enzymatic activity. The lactobacilli used in breadmaking generally lack extracellular proteinases and, thus, sourdough's proteolytic

activity is attributed to endogenous wheat carboxypeptidases and aspartic proteinases, both of which are active in acidic conditions (Gänzle et al., 2008). Acidification of the dough also increases protein solubility and depolymerization and has been shown to enhance gluten's susceptibility to proteolytic enzymes (Gänzle et al., 2008; Loponen et al., 2004; Thiele et al., 2004). Indeed, Thiele et al. reported that chemical acidification of wheat dough yielded a comparable level of gluten proteolysis to that found in sourdough, and a significantly higher degree of proteolysis than in neutral controls (Thiele et al., 2003). In summary, sourdough's acidic environment plays an important role in gluten degradation.

1.5.2.2 Accumulation of reducing power

In addition to acidifying the dough, heterofermentative lactobacilli increase free thiol levels and accumulate GSH as part of their carbon metabolism (Jänsch et al., 2007; Vermeulen et al., 2006). Over the course of dough mixing and baking, thiol exchange reactions occur. Small, highly reactive compounds, such as GSH, can reduce the intramolecular disulfide bonds of wheat proteins, essentially capping the cysteine residues necessary for inter- and intramolecular disulfide bonds (Joye et al., 2009a; Noctor et al., 2012). As proteins often rely on disulfide bonds to maintain their native structure, the subsequent loss of structure increases the protein's susceptibility to enzymatic hydrolysis by exposing its cleavage sites (Gänzle et al., 2008; Joye et al., 2009a).

Figure 1.1 illustrates chemical, enzymatic, and bacterial reactions involved in GSH metabolism and protein degradation during breadmaking. During dough mixing, oxygen is incorporated into the matrix and used by ascorbic acid oxidase (AAO) to oxidize ascorbic acid (AA) to dehydroaschorbic acid (DHAA). DHAA is the substrate for the enzyme glutathione

dehydrogenase (GSH-DH), which reduces DHAA back to AA in a paired reaction with the oxidation of glutathione (GSH) to its dimeric form, GSSG (Joye et al., 2009a). GSH-DH has a pH optimum of 7.5 and, thus, exhibits low activity in acidic conditions (Kaid et al., 1997). In straight dough, GSH is consumed by GSH-DH, rather than accumulated. Wheat is a natural source of ascorbic acid and glutathione, but these agents can also be added to commercial flour to modulate dough rheology. Indeed, AA and GSH exert opposing effects on dough rheology and bread quality: AA increases dough strength and loaf volume, while GSH weakens the dough, resulting in a softer bread (Grosch and Wieser, 1999; Joye et al., 2009b; Navrot et al., 2018).

Thiol metabolism in straight dough is affected by endogenous wheat enzymes and dough ingredients. Sourdough, however, is further influenced by bacterial metabolism. Many heterofermentative bacteria used in sourdough express the enzyme glutathione reductase (gshR), which generates GSH in a coupled reaction with NADH (Joye et al., 2009a). Increased abundance of free thiols, including GSH, during fermentation has been documented (Huang et al., 2020; Loponen et al., 2008; Tang et al., 2017; Vermeulen et al., 2006). As mentioned previously, GSH participates in non-enzymatic thiol exchange reactions with endogenous wheat proteins (PSSP). This contributes to protein depolymerization and enzymatic hydrolysis, leading to a soft, weaker dough structure (Cagno et al., 2002; Joye et al., 2009a; Loponen et al., 2008; Thiele et al., 2004, 2003).

The effect of redox agents on bread quality varies depending on the redox agent and flour quality. Adding ascorbic acid to straight dough produces greater dough strength and higher loaf volume (Grosch and Wieser, 1999). This is attributed to the consumption of GSH by GSH-DH, resulting in a stronger protein network than in sourdough. Fermentation with an *F. sanfranciscensis* mutant lacking gshR (*ΔgshR*) produced the highest loaf volume, while straight

dough controls produced an intermediate loaf volume and fermentation with *F. sanfranciscensis* wildtype produced the lowest loaf volume (Tang et al., 2017; Xu et al., 2018). Additionally, a higher proportion of polymeric gluten proteins were reported in sourdough fermented with *F. sanfranciscensis ΔgshR* compared to dough fermented with the wildtype strain (Xu et al., 2018). The combination of gshR activity and inactivation of GSH-DH at low pH during fermentation contributes to sourdough's characteristic softness due to depolymerization of the gluten network.

During the baking stage, cycles of polymerization and depolymerization of proteins continue as heat-induced disulfide exchange reactions continue to occur. (Joye et al., 2009a).

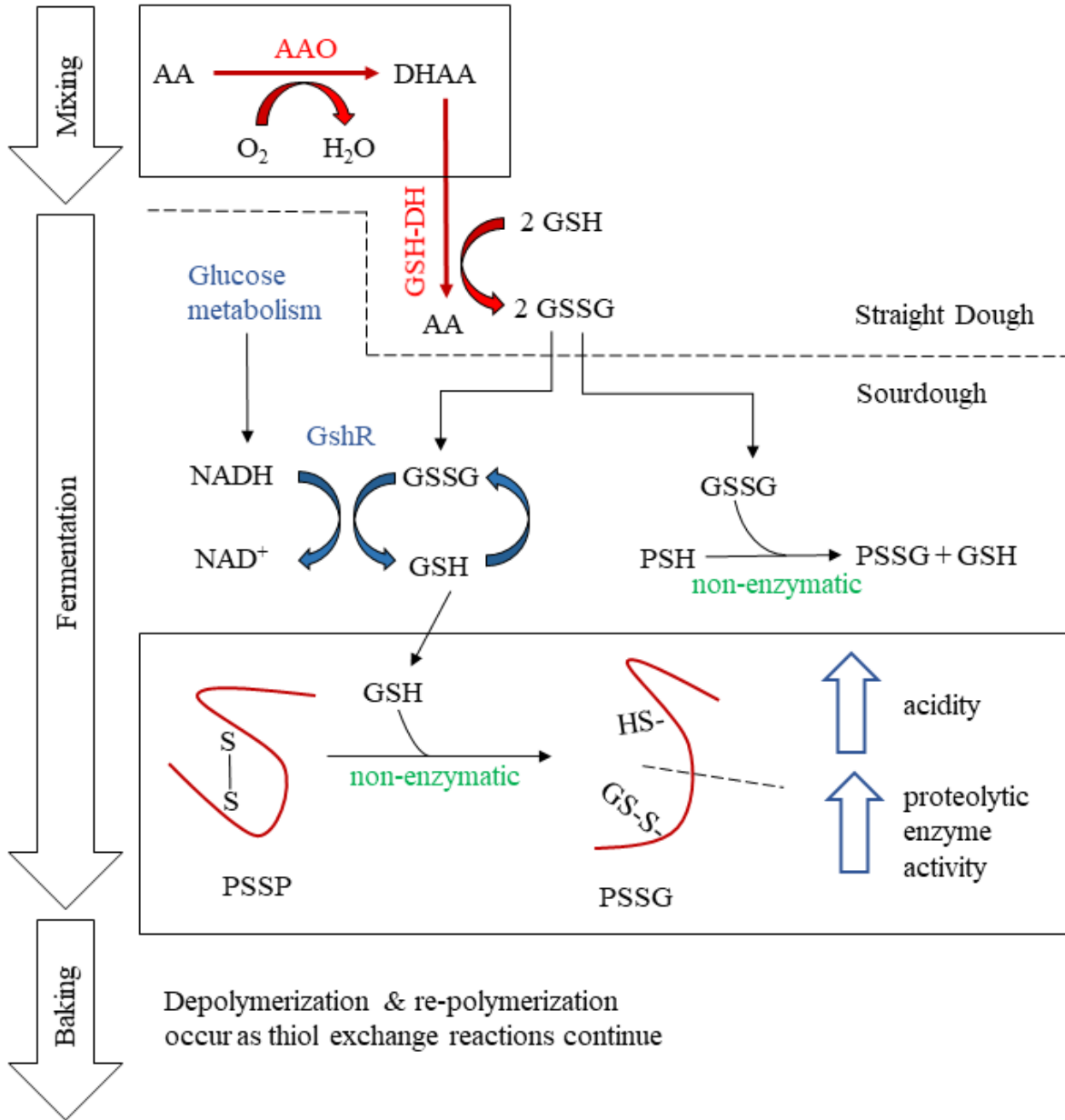**Figure 1.1** Glutathione metabolism during breadmaking. Red = wheat enzymes; blue = microbial

processes; green = chemical reactions. Red line = wheat protein. Abbreviations: AA = ascorbic acid;

DHAA = dehydroascorbic acid; AAO = ascorbic acid oxidase; GSH-DH = glutathione dehydrogenase;

GSH/GSSH = reduced/oxidized glutathione; GshR = glutathione reductase; S = sulfur on cysteine

residue; PSH/PSSP/PSSG = reduced/oxidized protein.

1.5.2.3 GSH accumulation is a strain-specific trait

Heterofermentative and homofermentative lactobacilli exert opposing effects on dough redox potential due to differences in their central carbon metabolism (Vermeulen et al., 2006). Homofermentative lactic acid bacteria metabolize glucose via glycolysis which yields 2 ATP/glucose. Heterofermentative lactic acid bacteria, by contrast, metabolize glucose using the phosphoketolase pathway (PKP). In absence of alternate electron acceptors, the PKP converts acetyl-phosphate to ethanol to regenerate 2 NAD+ with a yield of 1 ATP/glucose. In the presence of alternate electron acceptors, such as GSSG, ketones and aldehydes, the PKP converts acetyl-phosphate to acetate instead and generates ATP rather than NAD+. Instead, NAD+ is regenerated through the use of alternate electron acceptors. One of the enzymes involved in NAD+ regeneration is glutathione reductase (gshR) which produces 2 NAD+ in a paired reaction with the reduction of GSSG to 2 GSH. This shift yields 2.5 ATP/glucose instead of 1 ATP/glucose (Gänzle, 2015; Zheng et al., 2015). Thus, the role of gshR in heterofermentative bacteria is to maximize the energy yield from glucose metabolism.

1.5.3 Sourdough affects protein bioactivity

It has been established that sourdough affects protein structure through acidification and accumulation of low molecular weight thiols. Loss of structure often affects protein bioactivity by: (1) increasing protein susceptibility to enzymatic hydrolysis, and (2) altering epitope conformation. Many food allergens, including gluten and ATI, are stabilized against thermal and enzymatic degradation by disulfide bonds. Considering this, a literature review was conducted on how reducing agents - including sourdough - can affect the ability of food allergens to provoke an immune reaction. The results are displayed in Table 1.2.

The majority of studies found that the treatment of food allergens with reducing agents, namely the NTR system (TRX, NADPH and NADP-TRX reductase) and sourdough, significantly decreased food allergen antigenicity. Other reducing agents, such as GSH or DTT, did not have the same effect (Buchanan et al., 1997; del Val et al., 1999; Waga et al., 2008). Similarly, Kobrehel and colleagues report that ATI is reduced by the NTR system or DTT, but not by GSH (Kobrehel et al., 1991). This may suggest some proteins have dithiol specificity, or that glutathione alone is not enough for significant proteolysis.

Li et al. found that fermentation with *Lactobacillus delbruekii* significantly lowered the IgE binding capacity of egg white proteins, but the free thiol levels did not change. By contrast, Loponen and colleagues observed increased thiol levels and significant hydrolysis of the egg white protein ovotransferrin in wheat sourdough fermented with *F. sanfranciscensis*. The hydrolysis of ovotransferrin was dependent on use of heterofermentative lactobacilli and active wheat aspartic proteases (Loponen et al., 2008). In wheat sourdough, Huang et al reported elevated free thiol levels and reduced cytokine production in response to wheat ATI. This study also noted that starters containing multiple lactobacilli strains were more effective at lowering bioactivity than starters containing pure strains (Huang et al., 2020).

Other studies that do not specifically take into consideration redox reactions corroborate that sourdough can reduce or even abrogate the antigenicity of wheat allergens. Di Cagno et al. reported that fermentation with lactobacilli and fungal proteases rendered wheat baked goods nontoxic when ingested by patients with Celiacs disease. Prolonged exposure failed to provoke an immune response and they reported that gluten was reduced below 10 ppm (di Cagno et al., 2010). Caminero et al. observed that probiotic supplementation of lactic acid bacteria reduced pro-inflammatory responses to ATI in sensitized mice models (Caminero et al., 2019).

In conclusion, the literature suggests that reducing agents and sourdough has the potential to modulate food protein antigenicity. Ingredients for the NTR system, however, are expensive and not commonly used in food processing. Less expensive solutions, such as sourdough, are easier to implement as the resources for sourdough breadmaking are readily available and commonly accepted by consumers. This thesis aims to explore the feasibility of using sourdough to reduce allergen content in baked wheat breads.

Table 1.2 Literature review on the effect of reducing agents on the bioactivity of food protein allergens. Abbreviations: TRX = thioredoxin; GSH = glutathione; NTR system = thioredoxin, NADPH, NADP-TRX reductase; GSH system = GSH, glutathione reductase, NADPH; DTT = dithiothreitol; ATI = α-amylase/trypsin inhibitors.

Author	Protein	Reducing agent(s)	Methods & observations	Conclusions
del Val et al. 1999	β-lactoglobulin (β-LG, bovine milk)	TRX GSH	TRX reduced β-LG, increased it's sensitivity to enzymes, and reduced it's allergenicity in sensitized dog models. GSH did not have the same effect.	Reduction by TRX affected the structure and bioactivity of β-LG.
Buchanan et al. 1977	Wheat proteins	NTR system GSH system DTT + TRX DTT	The NTR system decreased allergic reactions to wheat in sensitized dog models. Other experimental conditions did not.	Reduction by the NTR system affects the functionality of wheat proteins.
Waga et al. 2008	Wheat prolamins	NTR system	Reduction of gliadins by the NTR system decreased immunoreactivity, as measured by ELISA using antigliadin antibodies.	Reduction by the NTR system affects the immunoreactivity of wheat proteins.
Li et al. 2013	Egg whites	Lactic acid bacteria fermentation	Fermentation with *Lactobacillus delbruekii* reduced the IgE-binding capacity of egg whites, as measured by ELISA. Other strains did not have an affect and free thiol levels did not change.	Fermentation with *L. delbrueckii* reduced the bioactivity of egg white allergens.
Huang et al. 2020	ATI (wheat)	Lactic acid bacteria fermentation	Sourdough degraded the wheat ATI tetramer and reduced the release of pro-inflammatory cytokines in human monocytes. Free thiol levels increased. GSH reductase had no effect.	Sourdough fermentation reduced the antigenicity of wheat ATI.

1.6   Analytical Methods

1.6.1   Analytical methods background

Mass spectrometry (MS) is a powerful tool for food analysis, particularly for low abundance

compounds in complex matrices. It operates by converting analytes to gas phase ions,

determining their mass-to-charge-ratio (*m/z*) and measuring their abundances. Mass spectrometry

can be used on its own, or in combination with gas or liquid chromatography for analyte

detection, identification, quantification, determination of structure and protein sequencing (Glish

and Vachet, 2003; Johnson et al., 2011).

Different types of mass analyzers are available, such as time of flight (TOF), quadrupole, Fourier

transform ion-cyclotron resonance (FT-ICR) and linear ion traps (LIT). Mass analyzers are used

alone or arranged in tandem for MS^n analysis. Identification and quantitation of analytes is often

carried out in product ion mode on a tandem mass spectrometer (MS/MS) which consists of

multiple mass analyzers, such as a triple quadrupole mass spectrometer (QQQ), or quadrupole-

time of flight mass spectrometer (QTOF). The tandem arrangement allows for the parent ion to

be selected by the first mass analyzer and then fragmented by collision with gas phase N_2. The

fragments are transmitted to a subsequent mass analyzer for detection. The resulting data is an

MS/MS spectrum that is characteristic of the analyte's structure (Glish and Vachet, 2003).

MS/MS allows for enhanced specificity because, in addition to the *m/z* of the parent ion, the

distinctive fragmentation pattern of the precursor is used to confirm its identity. MS^E is another

acquisition method that builds off MS/MS. In MS^E, the identities of the analytes are not

necessarily known beforehand, and precursor and fragment ions are generated simultaneously as

the instrument cycles through low and high energy states. The low energy states collect data on

the precursor ions present in the select mass window, while the high energy states collect their

MS/MS fragmentation data (Zhang et al., 2020). The precursor ions are retroactively assigned to their respective MS/MS spectra by software algorithms in a process known as deconvoluting (Lukas Krasny and H. Huang, 2021). MS^E allows for a large amount of data to be gathered from a single sample and for researchers to retroactively identify sample constituents.

Gas or liquid chromatography is often used prior to MS analysis in order to separate analytes from a complex mixture based on their chemical properties. Gas chromatography is appropriate for volatile analytes and separation occurs between an inert carrier gas and the stationary phase (Bubli et al., 2021). High pressure liquid chromatography separates compounds dissolved in liquid solvents and analytes partition between the liquid mobile phase and solid stationary phase (Reuhs, 2017). The way in which analytes partition along the column is determined by how they interact with the solid phase. These interactions include hydrogen bonding, hydrophobic interactions, electrostatic interactions, or Van der Waals forces (Ismail, 2017). Chromatography is often used as a precursor to mass spectrometry (GC-MS or LC-MS) to enhance the separation of compounds in a mixture and sensitivity.

LC-MS/MS combines the separation of sample constituents with the specificity of MS detection. For this thesis, a multiple reaction monitoring (MRM)-based MS acquisition method was applied. The experiment was carried out on an LC-QTRAP MS in which the first quadrupole mass analyzer (Q1) selects for the precursor ion and transmits it the second quadrupole mass analyzer (Q2). Q2 acted as a collision cell and fragments the ion by colliding it with neutral N_2 gas-phase molecules. The fragments are transmitted to the third quadrupole mass analyzer (Q3) where only the fragment ions were detected (Meng and Veenstra, 2011). In MRM, the only ion intensity recorded is from the analyte, resulting in reduced background noise and increased S/N which is advantageous for low abundance compounds. Hence, analyte identification is not only

achieved by establishing its elution time on the LC, but also based on the *m/z* of the molecular ion and its characteristic fragmentation pattern (Liebler and Zimmerman, 2013). An advantage of MRM is high specificity, or confidence in identification of the analyte. The structure of the target molecule must be established prior to analysis as the precursor ion and ion transitions must be designated before performing the experiment. In this manner, it is not possible to use MRM to analyze unidentified compounds. A second limitation of MRM is that only data pertaining to the select ion is collected. Thus, while it can detect trace amounts analytes in the presence of far more abundant compounds, one must keep in mind that the other compounds in the sample are not observed at all. For this reason, it is unsuited for certain types of applications, such as a broad, untargeted survey of a sample's constituents.

Analytes in food can be challenging to ascertain because they exist in small or trace amounts, have multiple structural permutations, or undergo conformational changes in during food processing. These challenges limit the efficiency of other analytical methods, such as immunoassays (Monaci and Visconti, 2009). The advantages of LC-MS/MS for food analysis include high sensitivity for low abundance compounds, the ability to observe structural changes, and the ability to monitor multiple compounds simultaneously. The high specificity allowed for by MRM is particularly important for low abundance analytes in complex natural mixtures. Furthermore, automation of sample introduction through LC or GC and of MS data acquisition allows for high throughput for large sample sets. A limitation of LC-MS/MS is that the instruments themselves tend to be expensive to purchase and maintain. Mass spectrometers can also respond very differently to different analytes, and, for this reason, absolute quantification requires the availability of highly purified standards (Johnson et al., 2011; Monaci and Visconti,

2009). Overall, due to the advantages of MS for peptide analysis in complex food matrices, LC-MS/MS was selected for use in this investigation.

1.6.2 Analytical methods literature survey

This section surveys the extraction and analytical methods used for GSH and CM3 in similar studies, while a more detailed comparison of their results is provided in Chapter 4. Additionally, a thorough discussion of the method development process pertaining to this thesis is located in Chapter 3.

1.6.2.1 Extraction methods

A variety of methods have been used to extract GSH from wheat and other biological sources. Reinbold et al. used tris (2-carboxyethyl) phosphine in 0.1% v/v formic acid to extract GSH from wheat flour (Reinbold et al., 2008). Other studies in wheat flour used solutions of triethylamine/formic acid or 5% w/v ice cold perchloric acid (Sarwin et al., 1992; Schofield and Chen, 1995). Tang et al. extracted GSH from sourdough with 1 mM EDTA and 0.1% v/v formic acid (Tang et al., 2017). Since GSH is commonly extracted from sources other than wheat as well, a brief literature survey of other studies was conducted. To extract GSH from yeast cells, Xiong et al. used various ethanol-water solutions and Ma et al. used hot water at 90 °C (Ma et al., 2010; Xiong et al., 2009). Squellerio et al. used 10% TCA containing 1 mM EDTA in a 1:1 v/v ratio to extraction GSH from human blood (Squellerio et al., 2012).

A second literature survey regarding ATI extraction methods from wheat flour was conducted. Prandi et al. extracted CM3 from wheat flour using a 0.5 M NaCl aqueous solution (Prandi et al., 2013). By contrast, Bose et al. explored different combinations of isopropanol, DTT and urea extraction solutions, accompanied by defatting and protein precipitation steps (Bose et al., 2020, 2019b, 2019a). Geisslitz et al. and Sielaff et al. extracted ATI from wheat flour using 50 mM

ammonium bicarbonate and 10 mM sodium bicarbonate in 0.5 M sodium chloride, respectively (Geisslitz et al., 2020, 2018; Sielaff et al., 2021). Huang et al. defatted wheat flour with methanol: diethyl ether (1:1, v/v), extracted ATI with 150 mM NaCl and precipitated the proteins in 20% ammonium sulfate (Huang et al., 2020).

1.6.2.2 Instrumental methods

Few studies have examined GSH using LC-MS/MS in the context of wheat or bread systems. Reinbold et al. and Tang et al. used LC-MS/MS to measure GSH content in wheat flour to measure its relationship to sulfur availability and its abundance over the course of a 24 h sourdough fermentation, respectively (Reinbold et al., 2008; Tang et al., 2017). Other studies have used spectrophotometric and HPLC-UV to measure free thiol content, though these analyses were not absolutely quantitative nor specific to GSH (Huang et al., 2020; Vermeulen et al., 2006). This thesis aims to develop an LC-MS/MS-based method for the absolute quantification of GSH in sourdough to measure how breadmaking affects its abundance.

Several studies have applied LC-MS-based methods to quantify ATI in wheat and related grains. Prandi et al. measured CM3 abundance in different wheat varieties and growing regions using three peptides unique to the protein at the MS1 level with LC-MS (Prandi et al., 2013). Bose et al. expanded on this by quantifying 18 ATI species using MRM-based LC-MS/MS, also across multiple wheat cultivars. (Bose et al., 2020). Also using LC-MS/MS, Geisslitz et al. reported the ATI content in wheat flour, and compared it to that of related grains, such as einkorn, emmer and spelt. (Geisslitz et al., 2020, 2018). Sielaff et al. similarly measured ATI isoforms in wheat flour and flour of related grains but using LC-MS[E]. (Sielaff et al., 2021). Huang et al. used fluorescence-tagged size exclusion chromatography to compare the ratio of tetrameric isoforms to monomeric isoforms in unbaked sourdough, before and after fermentation (Huang et al.,

2020). It is clear that there are several reports of using LC-MS/MS for absolute quantitation of ATI in the literature. However, these studies are limited to wheat flour, and do not explore on the effects of food processing on ATI content. To our knowledge, this investigation is the first report of ATI quantitation by LC-MS/MS in sourdough and during breadmaking.

1.7 Objectives and significance

Sourdough exerts a proteolytic effect on gluten proteins due to a combination of acidification and accumulation of reducing agents. Similar to gluten, wheat ATI are dependent on multiple disulfide bridges to maintain their structure and bioactivity. Due to the structural similarities between gluten and ATI, it was hypothesized that sourdough fermentation may be capable of breaking down ATI via a similar mechanism as it does gluten.

The objective of this thesis was to understand how sourdough fermentation affects ATI abundance. As CM3 is considered one of the most bioactive ATI compounds, it was selected as the focus of this study. Similarly, due to GSH's important role in protein depolymerization via disruption of intramolecular disulfide bridges, it was designated as a second target of study. The aims of this thesis were to (1) develop an LC-MS/MS method to quantify GSH in sourdough, (2) develop an LC-MS/MS method to quantify CM3 in sourdough, and (3) apply the methods over the course of the sourdough breadmaking process.

LC-MS/MS is a powerful and comprehensive tool for the analysis of complex food matrices. Application of LC-MS/MS to expand our knowledge of how bioactive proteins and peptides behave in a sourdough system has implications for the development of tools to increase the

tolerability of foods for wheat sensitive consumers. As far as we know, this is the first report to

quantify CM3 during breadmaking.

CHAPTER 2   MATERIALS AND METHODS

2.1   Wheat flour

Sourdough bread was baked using two pure wheat cultivars: Red Fife and Brennan. The cultivars were stored at −20 °C and ground into flour using a 0.5 mm screen on a Retsch ZM200 ultra centrifugal mill (Retsch, Germany). Commercial whole wheat flour was used for general method development.

2.2   Reagents and standard solutions

Iodoacetamide (IAM), HPLC grade formic acid, dithiothreitol (DTT),γ-L-Glutathione (GSH), DL-lactic acid 90% and acetic acid glacial 99.7% were obtained from Sigma Aldrich (Oakville, ON, Canada). Ammonium bicarbonate, LC-MS grade water and LC-MS grade acetonitrile were supplied by Fisher Scientific (Ottawa, ON, Canada). Deuterated glutathione (d_5-GSH) was purchased from SantaCruz Biotechnology (Dallas, TX, USA). Trypsin Gold Mass Spectrometry Grade and ProteaseMAX^{TM} Surfactant Trypsin Enhancer were obtained from Promega (Madison,WI, USA). Two custom, isotope labelled peptides corresponding to select CM3 peptides were synthesized by BaChem (Torrance, CA, USA): (1) H-Tyr-Phe-Ile-Ala-[^{13}C6;^{15}N]Leu-Pro-Val-Pro-Ser-Gln-Pro-Val-Asp-Pro-Arg-OH;(2) H-Ser-Gly-Asn-Val-Gly-Glu-Ser-Gly-[^{13}C6;^{15}N]Leu-Ile-Asp-Leu-Pro-Gly-Cys-Pro-Arg-OH. 4-20% Mini-PROTEAN TGX Precast Protein Gels were obtained from BioRad.

Standard solutions of GSH and d$_5$-GSH were dissolved in 50% acetonitrile and derivatized with IAM using the same procedure as outlined in section 2.6. Standard solutions for the CM3 isotope-labelled peptides were dissolved in 1% acetic acid, diluted with 0.1% formic acid, and derivatized with IAM following the same procedure as mentioned in the previous sentence.

2.3   Bacterial strains and culture conditions

Three strains of lactic acid bacteria were used: (1) *Fructilactobacillus sanfranciscensis* DSM20451 (isolated from sourdough), (2) the isogenic mutant *Fructilactobacillus sanfranciscensis* DSM2045118 Δ*gshR* which lacks the enzyme glutathione reductase, and (3) *Latilactobacillus sakei* TMW1.22 (LTH677, isolated from fermented sausage) (Ammor et al., 2005; Jänsch et al., 2007; Vermeulen et al., 2006). Strains were grown in modified de Man, Rogosa and Sharpe (mMRS) media with the carbohydrates autoclaved separately to limit Maillard reactions (Tovar and Gänzle, 2021) Cultures were grown at 30 °C in anaerobic conditions.

2.4   Sourdough fermentation

Lactic acid bacteria were streaked on mMRS agar and incubated for 24-48 h at 30 °C under anaerobic conditions. From these plates, subcultures were formed: mMRS broth was inoculated with single colonies. After incubation at 30 °C for 24 h, the cultures were centrifuged for 3 min to form a pellet, the supernatant was discarded, and the culture was washed with sterile water. The washing procedure was repeated twice, after which the cells were reconstituted with 10 mL

of sterile water and used to inoculate 10 g of wheat flour at a 1:1 ratio. Each inoculum was incubated at 30 °C for 24 h.

Sourdough samples were made using a two-step fermentation process. The inoculum was back slopped by mixing 1 g of inoculum with 4.5 g wheat flour and 4.5 mL tap water and incubated at 30 °C for 24 h. It was mixed with 20 g wheat flour and 20 mL tap water and incubated at 32 °C for 2.5 h. Bread dough was prepared by mixing the inoculum with 70 g wheat flour, 30 mL tap water, 2 g salt, 2 g sugar and 0.5 g active dry yeast. The dough was mixed for six minutes, rested for 1 hour at 32 °C, shaped and then proofed for 1 h also at 32 °C and baked in a forced convection oven for 12 min at 190 °C. Chemically acidified controls were acidified to pH 3.5±2 using a solution of acetic and lactic acid at a ratio of 1:4 and prepared identically to the sourdough bread. Straight dough controls were made by mixing 100 g of wheat flour with 55 mL tap water, 2 g salt, 2 g sugar and 0.5 g active dry yeast; all other steps were identical to the sourdough bread protocol. After baking, the breads were cooled before being sampled. Samples were collected from several different time points: from the flour, after mixing, after proofing and after baking (Tang et al., 2017; Xu et al., 2018).

2.5 Determination of cell counts and pH

The pH and cell counts of each sourdough and the pH of the breads were recorded (provided in the supplementary materials). One gram of each sample was dissolved in 9 mL of deionized water and analyzed using a pH meter. For cell counts, one gram of sample was dissolved in 9 mL of sterile deionized water and serial dilutions were made by diluting 100 μL of sample in 900 μL of sterile deionized water. A Whitley Automatic Spiral Platter (Don Whitley Scientific, England)

was used to plate the dilutions. The plates were incubated for 24-48 h at 30 °C before being counted.

2.6   Glutathione extraction and sample preparation

Flour and bread samples (100 g and 200 g, respectively) were extracted in 500 µL of 80% ethanol (EtOH). Samples were alkylated with iodoacetamide (IAM): the pH was adjusted to 8 using NH_4OH, samples were mixed with 10 µL 200 mM IAM and left in the dark for 30 min at room temperature, after which the pH was lowered to 4 with formic acid. Samples were sonicated for 10 min, centrifuged at 4 °C for 2 min and the supernatant was collected. This extraction was repeated, the supernatants were combined and then centrifuged for 15 min at 4 °C. The resulting supernatant was collected, filtered with 0.2 µm filter, diluted with 50% acetonitrile and combined with the derivatized internal standard (Reinbold et al., 2008; Tang et al., 2017; Xiong et al., 2009).

2.7   LC-MS/MS quantitation of glutathione

Glutathione was quantified by LC-MS/MS using a targeted multiple reaction monitoring (MRM) approach. Samples and standards were analyzed using an Agilent 1200 series HPLC system coupled to a 3200 QTRAP mass spectrometer (SCIEX, Redwood City, CA, USA) with a TurboIonSpray source operating in positive ion mode. For the separation on an Ascentis Express HILIC column (10 cm x 2.1 mm x 2.7 µm), eluant A was 0.1% formic acid in acetonitrile while eluant B was 0.1% formic acid in water. The following gradient was used: 0-10 min, 95-80% A; 10-10.1 min, 80-95% A; 10.1-20 min, 95% A. Data acquisition and peak integration was carried

out using Analyst 2.0 (Applied Biosystems, Foster City, CA, USA). The MRM transitions that were selected for the analyte and internal standard are reported in Table 2.1. GSH standards were diluted in 50% acetonitrile and used to make a five-point external calibration curve from 1 ppb to 50 ppb (shown in Chapter 3). To calculate the abundance of GSH in sourdough, the peak areas of the two select ions were summed, normalized to the internal standard, and transformed into a quantitative amount using the external calibration curve.

Table 2.1 Mass transitions and collision energies (CE) optimized for MRM detection of alkylated glutathione (IAM-GSH) and d_5-glutathione (IAM- d_5-GSH).

Analyte	Transition 1 (*m/z*)	CE	Transition 2 (*m/z*)	CE
IAM-GSH	365.1/236.0	17	365.1/290.0	19
IAM- d_5-GSH	370.1/236.0	25	370.1/295.0	25

2.8 Glutathione LC-MS/MS method validation

Sourdough made with commercial wheat and fermented with *F. sanfranciscensis* DSM20451 for 24 h at 30 °C was used for method validation. The precision of the extraction was calculated by analysis of 3 samples taken from the same wheat sourdough with each injected in duplicate. The results indicated good overall precision with a measured coefficient of variance of 3%.

Recovery was determined by spiking sourdough samples before and after extraction with 100 μL of at 1 ppm, 2.5 ppm and 5 ppm GSH solutions. Samples that were not spiked with standards were spiked with an equivalent amount of solvent (50% acetonitrile) to maintain identical extraction conditions. Recovery was calculated using the following equation:

$[(A−C)/(B−C)]×100$, where A = peak area of IAM-GSH in sourdough sample spiked before extraction; B = peak area of IAM-GSH in sourdough sample spiked after extraction; C = peak area of IAM-GSH in sourdough sample not spiked with standards. The average recovery was 81% ± 0.95.

The limit of detection (LOD) was defined as 3 times the S/N, while limit of quantification (LOQ) was defined as 10 times the S/N. Both were determined using the sum of the two mass transitions

for the alkylated glutathione standard dissolved in 50% acetonitrile. The LOD was 0.2 ppb and the LOQ was 1 ppb. A more detailed discussion of the method validation is provided in Chapter 3.

2.9   Identification of CM3 marker peptides

The amino acid sequence for CM3 was obtained from the literature (Figure 2.1) and previously isolated CM3 obtained from Huang et al. was used for method development and MS optimization (Altenbach et al., 2011; García-Maroto et al., 1990; Huang et al., 2020). *In silico* tools from prospector.ucsf.edu were used to predict and select CM3 marker peptides: MS-digest was used to generate a list of possible peptides generated by tryptic digestion; MS-isotope was used to predict their isotope distributions; and MS-product helped predict their MS/MS spectra. LC-QTOF was used to confirm the presence and identity of CM3 peptides in sourdough samples. The two most abundant peptides were selected as markers for quantitation: (1) YFIALPVPSQPVDPR, and (2) SGNVGESGLIDLPGCPR (Geisslitz et al., 2018).
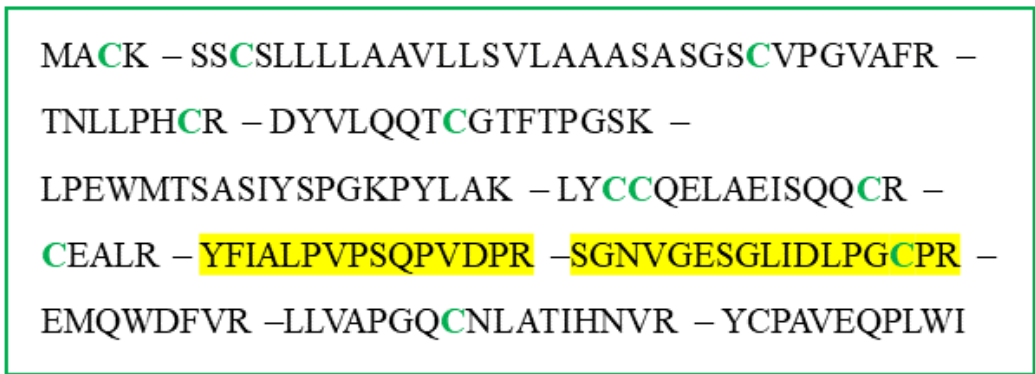

Figure 2.1 Amino acid sequence of CM3. The peptides produced by the trypsin digestion are indicated by the hyphens, the cysteine residues are bolded in green, and the two high intensity peptides selected for use in the quantitation method are highlighted.

2.10 Extraction and trypsin digestion of CM3

Flour and bread dough samples (50 g and 100 g, respectively) were weighed out and
reconstituted in 500 mL of 50 mM ammonium bicarbonate. Samples were alkylated with 10 μL
of 200 mM IAM and left in the dark for 30 min. They were then centrifuged for 25 min at room
temperature before the supernatant was collected. This extraction was repeated, and the
supernatants were combined, centrifuged for 15 min at room temperature. The resulting
supernatant was collected and digested with MS grade trypsin in preparation for mass
spectrometric analysis: samples were mixed with 44 μL of 0.1% ProteaseMAXsurfactant
enhancer and reduced with 100 μL of 100 mM DTT for 30 min a 30 °C. The reduced proteins
were alkylated with 10 μL of 200 mM IAM for 30 min in the dark and mixed with 1 μL 1%
ProteaseMAX^{TM} surfactant enhancer. Samples were digested with 10μL 0.5 μg/μL Trypsin Gold
at 37 °C for 1 h. To halt further reactions, the pH was lowered to < 4 with formic acid. (Geisslitz
et al., 2018)

2.11 LC-MS/MS quantitation of ATI CM3

The CM3 tryptic digest was analyzed on under turbospray positive mode by an Agilent 1200
series HPLC system coupled to a 3200 QTRAP mass spectrometer (SCIEX, Redwood City, CA,
USA). A multiple reaction monitoring (MRM) approach was used to quantify two select product
ions per analyte using optimized parameters, as described in Table 2.2. Eluant A was 0.1%
formic acid in acetonitrile while Eluant B was 0.1% formic acid in water. Samples were
separated on an ACE 3 AQ column (150 mm x 2.1 mm x 3μm) and eluted using the following
gradient: 0-6 minutes, 0% A; 6-20 minutes, 100% A;20-30 minutes, 100% A; 30-40 minutes, 0%
A. Data acquisition and peak integration was completed using Analyst 2.0 (Applied Biosystems,

Foster City, CA, USA). To calculate relative abundance, the peak areas of the two CM3 peptides were summed and normalized to the internal standard. External calibration standards using the isotope-labelled standards derivatized with iodoacetamide were diluted with 0.1% formic acid for a five-point calibration curve running from 0.05 ppm to 5 ppm to ensure that the instrument response was linear in the range studied.

Table 2.2 Mass transitions and collision energies (CE) used for MRM quantitation of CM3 marker peptides and their isotope-labelled internal standards.

Amino acid sequence	Transition 1 (*m/z*)	Transition 2 (*m/z*)	CE
SGNVGESGLIDLPGCPR	864.5/586.3	864.5/699.3	55
SGNVGESG[$^{13}C_6,^{15}N$]LIDLPGCPR	867.9/586.3	867.9/699.3	50
YFIALPVPSQPVDPR	849.9/495.2	849.9/272.1	36
YFIA[$^{13}C_6,^{15}N$]LPVPSQPVDPR	853.0/495.2	853.0/272.1	40

2.12   ATI CM3 LC-MS/MS method validation

Method precision was determined by assessing the CM3 peak areas of three samples extracted from the same wheat sourdough. The sourdough was made with commercial wheat and fermented with *F. sanfranciscensis* DSM20451 for 24 h at 30 °C. An overall % COV of 8.1% was achieved.

Recovery was determined by spiking sourdough samples before and after extraction with the previously isolated CM3 from Huang et al. diluted with 0.1% formic acid 1:50, 1:20 and 1:10 (Huang et al., 2020). Samples that were not spiked with the standard were spiked with an equivalent amount of solvent to maintain identical extraction conditions. The commercial wheat sourdough samples used in the recovery experiments were fermented with *F. sanfranciscensis*

DSM20451 for 24 h at 30 °C. Recovery was calculated using the following equation:

[(A−C)/(B−C)]×100, where A = peak area of CM3 in sample spiked before extraction; B = peak area of CM3 in sample spiked after extraction; C = peak area of CM3 in sample not spiked with any standards. The average percent recovery was 107% ± 1.4.

The limit of detection (LOD), defined as 3 times the S/N, and the limit of quantification (LOQ), defined as 10 times the S/N, are reported in Table 2.3. Both were determined using alkylated, isotope labelled CM3 marker peptide standards in 0.1% formic acid.

Table 2.3 Limit of detection (LOD) and limit of quantification (LOQ) for CM3 marker peptides

Peptide	LOD (ppb)	LOQ (ppb)
SGNVGESG[$^{13}C_6,^{15}N$]LIDLPGCPR	50	75
YFIA[$^{13}C_6,^{15}N$]LPVPSQPVDPR	100	200

2.13   Non-reducing SDS-PAGE analysis of ATI

100 g of bread dough (or 60 g of flour) was weighed out and reconstituted in 500 mL of 50 mM ammonium bicarbonate. Samples were alkylated with 10 μL of 200 mM IAM and left in the dark for 30 minutes. They were then centrifuged for 25 minutes at room temperature before the supernatant was collected. The extraction process was repeated for a total of two sequential extractions. The supernatants were combined, centrifuged for 15 minutes at room temperature and the resulting the supernatant was collected. Three biological replicates per wheat cultivar was analyzed. Samples were mixed with 4x loading sample buffer at a 1:4 ratio of buffer: sample (1 mL 1.5 M Tris, 600 μL 20% SDS, 3 mL glycerol, 0.18 mg bromophenol blue and 400 μL

deionized water) and separated using a non-reducing method on a 4-20% Mini-PROTEAN TGX

Precast Protein Gels, 15 well, 15 µL (BioRad, Mississauga, Ontario, Canada) with 1x running

buffer (10 g SDS, 303.3 g Tris and 144.1 g glycine dissolved in 1L of deionized water, and then

diluted 10x with deionized water). The proteins were stained with Coomassie Blue staining

solution (2.5g Coomassie Brilliant Blue R-250 in 450 mL methanol, 100 mL acetic acid and,

450 mL deionized water) and destained with Coomassie Blue destaining solution (450 mL

methanol, 100 mL acetic acid, and 450 mL deionized water). Thermo Scientific PageRuler Plus

Prestained Protein ladder, 10 to 250 kDa, (Fisher Scientific, Ottawa, Ontario, Canada) was used

for molecular markers. Running conditions were 150 V for 50 minutes. The protein band

intensities were measured using a BioRad ChemiDoc MP Imager (BioRad, Mississauga, Ontario,

Canada).

2.14   Statistics

Two-way ANOVAs and Tukey's HSD test (alpha = 0.05) were performed using R (version

4.0.5)

CHAPTER 3   RESULTS

3.1   Sourdough fermentation

Sourdough bread was fermented with three different lactobacilli. The pH and cell counts reported in Table 3.1 were used to verify that fermentation properly occurred. As expected, the pH of the ranged from 3.5 to 3.7 and the cell counts were in the range of 10^8, indicating that the lactobacilli had grown as expected. The colony morphologies confirmed that the expected strains were dominant, and the pH of the final breads ranged from 5.5 to 6.5.

Table 3.1 Average dough pH, bread pH and cell counts of the sourdough samples.

Flour	Fermentation	Average dough pH	Average bread pH	Average CFU/g
Red fife	*F. sanfranciscensis*	3.51 ± 0.15	5.48 ± 0.06	7.10 x 10^8
	F. sanfranciscensis ΔgshR	3.72 ± 0.28	5.67 ± 0.38	1.70 x 10^8
	L. sakei	3.45 ± 0.11	5.54 ± 0.19	6.70 x 10^7
	Chemically acidified	3.70 ± 0.03	5.93 ± 0.44	n/a
	Straight	n/a	6.25 ± 6.25	n/a
Brennan	*F. sanfranciscensis*	3.37 ± 0.03	5.82 ± 0.31	3.63 x 10^8
	F. sanfranciscensis ΔgshR	3.47 ± 0.01	5.87 ± 0.38	2.47 x 10^8
	L. sakei	3.47 ± 0.07	5.87 ± 0.43	1.12 x 10^8
	Chemically acidified	3.56 ± 0.03	6.27 ± 0.33	n/a
	Straight	n/a	6.61 ± 0.44	n/a

3.2 GSH quantitation

3.2.1 Instrumental method development

Initial instrumental method development was carried out using QTOF MS. The instrument parameters (declustering potential, exit potential, collision energy and exit potential) were optimized for the standard and the internal standard, and two high intensity fragments per compound were selected for MRM. The LC method was adapted from the literature and the samples were analyzed on a QTRAP MS. QTOF was selected for use in the initial stages of method development due to its higher sensitivity for the detection of low abundance compounds. QTRAP was used for the final quantitation due to its MRM capabilities and higher specificity, or the degree of confidence one has in identifying an analyte. Figure 3.1 shows the MS/MS spectra, fragmentation, and select ion transitions for derivatized and non-derivatized GSH and d_5-GSH.

Initially, the analyte was separated with reverse phase chromatography on a C18 column. However, because GSH is highly polar, it eluted rapidly from the C18 column with insufficient separation from the matrix, resulting in poor peak shape and background noise. Switching to hydrophilic interaction chromatography (HILIC) with a stationary phase of bare silica increased the retention time and improved peak shape (data not shown).
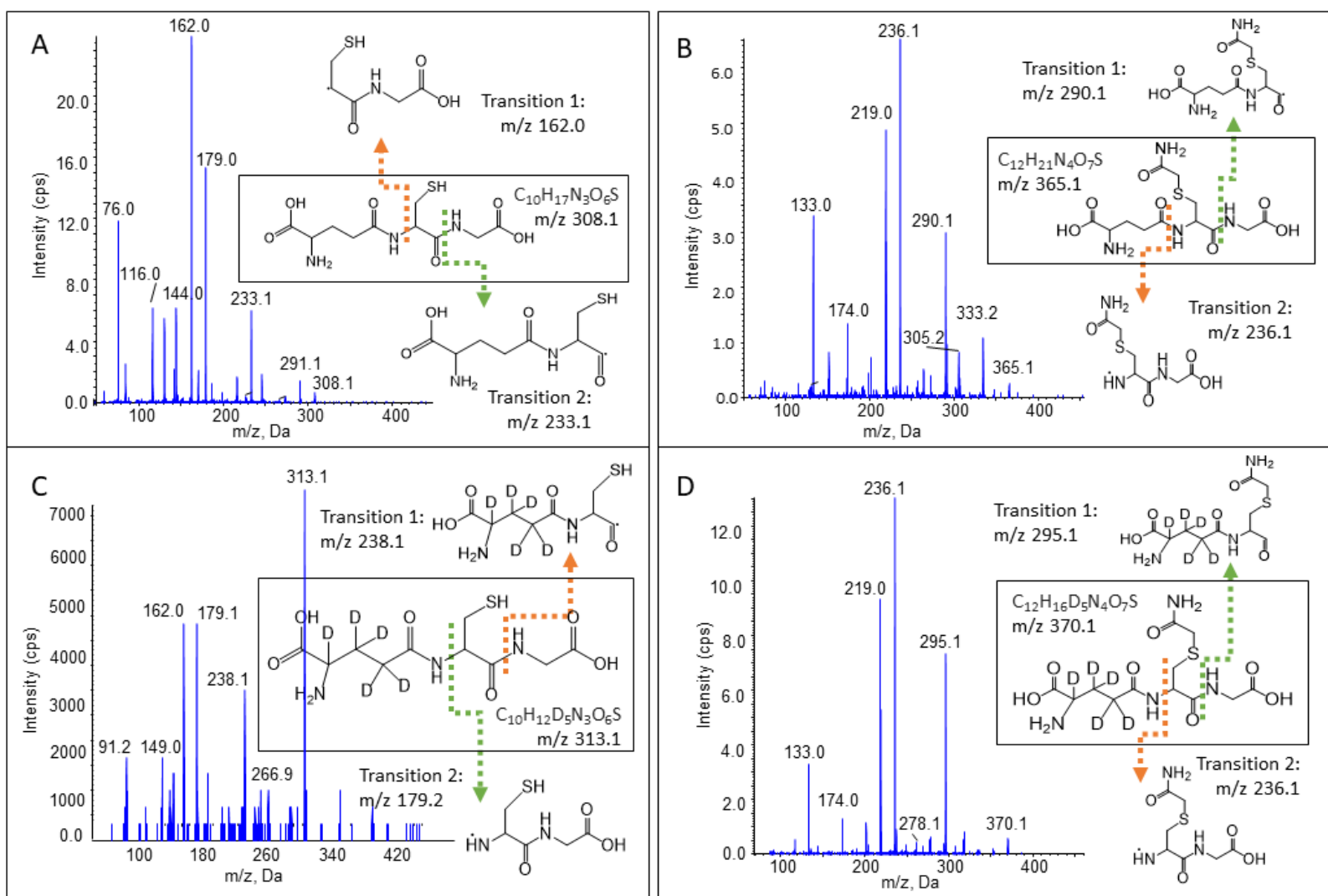
Figure 3.1 MS/MS spectra for GSH, d$_5$-GSH, their alkylated derivatives, and the selected ion transitions used for MRM. [A] GSH; [B] IAM-GSH; [C] d$_5$-GSH; [D] IAM- d$_5$-GSH.

Despite overall improvements on the HILIC column, the GSH peak was observed to split over time (Figure 3.2). This was solved by increasing the organic content of the sample solvent. In HILIC chromatography, a semi-immobilized water layer forms over the course of the run at the interface of the stationary and mobile phases, until the saturation point is reached. This layer is held in place by hydrogen bonds. Though the mechanism behind HILIC is not yet fully elucidated, chemists hypothesize that analytes partition between the semi-immobilized water layer and the mobile phase, rather than directly with the stationary phase. Thus, it is recommended that the sample solvent closely matches the initial conditions of the mobile phase and is high in organic content. Poor peak shape can occur if there is too much water in the sample solution as it can disrupt proper analyte partitioning or formation of the water layer (Chauve et al., 2010; Heaton and McCalley, 2016). By increasing the percent of acetonitrile in the sample solvent, the peak shape was greatly improved. The MS response to increasing amounts of acetonitrile in the sample solvent were analyzed and 50% acetonitrile was selected for use as the sample solvent because it yielded the highest single peak (Figure 3.3).

The optimized instrument method separated IAM-GSH and d_5-IAM-GSH on a HILIC column in 50% acetonitrile and quantified them using an MRM-based method on a QTRAP MS.
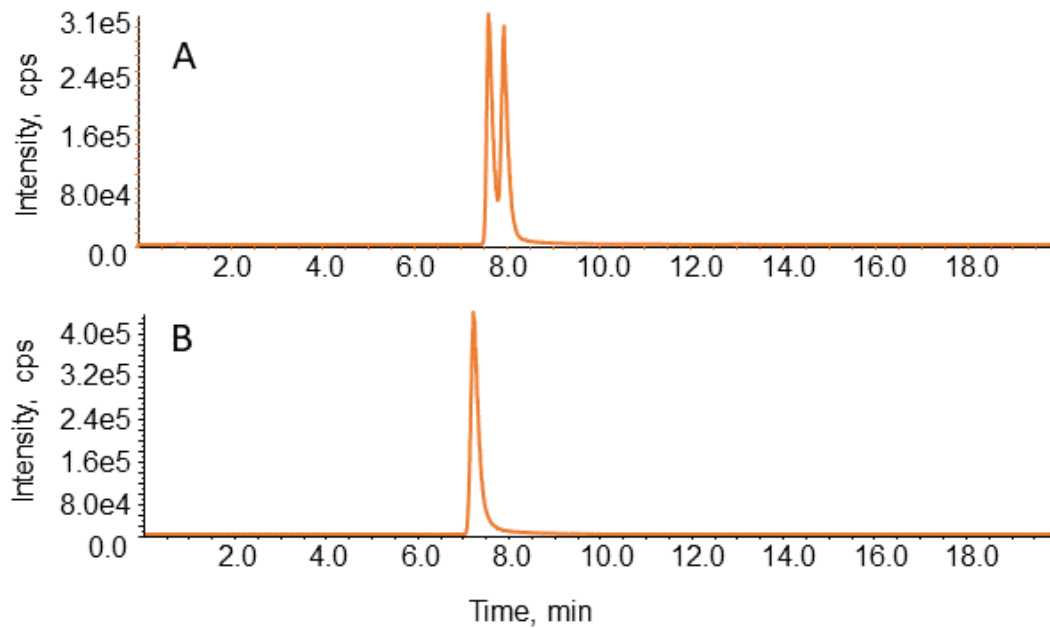
Figure 3.2 LC-QTRAP MRM traces of GSH dissolved in different solvents and separated on a HILIC column. The graphs represent the sum of two ions: m/z 308/179 and m/z 308/162. [A] GSH dissolved in 100% water. [B] GSH dissolved in 50% acetonitrile.
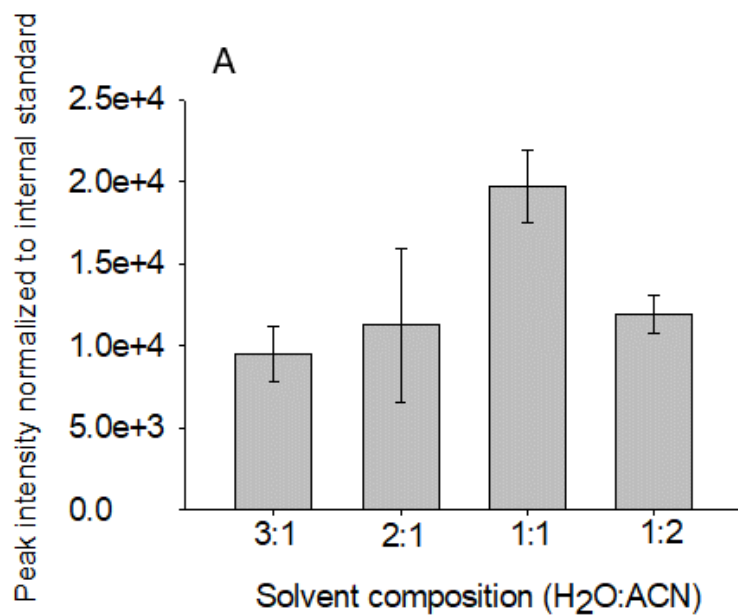

Figure 3.3 Effect of sample solvent composition on GSH peak area. Error bars represent the standard deviation and n = 2. Abbreviations: H_2O = water, ACN = acetonitrile.

3.2.2 Extraction optimization

After refining the instrumental method, the performance of the GSH extraction method was evaluated. Initially GSH was extracted in aqueous solutions of 1mM EDTA and 0.1% v/v formic acid, based on Tang et al. (Tang et al., 2017). However, the percent recovery was quite low, around 30-40%. To address this, the extraction process was re-evaluated and optimized. Adding alcohol to the extraction solvent was chosen based on a survey of the literature and in hopes of precipitating out unwanted proteins. (Xiong et al., 2009). The MS response to GSH extracted in different solutions were analyzed and the results are displayed in Figure 3.4. The extraction solutions tested were 25% methanol, 25% ethanol, 80% methanol and 80% ethanol. The extraction solvent that gave the highest GSH peak area was 80% ethanol.
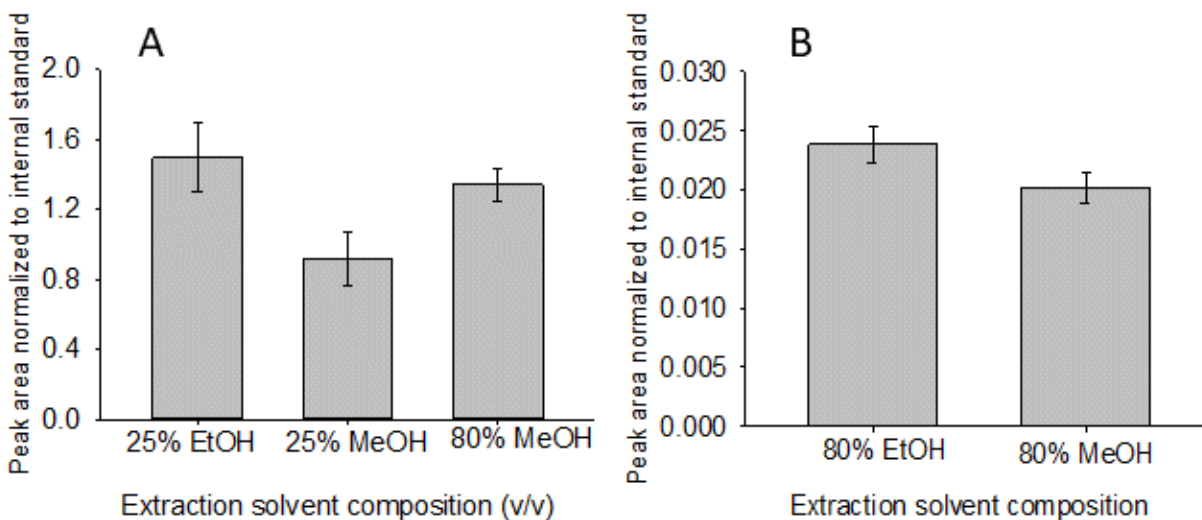

Figure 3.4 Optimization of the LC-MS/MS GSH extraction solution. [A] Effect of extraction solution compositions on GSH peak area. [B] Peak area of GSH in samples extracted in 80% EtOH or 80% MeOH. Error bars represent the standard deviation and n = 2. Abbreviations: EtOH = ethanol, MeOH = methanol.

To further improve recovery, GSH was derivatized with IAM to form IAM-GSH. GSH's cysteine residue readily undergoes thiol exchange reactions with itself and other proteins. The low recovery of the unmmodified analyte may have been due to losing GSH to polymerization with sourdough proteins. QTOF MS was used to confirm that the dimeric form of GSH, GSSG, was not observed in non-derivatized sourdough samples (data not shown). Derivatization of GSH with IAM attaches an amide group to the sulfur moiety on a cysteine residue, thereby preventing it from participating in redox reactions. This process is commonly referred to as alkylation and it reduced GSH loss during extraction by preventing polymerization. The alkylation procedure was adopted from a literature reference, which carried it out in an aqueous solution. However, the sample solvents used in this thesis contained significant amounts of ethanol or acetonitrile. To ensure that the alkylation reaction goes to completion in non-aqueous solvents, the MS response to IAM-GSH after alkylation in water, 50% acetonitrile, or 80% ethanol were compared. There was no significant difference between the IAM-GSH peak areas after alkylation in any of the solutions, confirming that the reaction goes to completion in all the solvents used in this experiment (Figure 3.5).
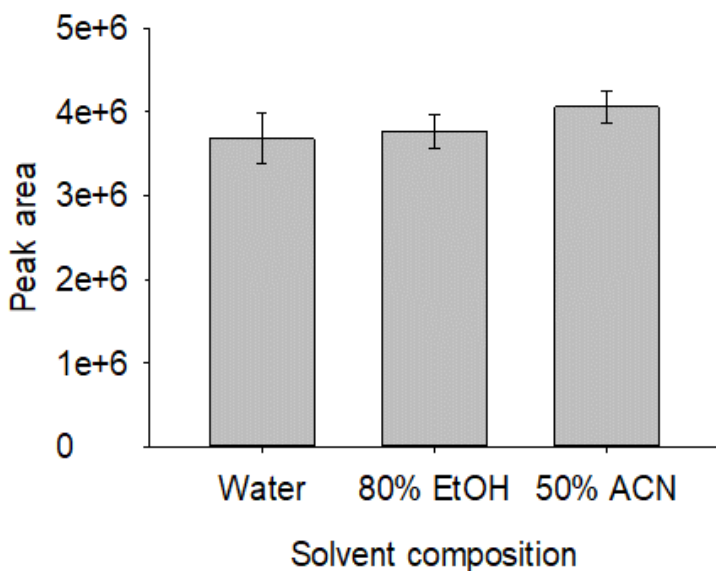
Figure 3.5 Peak area of IAM-GSH after alkylation in different solvents. Error bars represent the standard deviation and n = 2. Abbreviations: EtOH = ethanol, MeOH = methanol.

3.2.3 Method validation

The average percent recovery of the extraction process was determined by spiking sourdough samples with the standard at increasing concentrations (1 ppm, 2.5 ppm, and 5 ppm) before and after extraction (Figure 3.6 A). After method optimization, the percent recovery increased from around 30% to 81% ± 0.95. In other words, the extraction method extracts 81% of the GSH in the sourdough sample. Although 100% recovery is ideal, 81% recovery is acceptable in the context of food analysis and is within the range of recoveries published by AOAC (Kharandi et al., 2013; Martínez et al., 2015; New et al., 2020; Wood et al., 2021). GSH abundance was only of importance in relation to CM3 abundance and 81% recovery is sufficient to show general trends.

The precision of the method was determined by extracting GSH from three samples taken from the same sourdough. The consistency of the resulting peak areas was analyzed (Figure 3.6 B). Precision indicates the reproducibility of the method and is measured by coefficient of variance (COV): the ratio of the standard deviation to the mean. Percent COV gives a numerical indicator of how spread out a set of data points are: highly spread out data points indicates low precision and gives a high % COV. The % COV of the extraction method was 2.65. This relatively low % COV indicates that the method is reproducible and has an acceptable degree of precision.

The lower limits of the method were established by determining the LOD and LOQ. The LOD is the lowest amount of analyte that can be detected by the method and was defined as 3 times the S/N. The LOQ, by contrast, establishes the smallest amount of analyte that can be quantified by the method and was defined as 10 times the S/N. The amount of GSH in the samples were at least 20 times higher than the limit of detection and at least 4 times higher than the limit of quantification. This indicates that the method was suitable for the application.

GSH standards of known concentrations were used to make the calibration curve, which had a R^2 of 0.9969 (Figure 3.6 C). A calibration curve creates a linear regression model based on the instrumental response to standards of a known concentration. The equation produced by this model is used to deduce the concentration of an unknown based on established instrumental responses. The plot's R^2 value measures how well the differences in instrument response relates to differences in analyte concentration and a higher R^2 value indicates increasing linearity. The highest possible value of R^2 is 1. The $R^2 = 0.9969$ indicates that the instrument response is highly predictable in a linear fashion over the range of samples studied.
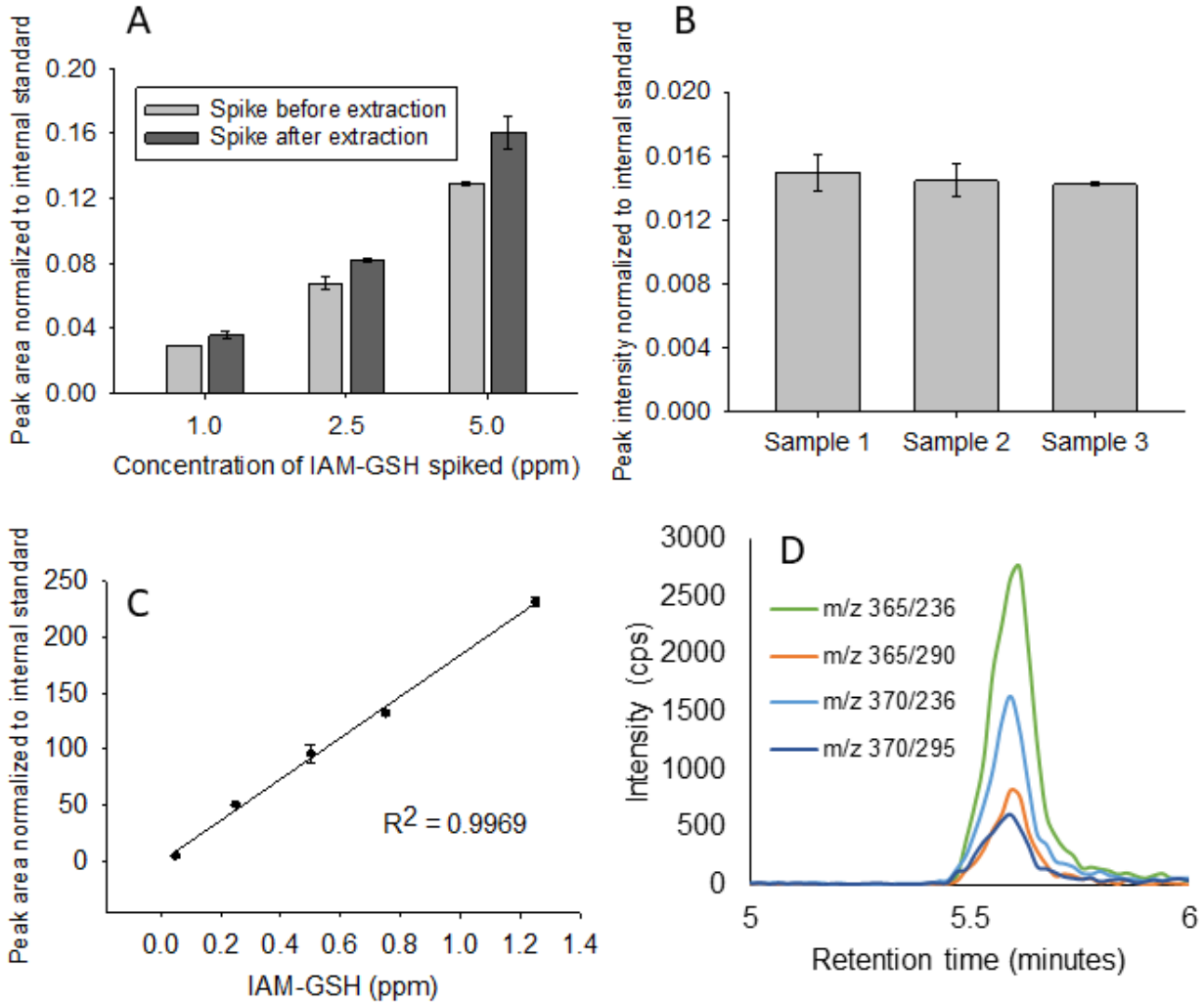
Figure 3.6 Validation of GSH LC-MS/MS quantitation method in sourdough breadmaking. [A] Peak area of IAM-GSH at different concentrations of spiked standard and spiked before and after extraction, [B] Peak area of IAM-GSH in extracted 3 times from the same sourdough, [C] IAM-GSH calibration curve. [D] Chromatogram of IAM-GSH (m/z 365) and IAM-d$_5$-GSH (m/z 370) in sourdough made with cv. Red Fife, fermented with *F. sanfranciscensis,* and taken after mixing. Error bars represent the standard deviation and n = 2.

3.2.4 Quantitative data

GSH was quantified in wheat flour and at several time points during sourdough breadmaking by LC-MS/MS (Figure 3.7). In cv. Red Fife and cv. Brennan flour, the amount of GSH measured was 35 and 56 nmol/g flour, respectively. In doughs made with cv. Red Fife, GSH decreased to approximately 4 nmol/g after mixing, remained around 3 nmol/g after proofing and increased significantly to about 11 nmol/g after baking across all treatment types. The doughs made with cv. Brennan followed a similar trajectory: GSH content dropped to roughly 4 nmol/g after mixing, remained around 4 nmol/g after proofing and rose significantly to about 17 nmol/g, regardless of fermentation. GSH content was significantly higher in doughs fermented with lactobacilli than in the controls in samples made with cv. Brennan, but not in samples made with cv. Red Fife.
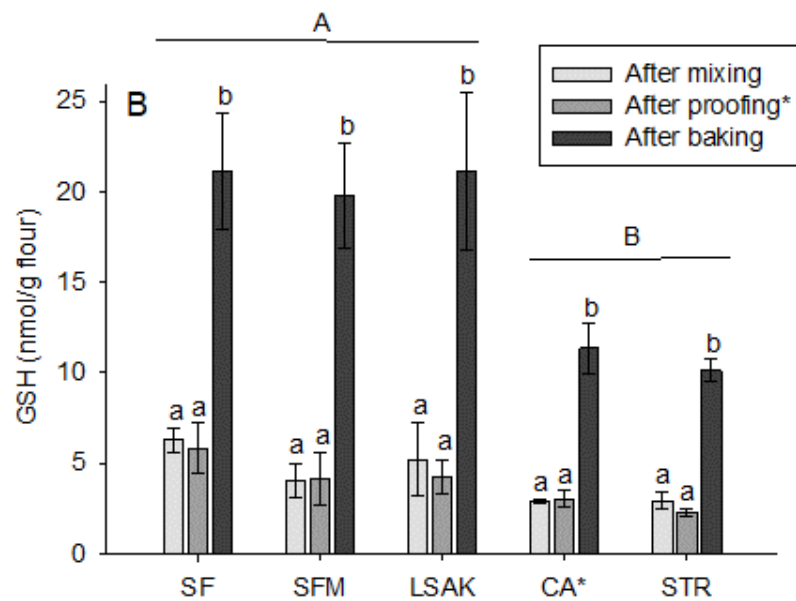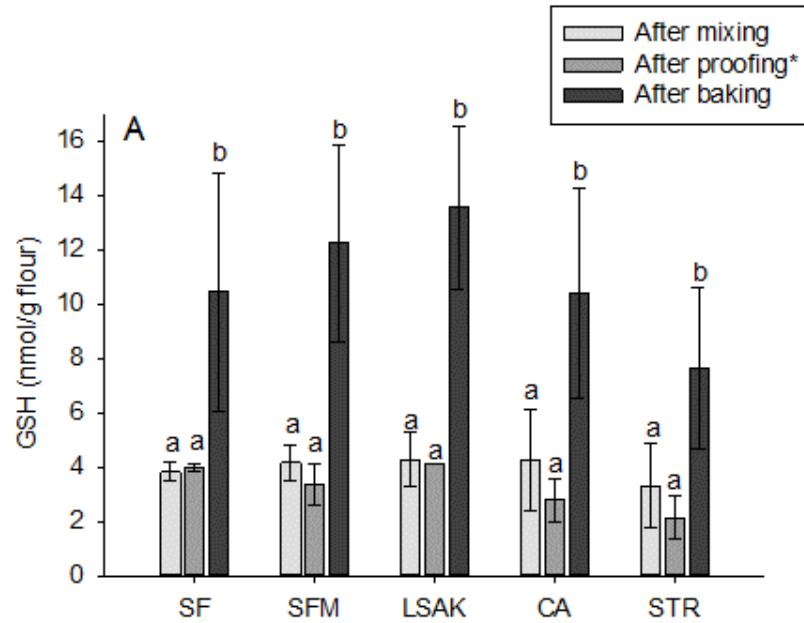
Figure 3.7 LC-MS/MS quantitation of GSH during sourdough breadmaking. Error bars represent the standard deviation and n = 3, except for *n = 2. The lowercase letters indicate differences within treatments and the uppercase letters indicate differences between treatments, both at $P<0.01$. [A] doughs made with cv. Red Fife; [B] doughs made with cv. Brennan. Abbreviations: SF = *F. sanfranciscensis*; SFM= *F. sanfranciscensis ΔgshR*; LSAK = *L. sakei*; CA = chemically acidified dough; STR = straight dough.

3.3 ATI CM3 quantitation

3.3.1 Optimization of trypsin digestion

Quadrupole mass analyzers typically have a mass limit of approximately *m/z* 3000. Thus, they cannot be used to analyze intact proteins whose *m/z* exceed the mass limit. Instead, these proteins must be digested enzymatically into peptides of appropriate sizes prior to MS analysis. Trypsin cleaves proteins after the carboxyl group of arginine or lysine and is commonly used in MS analysis as the peptides generated by it are highly predictable. CM3 was too large to analyze by QTRAP at 16 kDa and, thus, was subjected to trypsin digestion. The process was optimized using CM3 purified by Huang et al. and SDS-PAGE was used to confirm that the protein was digested completely (Huang et al., 2020). The protein to enzyme ratio was approximately 1:80 and the digestion was carried out at 30 °C for 1 hr. Initially, a small amount of intact CM3 remained after digestion (data not shown). This was solved by addition of Promega's ProteaseMAX^{TM} Surfactant, which is designed to enhance enzymatic digestion by solubilizing proteins and providing a denaturing environment. After application of the ProteaseMAX^{TM} Surfactant during trypsin digestion, the CM3 band at 16 kDa was no longer visible (Figure 3.8).
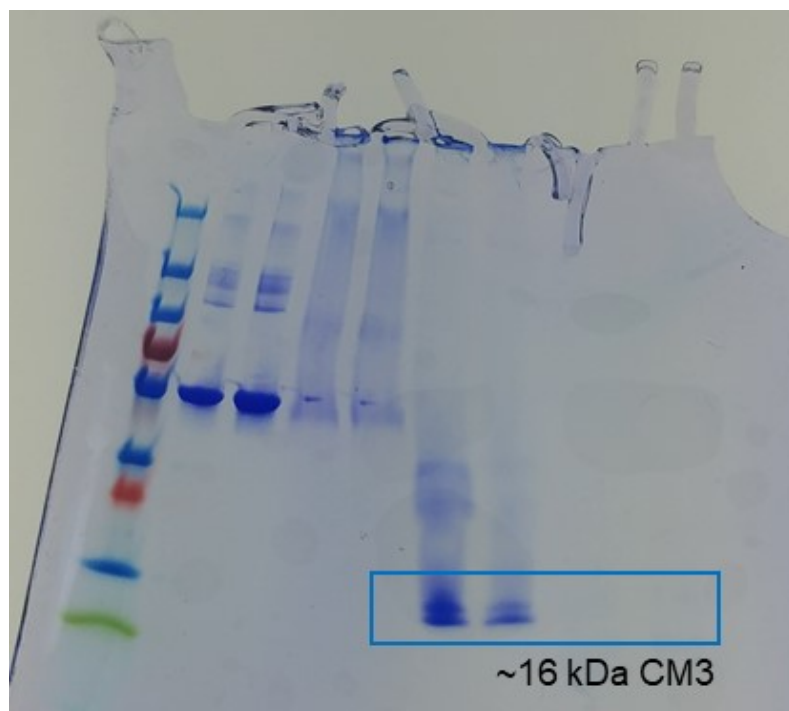
Figure 3.8 SDS-PAGE confirming that the digestion of CM3 with trypsin went to completion. Lanes left to right: (1): protein ladder; (2-3): bovine serum albumin (BSA); (4-5): BSA + trypsin; (6-7): CM3; (8-9): CM3 + trypsin.

3.3.2 Instrumental method development

The amino acid sequence for CM3 was obtained from the literature and *in silico* tools from prospector.ucsf.edu were used to predict the peptides produced by trypsin digestion and their MS/MS fragments (García-Maroto et al., 1990). Initial method development was carried out using purified CM3 extracted from wheat by Huang et al. and commercial wheat sourdough fermented with *F. sanfranciscensis*. (Huang et al., 2020). QTOF MS was used for identification of the CM3 peptides and their respective fragments due to its high sensitivity. Two high intensity peptides unique to CM3, YFIALPVPSQPVDPR and SGNVGESGLIDLPGCPR, were selected for MRM-based quantitation, as well as two high intensity fragments per peptide. The MS parameters (declustering potential, exit potential, collision energy and exit potential) were

optimized for both analytes and their isotope-labelled standards. Figure 3.9 shows the amino acid

sequences and predicted fragmentation patterns for the select CM3 peptides. The MS/MS spectra

of the peptides and their isotope-labelled internal standards are shown in Figure 3.10.

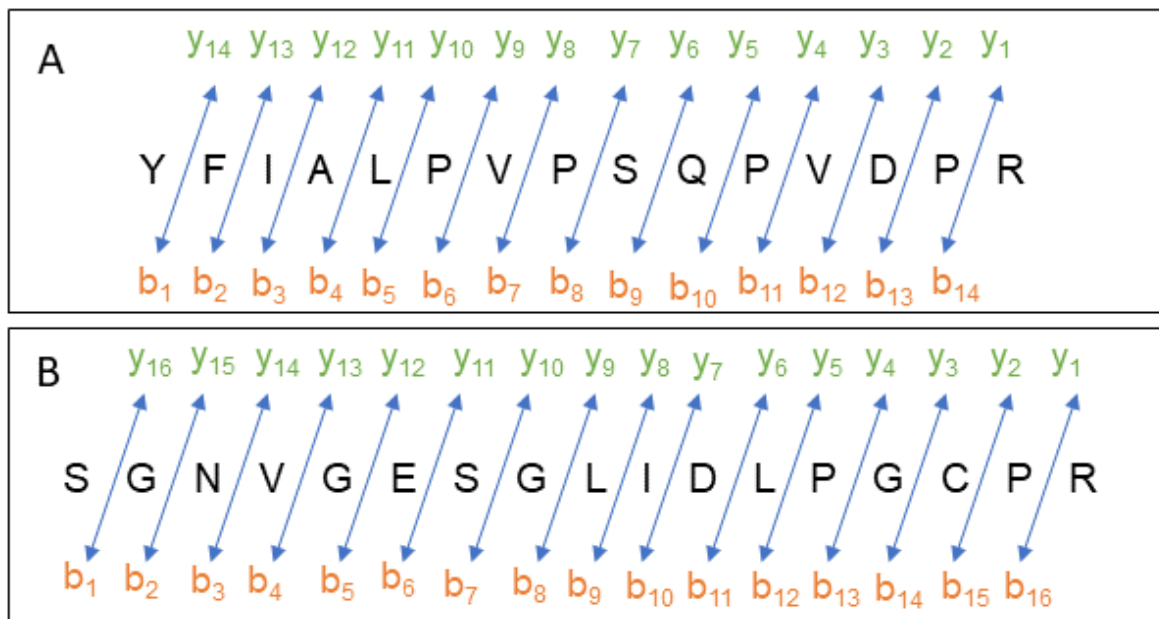**Figure 3.9** Annotated predicted fragmentation pattern of two high intensity CM3 peptides. [A]
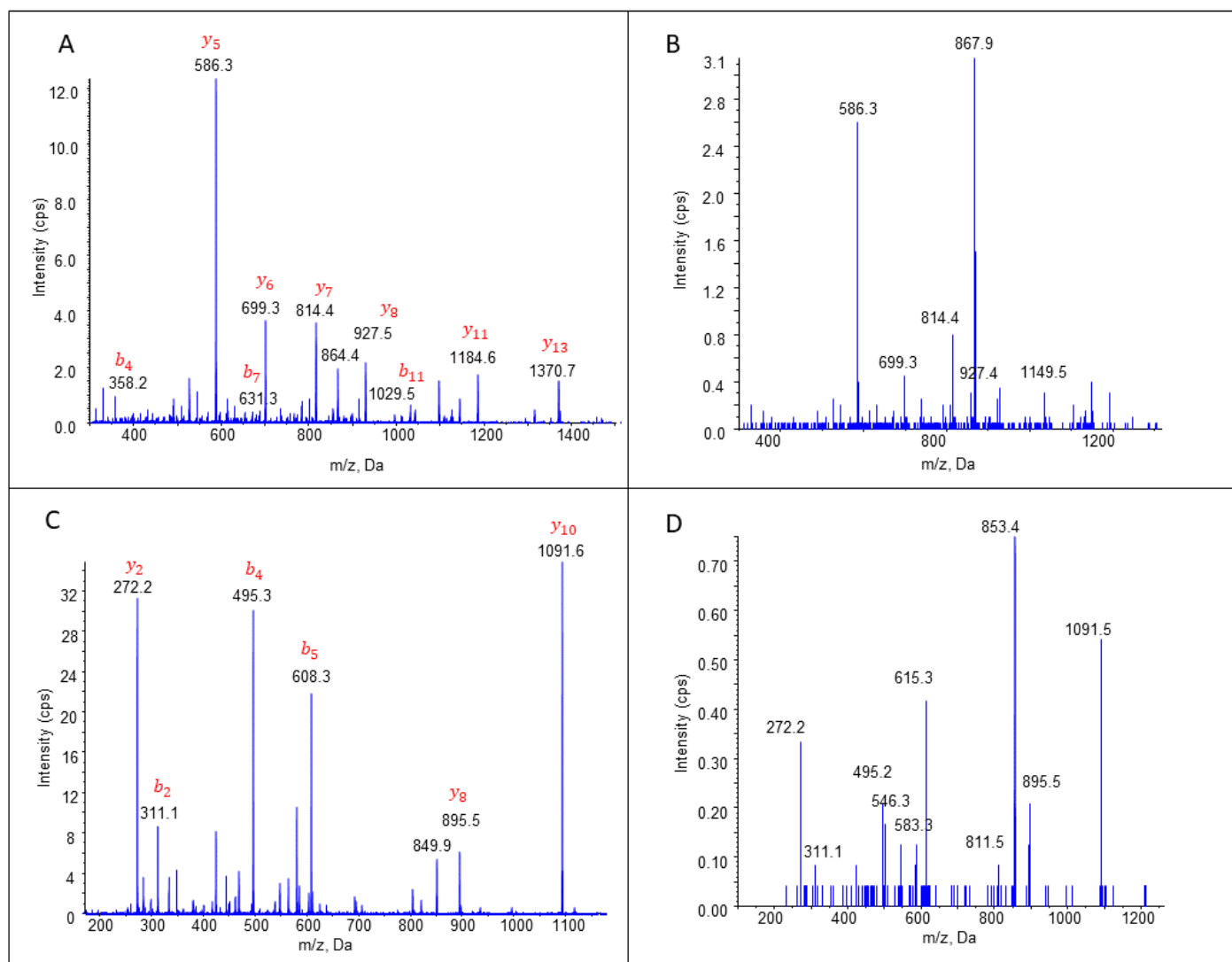
YFIALPVPSQPVDPR. [B] SGNVGESGLIDLPGCPR.

Figure 3.10 MS/MS spectra of two CM3 peptides and their internal standards. Annotations in red correspond to predicted fragments in Figure 3.6.

[A] SGNVGESGLIDLPGCPR, [B] SGNVGESG[^{13}C$_6$,^{15}N]LIDLPGCPR, [C] YFIALPVPSQPVDPR, [D] YFIA[^{13}C$_6$,^{15}N]LPVPSQPVDPR.

An extraction method for CM3 from wheat flour and an LC method were adapted and optimized from the literature for use in sourdough (Geisslitz et al., 2018). Figure 3.11 shows extracted ion chromatograms (XIC) of the two high intensity CM3 peptides separated on a C18 column.

The quantitation of CM3 was ultimately carried out using LC-QTRAP MS and an MRM-based acquisition method for enhanced specificity. MRM chromatograms of the two CM3 peptides and their isotope-labelled standards in sourdough is provided in Figure 3.12.
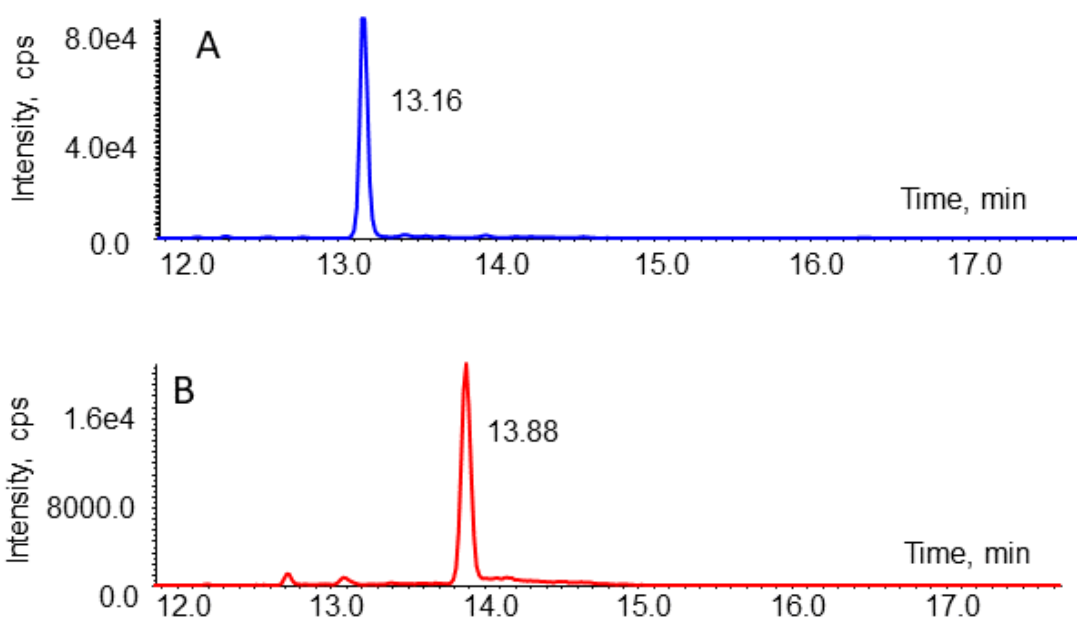

Figure 3.11 LC-QTOF chromatograms showing the separation of two high intensity CM3 peptides after trypsin digestion: [A] SGNVGESGLIDLPGCPR and [B] YFIALPVPSQPVDPR.
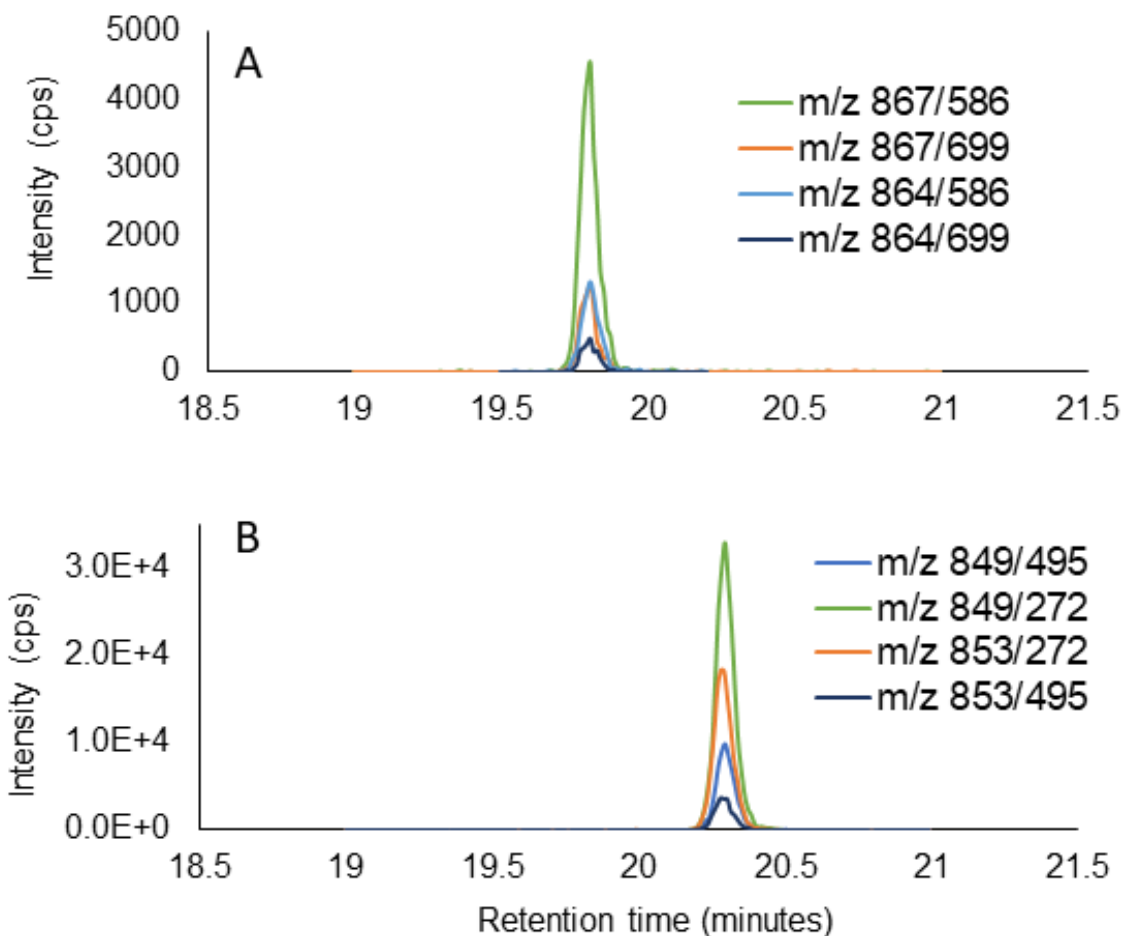
Figure 3.12 LC-QTRAP MRM chromatograms of alkylated CM3 peptides and their isotope-labelled internal standards in sourdough made with cv. Red Fife, fermented with *F. sanfranciscensis* and taken after mixing. [A] SGNVGESGLIDLPGCPR (*m/z* 864) and SGNVGESG[$^{13}C_6$,^{15}N]LIDLPGCPR (*m/z* 867). [B] YFIALPVPSQPVDPR (*m/z* 849) and YFIA[$^{13}C_6$,^{15}N]LPVPSQPVDPR (*m/z* 853).

3.3.1 Method validation

Validation of the method, including recovery and precision, is shown in Figure 3.13. The closest approximate to a standard that was available was CM3 that had been extracted from wheat by Huang *et al.* and the exact purity was unknown (Huang et al., 2020). In order to evaluate the method's percent recovery over a range of concentrations, the extract was diluted 10 times, 20 times and 50 times with 0.1 % formic acid. Sourdough samples were spiked with the diluted

extract before and after extraction, and the resulting peak areas were assessed. The percent recovery was ascertained to be 107% ± 1.4. This indicates that the method extracts 107% of the analyte in the solution and has good recovery. Although it exceeds 100%, the recovery is within the expected range, as 60-120% recovery is considered acceptable in food analysis (AOAC SMPR 2016.002, AOAC SMPR 2017.021). Furthermore, 107% recovery is well within the range of other methods published by AOAC (McKie and McCleary, 2016; New et al., 2020; Wood et al., 2021). Some factors that may contribute to a greater than 100% recovery include evaporation of solvents and manual errors.

To evaluate the method's precision, three samples were taken from the same sourdough, CM3 was extracted from them, and the resulting peak areas were compared. As mentioned before, precision expresses the reproducibility of a method and how close a set of data points are to each other. The peak areas were expected to be very similar and have a low % COV as the samples originated from the same dough. The % COV for the method was 8.1, which is within the acceptable range and demonstrates good reproducibility.

The lower limits of the method were ascertained using the isotope-labelled internal standard (Table 2.3). The LOD was defined as 3 times the S/N and the LOQ was defined as 10 times the S/N. These values indicate the minimum concentration of analyte that must be present for accurate detection and quantitation. The LOD and LOQ were in the order of 10^0, while the analyte concentrations in the samples were in the order of 10^1 or higher, confirming that the method was suitable for the purpose.

An external calibration curve made with the internal standard was used to ensure a linearity across the range studied. As mentioned before, a calibration curve is used to deduce analyte concentration based on a known instrument response. Although absolute quantitation was not

carried out, it was important to confirm that the instrument response was linear over the range of

the samples studied. This ensured that any variance in instrument response could be attributed to

the sample, rather than to instrument performance. For this method, $R^2 = 0.9981$, which indicated
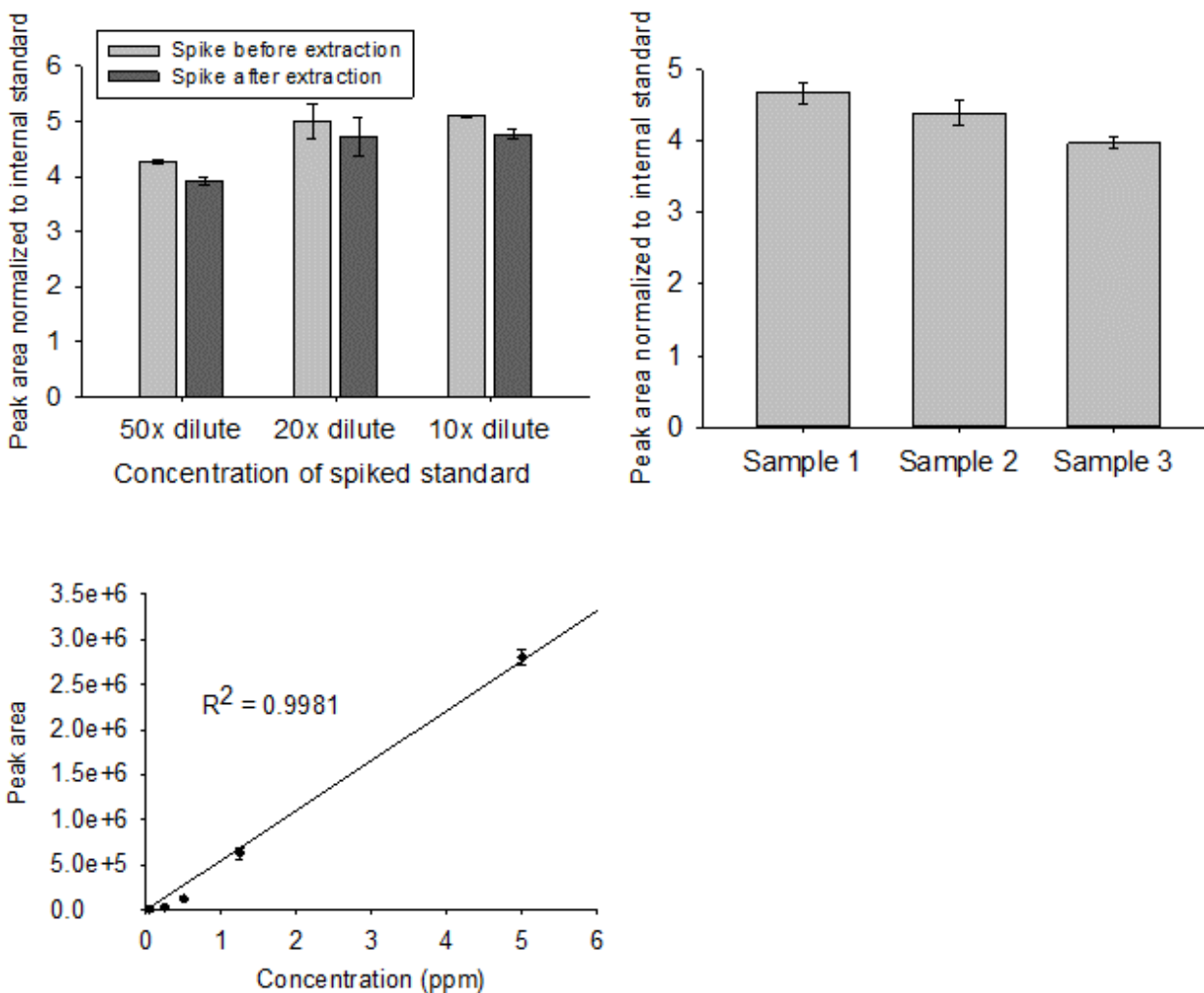
an acceptable degree of linearity.

Figure 3.13 Validation of CM3 LC-MS/MS method in sourdough. [A] Recovery at different

concentrations; percent recovery = 107%. [B] Extraction precision; % COV = 8.09. [C] Calibration curve

made using isotope-labelled standard. Error bars represent the standard deviation and n = 2.

3.3.2 Relative abundance data

LC-MS/MS methods were developed and applied to measure the abundance of ATI CM3 during sourdough breadmaking (Figure 3.14). The relative abundance of CM3 was similar after proofing and decreased significantly after baking in all doughs made with both cv. Red Fife and cv. Brennan, regardless of fermentation type. Differences between treatments were only observed in cv. Brennan samples: CM3 abundance in samples fermented with *L. sanfranciscensis ΔgshR* was higher than both of the controls and the CM3 abundance in samples fermented with *F. sanfranciscensis* was greater than the straight control.

To understand how fermentation affects the proportion of tetrameric and monomeric ATI, undigested samples were analyzed using non-reducing SDS-PAGE. ATI isoforms were identified by their molecular weights and by cross-referencing with the literature. Figure 3.15 shows an example gel with the ATI bands annotated. The band intensities of both tetrameric and monomeric isoforms were significantly reduced after baking (Figure 3.16). This corroborates the LC-MS/MS data in that overall baking was the greatest determinant of ATI band intensity.
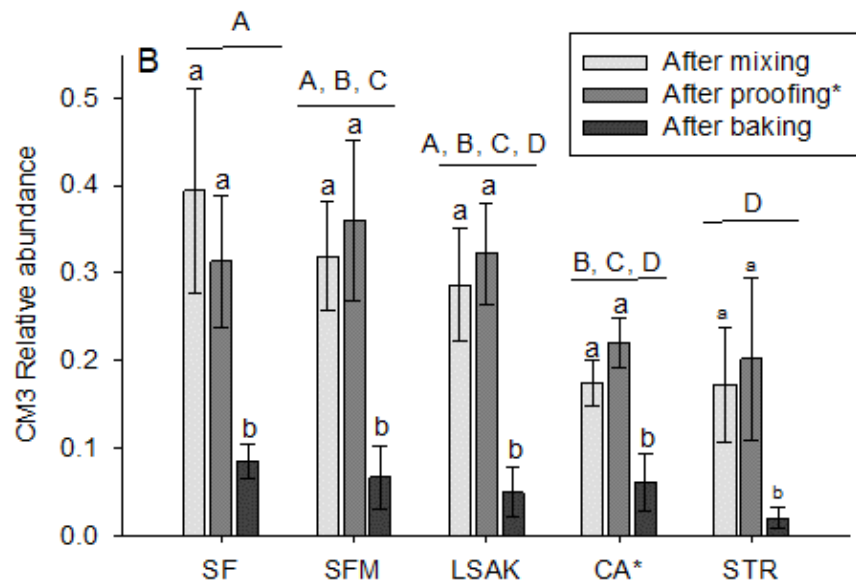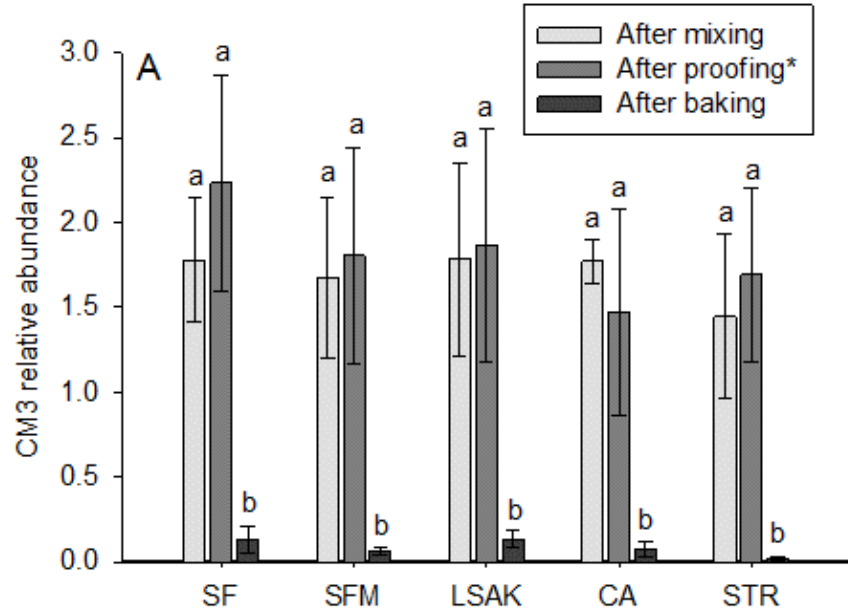
Figure 3.14 Relative abundance of CM3 during sourdough breadmaking, as determined by LC-MS/MS.

The error bars represent the standard deviation and n = 3, except for *n = 2. The lowercase letters

represent differences within treatment a nd the uppercase letters indicate differences between treatments,

both at $P<0.01$. [A] doughs made with cv. Red Fife; [B] doughs made with cv. Brennan. Abbreviations:

SF = *F. sanfranciscensis*; SF *ΔgshR* = *F. sanfranciscensis ΔgshR*; LSAK = *L. sakei*; CA = chemically

acidified dough; STR = straight dough.
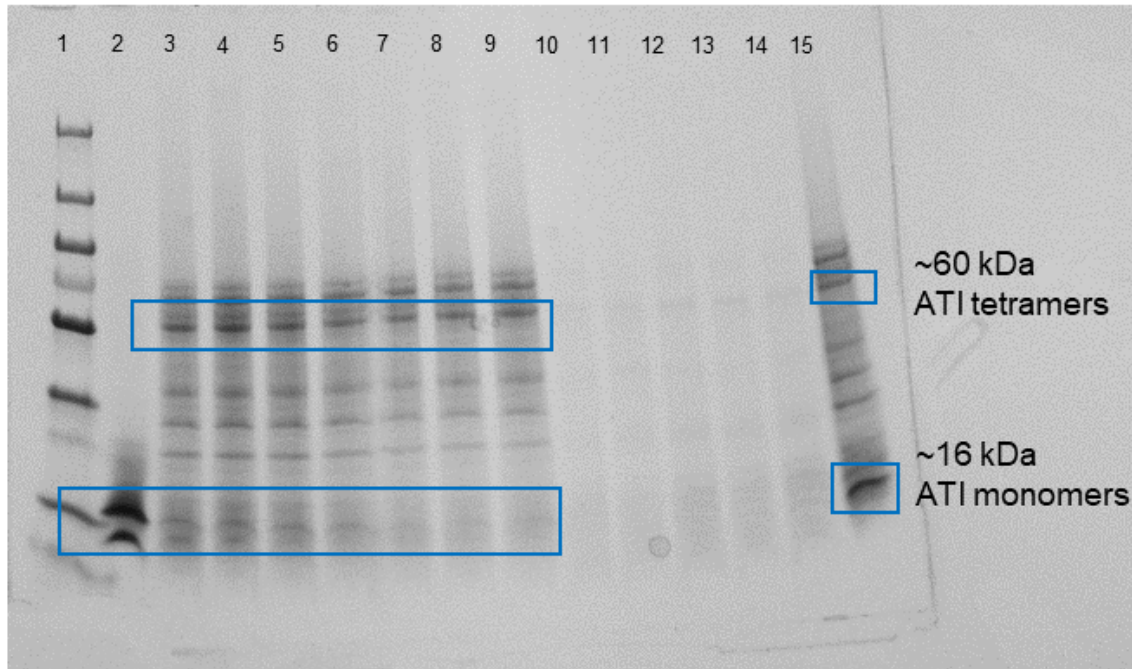
Figure 3.15 SDS-PAGE separation of ATI extracts from sourdoughs made with cv. Red Fife during breadmaking. All samples shown were extracted from doughs or bread prepared from the wheat cultivar Red Fife. Lanes from left to right: (1) protein ladder, (2) extracted ATI CM3, (3) chemically acidified, after mixing; (4) straight dough, after mixing; (5) *F. sanfranciscensis* after proofing; (6) *F. sanfranciscensis ΔgshR* after proofing; (7) *L. sakei* after proofing; (8) chemically acidified after proofing; (9) straight dough after proofing; (10) *F. sanfranciscensis* after baking; (11) *F. sanfranciscensis ΔgshR* after baking; (12), *L. sakei* after baking; (13), chemically acidified after baking; (14), straight dough after baking, (15), *F. sanfranciscensis* after mixing.
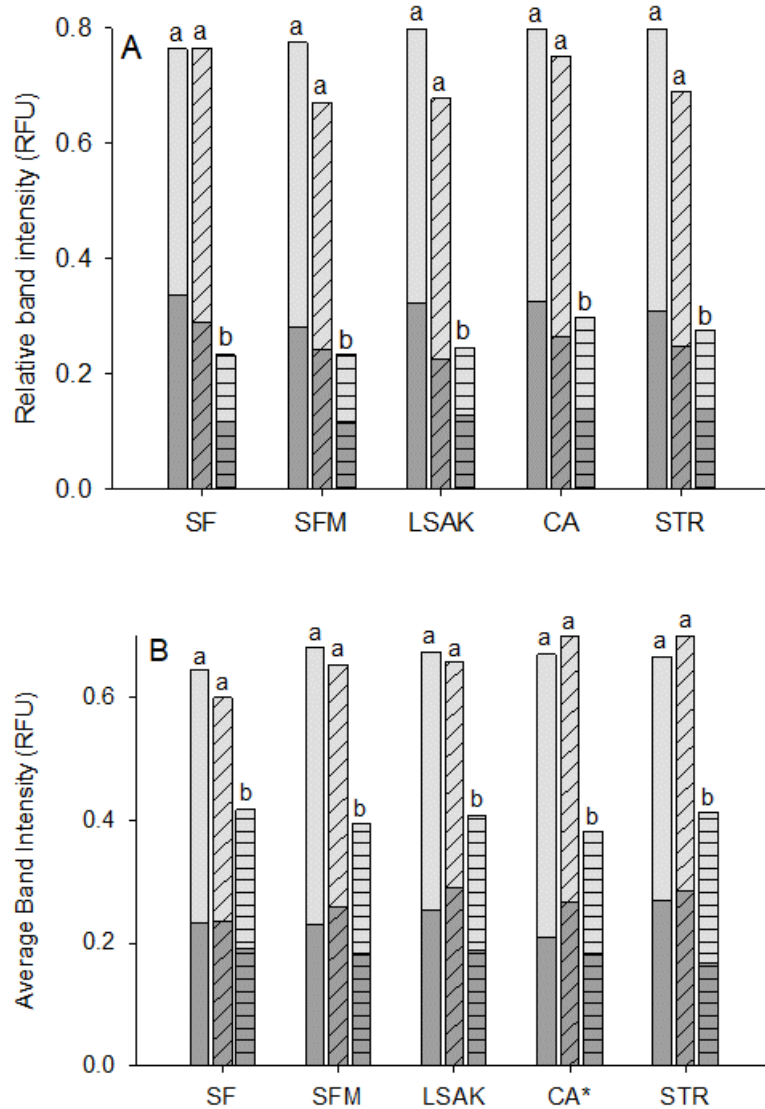
Figure 3.16 Relative band intensity of ATI proteins separated by SDS-PAGE during sourdough breadmaking. The error bars represent the standard deviation, and the letters indicate significant differences within treatments at *P*<0.01. [A] doughs made with cv. Red Fife; [B] doughs made with cv. Brennan. Bar graph key: light grey = ATI tetramers, dark grey = ATI monomers; no lines = after mixing, diagonal lines = after proofing*, horizontal lines = after baking. Abbreviations: SF = *F. sanfranciscensis*; SF *ΔgshR* = *F. sanfranciscensis ΔgshR*; LSAK = *L. sakei*; CA = chemically acidified dough; STR = straight dough; RFU = relative fluorescence units.

CHAPTER 4   DISCUSSION

Sourdough is a widely used method of breadmaking that yields breads with favorable organoleptic properties. The amount of inoculum used for fermentation typically ranges from 10-20% and the baking process used in this experiment was designed to mimic conditions used in industry. Fermentation length determines what aspect of the process is emphasized: longer fermentation times favor acidification, while the shorter fermentation length used in this study emphasized microbial metabolic activity (Gänzle, 2014; Arora et al., 2021).

The present study explored how sourdough affects bioactive wheat proteins and peptides using LC-MS/MS. Previous studies have documented the abundance of GSH in wheat flour, indicating that it varies widely between cultivars and due to nutrient availability. Sarwin et al. reported 16-41 nmol GSH/g and Schofield and Chen reported 18-81 nmol GSH/g across a range of pure wheat cultivars (Chen and Schofield, 1996; Sarwin et al., 1992; Schofield and Chen, 1995). Similarly, in wheat cv. Star, Reinbold et al. observed 84-135 nmol GSH/g flour and noted that GSH abundance increased with increasing sulfur availability in the soil (Reinbold et al., 2008). Our study reported 35 and 55 nmol GSH/g, which is comparable with the current literature. Over the course of a 24-hour fermentation of commercial flour with lactobacilli, Tang et al. observed GSH concentration to be around 6-24 μmol/kg which is similar to the values reported in our study after mixing (Tang et al., 2017). In all treatments, GSH abundance remained similar in doughs after mixing and after proofing but increased significantly in the breads after baking. This suggests that the overall baking process was the most significant determinant of GSH abundance in wheat bread, including sourdough bread. This is supported by a previous study in which free thiol content in sourdough was not found to differ significantly between mixing and

proofing (Xu et al., 2018). GSH content was higher in doughs fermented with lactobacilli than in the controls in samples made with cv. Brennan, but it did not differ between lactobacilli fermentations. This suggests a mechanism other than microbial glutathione reductase activity and thiol exchange reactions affected GSH abundance.

The relative abundance of CM3 decreased significantly after baking regardless of fermentation. This suggests that the overall baking process exerted a greater effect on its abundance than fermentation by lactobacilli. A few studies have linked lactobacilli with reduction in ATI-related pro-inflammatory responses. Caminero et al. reported that probiotic supplementation with lactobacilli reduced ATI-induced inflammation in sensitized mouse models (Caminero et al., 2019). It is important to note that this study was performed *in vivo*, so it is undetermined if these results relate to ATI degradation in bread. During sourdough fermentation, Huang et al. reported the reduction of ATI tetramers to monomers by SEC, as well as reduced protease inhibition and increased free thiol content (Huang et al., 2020). While sourdough may reduce tetrameric ATI to monomeric forms, the latter are still bioactive, and our study suggests that the abundance of the monomer CM3 was not affected by sourdough any more than it was in straight or acidified controls. Other studies have reported reductions in ATI bioactivity and enzyme inhibition in baked straight dough which supports the idea that ATI abundance diminishes during baking. Simonato et al. observed that the 16 kDa ATI protein is recognized by IgE immunoblotting in unheated flour, but not in baked bread (Simonato et al., 2001). Kostekli and Karakaya reported that fermentation and baking reduced trypsin inhibitory activity in various flours (Kostekli and Karakaya, 2017). Zevallos et al. also observed a decrease in IL-8 release from THP-1 cells, as measured by ELISA, from ATI in wheat bread when compared to wheat flour (Zevallos et al.,

2017). It is important to note that food processing and heat treatment may also affect the solubility and extractability of wheat proteins.
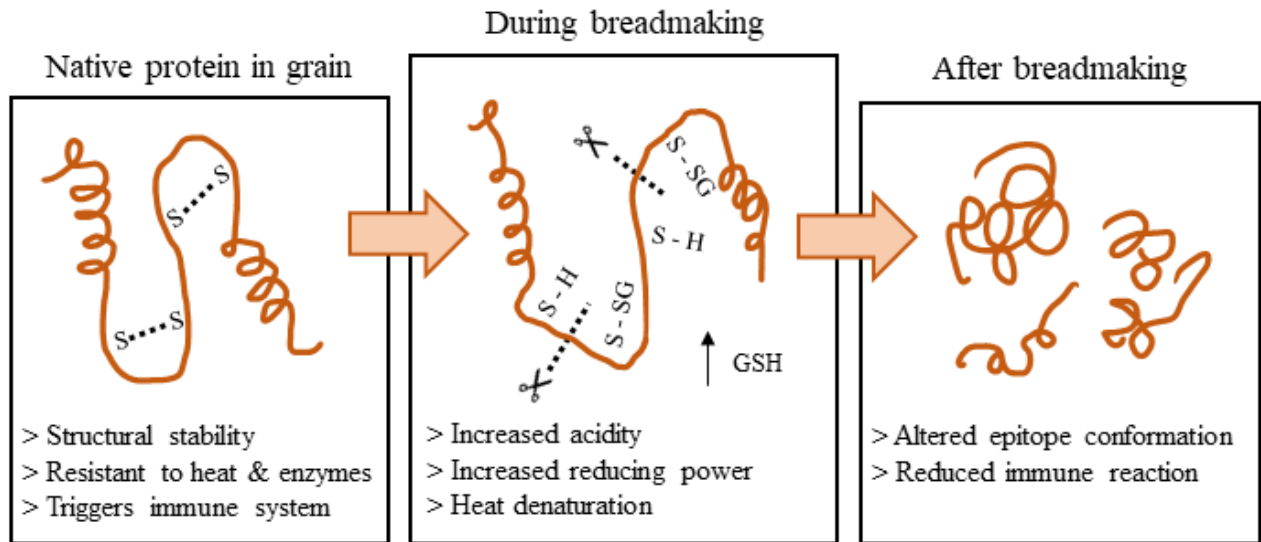
Figure 4.1 Sourdough fermentation may affect protein structure and function. Key: Orange line = wheat protein; S = sulfur on cysteine residues; S-H = reduced cysteine ; S-SG = cysteine reduced by GSH; GSH = glutathione; Scissors = proteolytic enzymes.

The mechanisms that can affect the structure and abundance of proteins during sourdough breadmaking are illustrated in Figure 4.1. Native protein structure can be disrupted by acidification, redox reactions, and heat denaturation. Redox reactions refer to reduction by low molecular weight thiols, such as GSH, as well as thiol-mediated reactions with other proteins. Acidification plays an important role in gluten degradation, but the lack of difference between chemically acidified and straight doughs in our study indicate that acidity had little effect on CM3 abundance (Thiele et al., 2003; Loponen et al., 2004; Thiele et al., 2004). In addition to acidity, redox agents have been shown to modulate gluten polymerization via disulfide exchange reactions. Vermuelen et al. observed that heterofermentative and homofermentative lactobacilli exert opposing effects on gluten proteins, attributed to differences in thiol metabolism (Grosch

and Wieser, 1999; Vermeulen et al., 2006; Navrot et al., 2018). However, there were no significant differences in abundance of CM3 or GSH between *F. sanfranciscensis* and its *ΔgshR* mutant, suggesting that the presence of glutathione reductase was not a significant determinant. Similarly, we observed no significant differences between breads fermented with the heterofermentative *F. sanfranciscensis* and the homofermentative *L. sakei*. ATI structural changes during baking, such as aggregation, denaturation or thiol-mediated linkages to other proteins remains unknown. In general, the baking process had a greater effect on GSH and water soluble CM3 abundance than acidification and differences in thiol metabolism.

Overall, our study demonstrated that the content of extractable ATI decreases substantially after baking. However, it is important to note that our experimental design could not determine whether this decrease after baking relates to proteolysis in the initial stages of the baking process, ATI structural changes such as aggregation and denaturation, which may reduce extractability in the absence of chaotropic salts, or thiol-mediated linkages to other proteins. Whether the decrease in ATI concentration, or a decrease in extractability in absence of chaotropic salts and reducing agents, impacts its biological activity in the context of NCWS remains subject to future investigations. Our results emphasize, however, that studies that aim to determine the impact of fermentation processes in breadmaking on the quantity and biological activity of ATI should consider the baking process as a key determinant of ATI in bread.

Beyond ATI, sourdough has been shown to modulate other wheat constituents linked to NCWS, including FODMAPs and wheat germ agglutinin (WGA). FODMAPs are naturally occurring undigestible carbohydrates that are readily fermented by colonic microbiota. The resulting gas production and osmotic activity is linked to distension, bloating and abdominal discomfort in certain subpopulations (Shepherd et al., 2014). A low FODMAP diet alleviates these symptoms

but can be challenging due to lack of dietary fiber (Halmos et al., 2014; Roest et al., 2013).

Sourdough's ability to break down undesirable FODMAPs while preserving the dietary fiber in bread is currently under investigation. Yeasts are capable of limited fructan degradation and certain lactobacilli strains express extracellular fructanases, such as *Lactobacillus crispatus* and *L. amylovorus* (Loponen and Gänzle, 2018). Indeed, Li et al. reported that fermentation with *L. crispatus* reduced the fructan content of bread by over 90% (Li et al., 2020). Similar to ATI, WGA has been linked to NCWS, but the pathophysiology is not yet well understood (Punder and Pruimboom, 2013). WGA has been shown to trigger the release of pro-inflammatory cytokines in human cells and increase the permeability of the epithelial layer (Pellegrina et al., 2009). Similar to gluten and ATI, they are stabilized against denaturation by multiple disulfide bonds. Tovar and Gänzle reported that fermentation of wheat flour with *L. sanfranciscensis* and *L. sakei* reduced WGA content in sourdough bread. They also related this effect to redox reactions in the dough (Tovar and Gänzle, 2021). Overall, research indicates the possibility of using sourdough to create NCWS-tolerable foods but the applicability of it remains inconclusive due to insufficient research. Further investigation is merited to elucidate the pathophysiology of wheat constituents and how they are affected by food processing.

Sourdough influences wheat components and bread qualities beyond NCWS-related compounds. As mentioned before, the proteolytic effect of sourdough on gluten is well-documented (di Cagno et al., 2010, 2002; Loponen et al., 2004; Thiele et al., 2004, 2003). Beyond proteins, sourdough influences the texture and flavor of bread via carbohydrate and peptide metabolism. As a part of biofilm formation, lactic acid bacteria produce exopolysaccharides which have positive effects on loaf texture and volume and contributes to dietary fiber (di Monaco et al., 2014; Gänzle, 2014; Xu et al., 2020). In addition to degrading proteins, sourdough produces

taste-active peptides, such as glutamate and ornithine, and peptides exhibiting positive forms of bioactivity, such as antihypertensive effects (Gänzle, 2014; Gänzle et al., 2008). Sourdough also removes undesirable, non-protein plant components. Wheat naturally contains phenolic acids, which have anti-nutritive or bitter-tasting effects. Some phenolics may also have positive, antioxidative properties. Certain lactobacilli express reductases and decarboxylases capable of releasing and metabolizing bound phenolic acids (Gobbetti et al., 2019; Ripari et al., 2019). Phytate is another anti-nutritive wheat constituent that chelates with divalent cations, such as calcium and magnesium, and reduces their bioavailability. Sourdough degrades phytate by acidifying the dough to the pH optimum of endogenous cereal phytases, thereby increasing the bioavailability of minerals (Poutanen et al., 2009). Fungal spoilage in bread is a challenge faced by the baking industry. Certain lactobacilli are capable of producing antifungal compounds, such as organic acids and hydroxy fatty acids, which have been shown to delay fungal spoilage. The organic acids produced include formic and acetic acids, while certain strains have demonstrated the ability to convert linoleic acid to antifungal hydroxy fatty acids, such as ricinoleic or coriolic acid. (Black et al., 2013; Gänzle, 2014; Quattrini et al., 2019; Rizzello et al., 2011). In summary, the metabolic diversity of lactobacilli allows sourdough to modulate a wide variety of bread qualities, although research on this front is still emerging.

4.1   Directions for future research

This thesis demonstrated that the extractable ATI CM3 abundance decreased during breadmaking, but it did not determine the mechanism behind it. Future studies might seek to understand the structural changes made to ATI during breadmaking, such as aggregation, denaturation, and the effect of chaotropic agents.

Regarding the methods used in this experiment, it remains unknown whether breadmaking reduces the abundance of other isoforms of ATI beyond CM3. Expansion of the LC-MS/MS method used in this thesis to include other ATI isoforms would bolster our understanding of sourdough's proteolytic effects. Further refinement of the GSH extraction method to increase the percent recovery would also improve the method. This could be done by exploring different extraction solutions and methods or incorporation of solid phase extraction (SPE). Additionally, obtaining highly purified standards for the CM3 peptides would allow for absolute quantitation of CM3, rather than relative abundance.

The current lack of documentation relating ATI abundance to bioactivity limits the ability to draw definite conclusions regarding management strategies for NCWS. For example, few studies have combined quantitative ATI assays with immunoassays or enzyme inhibition assays. Future experiments should consider comparing ATI immunoreactivity and enzyme inhibition to quantitative data in wheat flour and wheat-based foods. This would allow us to understand whether ATI bioactivity in food is dose-dependent, or if it is affected by adjuvants in the food matrix. On a wider scale, the pathophysiology of ATI in NCWS remains unclear. Further research to elucidate how non-gluten wheat proteins trigger adverse symptoms in humans is necessary for drawing conclusions on how to mitigate their effects.

4.2   Concluding remarks

In conclusion, the chemistry of sourdough is influenced by bacterial enzymes and metabolites which distinguishes it from straight dough processes. Fermentation presents a potentially customizable variable to modulate constituents of the dough. Application of LC-MS/MS in food analysis allows for increased understanding of how fermentation and food processing affects

allergen content and for robust measurement of low abundance compounds with high specificity. This thesis expands our knowledge of how sourdough breadmaking affects bioactive proteins and peptides and suggests that the baking process is an important factor to consider when investigating ATI abundance in wheat-based foods.

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