Rheology and NMR Spectroscopy Studies of Commercial Wheat Gluten Doughs

BY

Rosa Elena de la Luz Gómez-Haro Aceves

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Food Science
University of Manitoba
Winnipeg, Manitoba

© Copyright by Rosa Elena de la Luz Gómez-Haro Aceves 2004

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Rheology and NMR Spectroscopy Studies

of Commercial Wheat Gluten Doughs

BY

Rosa Elena de la Luz Gómez-Haro Aceves

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

Rosa Elena de la Luz Gómez-Haro Aceves © 2004

Permission has been granted to the Library of the University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film, and to University Microfilms Inc. to publish an abstract of this thesis/practicum.

This reproduction or copy of this thesis has been made available by authority of the copyright owner solely for the purpose of private study and research, and may only be reproduced and copied as permitted by copyright laws or with express written authorization from the copyright owner.

I hereby declare that I am the sole author of this thesis.

I authorise the University of Manitoba to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Rosa Elena de la Luz Gómez-Haro Aceves.

I further authorise the University of Manitoba to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Rosa Elena de la Luz Gómez-Haro Aceves.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS....i
LIST OF TABLES....ii
LIST OF FIGURES....iv
LIST OF APPENDICES....vii
GENERAL ABSTRACT....viii
1. GENERAL INTRODUCTION....1
2. LITERATURE REVIEW....5
2.1 IMPORTANCE OF GLUTEN....5
2.1.1 Wheat Production....5
2.1.2 Wheat Products....5
2.1.3 Gluten Manufacture....7
2.1.4 Uses of Gluten....9
2.1.5 Functionality of Gluten....10
2.1.5.1 **Composition of Gluten**....10
2.1.5.2 **Viscoelasticity of Gluten**....12
2.2 GLUTEN STRUCTURE....13
2.2.1 Gluten and Its Physicochemistry....13
2.2.1.1 **Amino Acid Composition**....13
2.2.1.2 **Physicochemical Characteristics**....14
2.2.1.3 **Gluten Conformational Models**....14
2.2.2 Effects of Processing and Ingredients on Gluten Structure....16
2.2.2.1 **Presence of Air**....17
2.2.2.2 **Presence of Acids**....18
2.2.2.3 **Presence of Salts**....19
2.3 RHEOLOGY OF GLUTEN....21
2.3.1 Empirical Rheology....23
2.3.2 Fundamental Rheology....24
2.3.2.1 **Oscillatory Tests**....26
2.3.2.2 **Ultrasound**.... 27

2.4 SPECTROSCOPY OF GLUTEN....31
2.4.1 Basic Concepts of NMR....31
2.4.1.1 **Spin and Magnetic Moment**....31
2.4.1.2 **Relaxation Times (T_1 & T_2)**....35
2.4.1.3 **The Spin Echo Sequence**....37
2.4.1.4 **Pulsed-Gradient-Spin-Echo Sequence (PGSE)**....39
2.4.2 NMR Studies of Gluten Proteins....42
3. MANUSCRIPT I. **Effect of Mixing Conditions and Fermentation Process Products on the Mechanical Properties of Wheat Gluten Assessed by Rheological Measurements**....44
ABSTRACT....44
3.1 INTRODUCTION....46
3.2 MATERIALS AND METHODS....49
3.2.1 Materials....49
3.2.2 Methods....49
3.2.2.1 **Gluten Dough Preparation**....49
3.2.2.2 **Adjusting Gluten Dough pH**....50
3.2.2.3 **Mixing Times for the Development of Gluten Doughs**....51
3.2.2.4 **Mixing Gluten Dough Under Different Headspace Conditions**....52
3.2.2.5 **Preparation of Gluten Doughs Samples for Rheological Measurements**....53
3.2.3 Dynamic rheological measurements at small deformations....54
3.2.4 Rheological parameters reported....55
3.2.5 Statistical analysis....55
3.3 RESULTS....57
3.3.1 Effect of mixing time on the pH of gluten doughs....57
3.3.2 Effects of acids on mixing time of gluten doughs....59
3.3.3 Effects of pH on mechanical properties of gluten doughs....61

3.3.4 Effects of pH on mechanical properties of gluten doughs when mixed under vacuum....65
3.3.5 Effects of different acids on properties of gluten doughs....67
3.4 DISCUSSION OF RESULTS....69
3.5 CONCLUSIONS....81
4. MANUSCRIPT II. **Ultrasonic Determinations of Gluten Doughs as Affected by Acids, Salts and their Combination**....82
ABSTRACT....82
4.1 INTRODUCTION....84
4.2 MATERIALS AND METHODS....88
4.2.1 Materials....88
4.2.2 Methods....88
4.2.2.1 **Gluten Dough Preparation**....88
4.2.2.2 **Acidic Doughs**....90
4.2.2.3 **Salty Doughs with and without Addition of Acid**...91
4.2.3 Density measurements....93
4.2.4 Low-intensity ultrasound measurements....94
4.2.5 Ultrasound parameters for gluten doughs....95
4.2.6 Statistical analysis....101
4.3 RESULTS....102
4.3.1 Effects of acids and salts on gluten dough development....102
4.3.2 Effects of acids and salts on gluten physicochemistry....104
4.3.3 Effects of acids and salts on the ultrasonic properties of gluten doughs....105
4.4 DISCUSSION OF RESULTS....111
4.5 CONCLUSIONS....117
5. MANUSCRIPT III. **^{1}H NMR Spectroscopy of Gluten Proteins Under Acidic Conditions**....119
ABSTRACT....119
5.1 INTRODUCTION....121
5.2 MATERIALS AND METHODS....125

5.2.1 Materials 125
5.2.2 Methods 125
5.2.2.1 **Gluten Dough Preparation** 125
5.2.2.2 **pH Measurements and Mixing of the Doughs** 126
5.2.2.3 **^{1}H NMR Measurements** 127
5.2.3 ^{1}H NMR determinations 128
5.3 RESULTS 131
5.3.1 ^{1}H NMR spectra for gluten doughs 131
5.3.2 2-D Diffusion (D) / spin-spin (T_2) relaxation correlation of gluten doughs 139
5.3.3 1-D spin-spin (T_2) relaxation analysis for gluten doughs 143
5.4 DISCUSSION OF RESULTS 146
5.5 CONCLUSIONS 151
6. GENERAL DISCUSSION AND CONCLUSIONS 152
LITERATURE CITED 158

ACKNOWLEDGEMENTS

Special thanks to the following for their support during my masters programme:

- To God.
- *Gracias madre por ser un ejemplo a seguir, por tu inmenso amor, tu apoyo incondicional y tu integridad.*
- To México and Canada.
- To my family and ALL my friends.
- To Dr. Martin Scanlon for his patience, help, constant encouragement, and guidance as well as for always having an answer, attributes that made him an excellent advisor.
- To Prof. Paul Callaghan for his time, kindness, guidance and for sharing with me his love of science.
- To Dr. Penny Hubbard for working with me side by side and sharing her knowledge and friendship.
- To Dr. John Page for his time, kindness, guidance and encouragement.
- To Dr. Sapirstein for his advice, corrections and guidance, as well as for allowing me to use his library anytime I needed it.
- To the University of Manitoba and specially to the people at the Department of Food Science, mainly to Dr. Arntfield and Yvonne Halden; to Hussein Elmehdi for his guidance and sharing with me his expertise in ultrasound matters; to the Ultrasound team in the Department of Physics and Astronomy; and to Hsing Chang from the Department of Mathematics.
- To the MacDiarmid Institute *for Advanced Materials and Nanotechnology* at the Victoria University of Wellington in lovely New Zealand for kindly hosting and guiding me as teachers.
- The institutions listed below are gratefully acknowledged for their financial support:
    - Alberto and Dolores Andrade Private Fellowship Foundation.
    - University of Manitoba Graduate Fellowship.
    - William B. Malchy Graduate Fellowship.
    - University of Manitoba One-Year Tuition Fee Waiver for Visa Students
    - NSERC (Natural Sciences and Engineering Research Council of Canada).
    - ARDI (Agricultural Research and Development Initiative of the Province of Manitoba).

"Solamente hay un éxito: disfrutar la vida" (Relgha)

LIST OF TABLES

Table 1. Common uses of gluten in addition to the baking and food filming industry.......... 10

Table 2. Amino acid composition of gliadin, glutenin and gluten (mol/10^5 g of protein).......... 13

Table 3. Studies done on gluten doughs by oscillatory tests.......... 27

Table 4. Acid solutions used for bringing down the pH of gluten dough.......... 51

Table 5. Optimal Mixing Time (OMT) of gluten dough samples.......... 52

Table 6. Effect of mixing time on gluten dough pH made with acetic acid.......... 57

Table 7. Effect of mixing time on gluten dough pH made with hydrochloric acid.......... 58

Table 8. Mixing times of gluten doughs when acid was added to the formula.......... 60

Table 9. Viscoelastic parameters of acid gluten doughs mixed to a fixed time.......... 62

Table 10. Viscoelastic parameters of optimally mixed acidified gluten doughs.......... 64

Table 11. Viscoelastic parameters of acidified gluten doughs optimally mixed with the GRL200 mixer under atmospheric conditions.......... 65

Table 12. Viscoelastic parameters of acidified gluten doughs optimally mixed with GRL200 (vacuum conditions).......... 66

Table 13. Effect of type of acid on the mechanical properties of optimally developed gluten doughs with a pH of 4.3.......... 67

Table 14. Hydrogen ions (H^+) sorbed into the gluten matrix during mixing.......... 68

Table 15. Side-chain charging of amino acids.......... 72

Table 16. Acid solutions used for bringing down the pH of gluten dough.......... 91

Table 17. Concentration of chloride salts used for gluten dough preparation.......... 92

Table 18. A typical example for comparing the three methods when obtaining the ultrasonic velocity and attenuation coefficient of the gluten dough.......... 100

Table 19. Effect of salts on mixing time of gluten doughs.....103

Table 20. Density of acetic acid gluten doughs.....104

Table 21. Density of hydrochloric acid gluten doughs.....104

Table 22. pH and density of salty gluten doughs, with and without addition of acetic acid.....105

Table 23. ^{1}H NMR chemical shift (ppm)[a] for wheat gluten doughs (average standard deviation in chemical shift = ±0.04).....134

Table 24. ^{1}H NMR chemical shift (ppm)[a] for Canadian wheat gluten dough (average standard deviation in chemical shift = ±0.04).....137

Table 25. ^{1}H NMR chemical shift (ppm)[a] for Australian wheat gluten dough (average standard deviation in chemical shift = ±0.04).....138

Table 26. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs.* spin-spin (T_2) relaxation time for wheat gluten doughs in the region from 3 to 6 ppm of the ^{1}H NMR spectra.....139

Table 27. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs.* spin-spin (T_2) relaxation time for wheat gluten doughs in the region from 0.6 to 1.6 ppm of the ^{1}H NMR spectra.....140

Table 28. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs.* spin-spin (T_2) relaxation time for Canadian gluten doughs in the region from 0.6 to 1.6 ppm of the ^{1}H NMR spectra.....142

Table 29. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs.* spin-spin (T_2) relaxation time for Australian gluten doughs in the region from 0.6 to 1.6 ppm of the ^{1}H NMR spectra.....143

Table 30. Presence/absence of spin-spin (T_2) relaxation time components for Canadian gluten doughs as the result of acid addition.....145

Table 31. Presence/absence of spin-spin (T_2) relaxation time components for Australian gluten doughs as the result of acid addition.....145

Table 32. Possible assignments of ^{1}H NMR chemical shifts for gluten proteins.....146

Table 1a. Concentration of hydronium ions (H_3O^+) coming from acetic and hydrochloric acid.....179

Table 2a. Hydronium ions (H_3O^+) sorbed into the gluten matrix during mixing.....181

LIST OF FIGURES

Figure 1. Martin (or dough) process for obtaining gluten (Adaptation from Wadhawan, 1988)....8

Figure 2. Depiction of equilibrium magnetisation moment, M_z (Knowles *et al.*, 1976)....33

Figure 3. The RF coil placed around the *x* axis (Hornak, 2003)....34

Figure 4. Production of the magnetisation moment, M_{xy} (Knowles *et al.*, 1976)....35

Figure 5. Description of the pulsed NMR experiment (Bovey *et al.*, 1989)....36

Figure 6. The spin echo experiment (Bovey *et al.*, 1989)....38

Figure 7. Gradient magnetic field (Hornak, 2003)....39

Figure 8. Diffusion of the molecules as measured by PGSE sequence, as the intensity of the gradient increases, the echo intensity decreases as the result of molecular diffusion. ^{13}C-PFG NMR spectra of $^{13}CCl_4$. (Price, 1997)....42

Figure 9. Effect of acetic and hydrochloric acid on the mixograms of gluten dough with a pH of 4.3....59

Figure 10. Stress-sweep plots of control and acidified gluten doughs....63

Figure 11. Molecular interpretation of gluten development during mixing (Létang *et al.*, 1999)....69

Figure 12. Possible effects of pH on wheat gluten proteins (Adaptation from Galal *et al.*, 1978)....72

Figure 13. Hydrogen ions (H^+) included in the gluten matrix during mixing (Adapted from Belton, 1999)....73

Figure 14. Assembly of gluten sample in between glass plates for ultrasound measurements, side view....90

Figure 15. Block diagram of experimental set-up of the ultrasound equipment. PG is the generated ultrasonic pulse to be sent through the sample, PR is the received pulse coming from the sample....95

Figure16. Typical ultrasonic waveform after passing through the gluten dough....96

Figure 17. Typical results for the thickness dependence of the transit time, from which the ultrasonic velocity was determined........................98

Figure 18. Typical results for the thickness dependence of the transmitted amplitude, from which the attenuation coefficient of gluten dough was obtained..100

Figure 19. Effect of type and amount of acid on ultrasonic velocity through gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all dough pH values obtained with the same acid)..106

Figure 20. Effect of type of and amount of acid on the attenuation coefficient (alpha) of gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all dough pH values obtained with the same acid)..107

Figure 21. Effect of type of salt on ultrasonic velocity through gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all types of salt with or without acetic acid)............108

Figure 22. Effect of salt type on the attenuation coefficient (alpha) of gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all types of salt with or without acetic acid)............108

Figure 23. Effect of type of acid and pH on the loss longitudinal modulus (beta") of gluten dough..109

Figure 24. Effect of salt type with and without addition of acetic acid on the loss longitudinal modulus (beta") of gluten dough.................110

Figure 25. Positioning of the gluten sample within the superconducting magnet (Adaptation from Godefroy *et al.*, 2003)..........................127

Figure 26. Essential components of PGSE NMR diffusion experiments (a). Schematic representation of a CPGM sequence followed (and preceded) by the PGSE sequence used for these experiments (b) (Adapted from Antalek, 2002 & Callaghan *et al.*, 2003[a]). See text for details of symbols...129

Figure 27. ^1H NMR spectra for Australian gluten hydrated to 125% with H_2O, where no gradient was applied (a) and where gradient was maximised (b). Note change in scale of intensity axis..........132

Figure 28. ^1H NMR spectra for Canadian gluten hydrated to 140% with D_2O, where no gradient was applied (a) and where gradient was maximised (b)..133

Figure 29. ^1H NMR spectra for Canadian gluten with no gradient (a) and maximum gradient (b), showing the effect of acetic and hydrochloric acid. Note change of scale of intensity axis............136

Figure 30. D *vs.* T_2 distribution maps for Australian gluten dough hydrated with water (a) and Canadian gluten dough hydrated with D_2O (b). Bar on right of graphs denotes intensity......................................141

LIST OF APPENDICES

APPENDIX 1. **Assessment of the amount of hydronium ions (H^+) incorporated in the gluten matrix during mixing**..................................180

GENERAL ABSTRACT

A comprehensive understanding of the properties of gluten proteins is of relevance due to our need to optimise their technological applications in either the food industry or in the packaging industry. Unique viscoelastic characteristics of the gluten are attributed to the molecular structure of its proteins. Gluten proteins are stabilised by covalent and non-covalent links (i.e., hydrogen bonds, electrostatic and hydrophobic interactions). These links are affected by processing conditions such as by the addition of acids and salts, or by the entrainment of oxygen or generation of carbon dioxide. In turn, the nature and the extent of these links affect the structure of the gluten proteins and alter its functional properties. However, a full understanding of the conformational changes in gluten proteins as affected by processing conditions is still not accomplished. The effects of acids and salts were assessed in this study by changes in the rheological properties of the gluten dough. Two acids were used: acetic and hydrochloric, to drop the pH of the gluten dough in five decrements from 6.3 to 4.3. Experiments analysing the effects of salts were conducted by adding sodium, magnesium or aluminium chloride at equivalent ionic strength (different concentrations). The effect of chloride salts in combination with acetic acid was also analysed. Rheological properties of the gluten dough were assessed by fundamental rheological techniques -oscillatory shear test at low-strain and low-intensity ultrasound. G' and tan δ were the parameters analysed when studying the effect of treatment on the shear

rheology of acidic gluten doughs by small-stress oscillatory shear tests (from 0.5 to 10 Pa). Analysis of low-intensity ultrasound signals (generated by transducers at a nominal frequency of 50 kHz) was performed on the acidic and salty doughs. Ultrasonic parameters that were analysed included ultrasonic velocity, attenuation coefficient, longitudinal modulus and loss longitudinal modulus. Pulsed-gradient-spin-echo ^{1}H NMR was used for identifying at a molecular level the conformational changes in Australian and Canadian gluten doughs at a pH of 4.3 (pH adjusted with the addition of acetic and hydrochloric acid) in comparison to gluten doughs made up with distilled water. NMR parameters included the analysis of chemical shift, spin-spin (T_2) relaxation times and molecular diffusion coefficient. From the fundamental rheological analysis it was surmised that the addition of acids leads to a less elastic dough as the pH is lowered (decrease of G' values). Ultrasonic determinations highlighted the relevance of type of acid in affecting gluten structure, as seen by large differences in ultrasonic velocity. The effects of acids on gluten rheology were most evident at the lowest pH of the doughs (pH of 4.3). The effect of salts' addition was noticed when combined with acetic acid. Concentration of chloride ions and the type of cation seemed relevant in understanding the conformational changes induced in the gluten. From the NMR results the significance of glutamine residues in stabilising the structure of gluten was corroborated. The analysis of T_2 relaxation times showed that addition of acids increased the hydration of the gluten polymers and promoted the exposure of hydrophobic side-residues. Acids and salts affect the structure of gluten proteins by exerting electrostatic effects on non-covalent bonds (i.e., hydrogen

bonds and hydrophobic interactions), and glutamine residues play an important role in the maintenance of the structure. Further research on the effect of acids on gluten structure is recommended due to the importance of gluten protein to the food industry, the packaging industry and cereal science.

1. GENERAL INTRODUCTION

The relationship between chemical components of the wheat grain and their functionality is of relevance to cereal scientists (MacRitchie, 1999). Special attention has been put on wheat storage proteins (gluten proteins) due to their unique viscoelastic properties when hydrated (Popineau *et al.*, 1994). These interesting and technologically important rheological properties are in large part dictated by the molecular structure of the proteins. Gluten proteins have been mainly used by the bakery industry (Magnuson, 1985), although lately, the packaging and coating industry has found in gluten a potential material with numerous advantages (Cuq *et al.*, 1998; Redl *et al.*, 1999). Therefore, elucidation of gluten structure should help in the understanding of gluten functionality, and this would have wide applications in the processing industry, as well as in cereal science (MacRitchie, 1999).

Different processing conditions (e.g., pH and vacuum mixing) as well as additives (e.g., salts) have been shown to affect the structural properties of gluten and thus its functional properties (Baker & Mize, 1937; Preston, 1981; Eliasson & Larsson, 1993). Fermentation is one of the main process steps of the breakmaking process, and in this process the pH of the dough can be lowered to values of about 4-4.3 (in the case of sourdoughs) (Clarke *et al.*, 2002). When gluten films are created, the wet process is usually followed by a step where pH adjustment takes place. The response of gluten to changes in pH plays an important role in determining the functionality of the final product

(Gontard *et al.*, 1992). Salts are commonly added to flour doughs in the breadmaking process and to gluten during the film-forming process. Salts have been shown to improve the flavour and the loaf volume of breads, as well as to improve the functional properties of packaging materials (Eliasson & Larsson, 1993; Balla *et al.*, 1998). Therefore, gluten responses to changes in pH and addition of salts were studied in this thesis. Acetic and hydrochloric acid were added to adjust the pH of the gluten dough, while sodium, magnesium and aluminium chloride salts were used as ionic additives.

The structural changes found in gluten after acid addition have been mainly attributed to electrostatic repulsions between protein polymers as the result of an increase in positive charges along the gluten structure (Bennett & Ewart, 1962; Elmehdi *et al.*, 2003). These repulsions lead to breakage of hydrogen bonds and the exposure of hydrophobic amino acid side chains (Galal *et al.*, 1978). Salts in general, have an electrostatic effect on gluten proteins when added to flour doughs at low concentrations (< 0.05 M) (Von Hippel & Schleich, 1969; Preston, 1989). When salts are added at higher concentrations, the changes in gluten conformation are attributed to specific-ion interactions with the water in the dough (Kinsella & Hale, 1984; Butow *et al.*, 2002). The effects of both acids and salts on gluten properties have been related to changes in hydrogen bonding and hydrophobic interactions within the gluten polymers (Galal *et al.*, 1978; Preston, 1981; Butow *et al.*, 2002).

Fundamental rheological measurements have proved to be useful in the characterisation of gluten doughs. Furthermore, oscillatory shear tests at low strain provide rheological information about the sample without disturbing its

structure (Dreese *et al.*, 1988[b]; Janssen *et al.*, 1996[a]; Georgopoulos *et al.*, 2004). Low-amplitude ultrasounic waves can also as be used as non-destructive rheological probe with several advantages for characterising the properties of food materials (Samari, 1994; McClements, 1997). Flour doughs have been studied with ultrasound techniques, where the determination and interpretation of measurements of ultrasonic velocity and attenuation coefficient has helped in the characterisation of the sample (Létang *et al.*, 2001; Elmehdi *et al.*, 2003; Ross *et al.*, 2004; Elmehdi *et al.*, 2004). Therefore, these two rheological techniques were used in this thesis in order to measure at different rates of testing the properties of gluten doughs as affected by acids, salts or their combination.

Proton nuclear magnetic resonance (^1H NMR) represents a rich spectroscopy technique with the potential to provide a deeper analysis of gluten proteins so that structural information is obtained (Umbach *et al.*, 1992; Calucci *et al.*, 2003; Esselink *et al.*, 2003[a]). Moreover, pulsed gradient spin echo (PGSE) NMR provides information about the translational diffusion of the molecules that allows the correlation of the results with specific components of the sample (Antalek, 2002). Three NMR parameters that allow the characterisation and study of chemical components in a specimen are chemical shifts, spin-spin (T_2) relaxation times and diffusion coefficients (D) (Gil *et al.*, 1997; Godefroy & Callaghan, 2003). NMR studies in flour and gluten doughs have provided cereal scientists with a better understanding of gluten structure as well as knowledge of the interactions of gluten proteins with water, starch and lipids (Cherian & Chinachoti, 1996; Gil *et al.*, 1997; Callaghan & Gil, 1999;

Esselink *et al.*, 2003 [ab]). Therefore PGSE NMR experiments were performed in this thesis with the aim of directly probing the effects of acids on gluten structure.

In summary, then, the purpose of this thesis is to examine the effect of processing and ingredients on molecular structure, and determine the effect of these structural changes on the rheological properties of gluten doughs. The addition of acids, salts or their combination will lead to conformational changes in the gluten structure that can be assessed by ^{1}H NMR. These structural changes are reflected in the rheological properties of the gluten dough and thus on its functionality. Fundamental and empirical rheological determinations (notably by both small strain dynamic shear rheometry and low-intensity ultrasound) will then assess the effects of conformational changes in the gluten dough. The quantitative information provided by the thesis should be applicable in the cereals processing industry.

2. LITERATURE REVIEW

2.1 IMPORTANCE OF GLUTEN

2.1.1 Wheat production

Cereals, the name of which comes from *Ceres* –the ancient Greek goddess of food, constitute the primary food energy supply aming the human diet and hence cereals have great importance. For more than 24 centuries, cereal production has been humanity's most important agricultural activity (Serna, 2001). In 2003, the world production of cereals was about 2 billion Metric tonnes (Mt) of which, wheat occupied 3rd place (after maize and rice), contributing 27% of the total (FAO, 2004). World wheat production in 2003 was about 560 million Mt, with Canada contributing about 24 million Mt (4%) and thus occupying 7th place, preceded by China (which occupies 1st place in production), India, U.S.A., Russian Federation, France and Australia (FAO, 2004).

2.1.2 Wheat products

The main parts of a cereal kernel are: bran, endosperm and germ. From a nutritional point of view the wheat kernel is one of nature's most complete foods, although cereal proteins are not of excellent nutritional quality, since they are low in lysine and arginine. The bran comprises about 14% of the kernel, holding the fibre, some minerals, most of the vitamins and 19% of the total protein. The endosperm, which constitutes about 85% of the total weight of the

kernel, holds the starch, most of the proteins (about 70% of the total protein), and some vitamins. The germ, holds a large amount of lipids, some proteins (about 8% of the total kernel), and a few of the vitamins (Serna, 2001; Kent, 1983).

Wheat as a cereal is outstanding because it is the basic raw material for one of the most ancient, globally widespread and highly commercialised food products: bread. In 2002, wheat flour production in Canada was about 2 million Mt, and wheat flour consumption was about 53 kg/year *per* person (Minister of Industry, 2003). Wheat owes its functionality to its main components, and although each component contributes towards a good quality end-product, wheat's storage proteins, collectively known as gluten, seem to be the most important ones. In spite of the research that has been carried out for characterising these proteins for the last 90 years, a full understanding of their characteristics has not been completely elucidated. This lack of knowledge has been generally attributed to the complexity of the gluten composition and its physical properties (Lásztity, 1984; Eliasson & Larsson, 1993).

Wheat proteins can be classified as either non-storage or storage proteins, representing about 20 and 80% of the total protein content, respectively. The non-storage proteins, which are referred to as metabolically active proteins (e.g., enzymes), comprise the soluble proteins, albumins (soluble in water) and gobulins (soluble in salt solutions). The storage or gluten proteins, which have the purpose in the plant of supplying the growing seed with nitrogen, carbon and sulphur, comprise the gliadins (soluble in aqueous ethanol) and the

glutenins (soluble, or rather dispersed, in dilute acid or alkali) (Eliasson & Larsson, 1993).

2.1.3 Gluten manufacture

Gluten was first isolated by Beccari at the University of Bologna, Italy, in 1745 by washing and gently working a flour dough under a small stream of water. The residual dough after washing, was then essentially free of the starch and water solubles (proteins, lipids and carbohydrates) (Shewry *et al.*, 2001). By 1994, world-wide commercial production of wheat gluten from wheat was of the order of 300,000 Mt *per* year (Hoseney, 1994). Industrially, gluten can be made from either wheat or wheat flour; if starting from wheat, the kernel will go through a dry flour milling process. There are six different processes for obtaining gluten from wheat: the Martin or dough process, the batter process, the aqueous dispersion processes, the chemical dispersion processes, the far-mar-co wet process, and the non-aqueous separation of wheat starch and gluten process. The Martin process is the most popular one and consists of four basic steps: mixing flour and water into a dough, washing out the starch, drying the remaining gluten, and milling the gluten (Fig. 1) (Wadhawan, 1988).

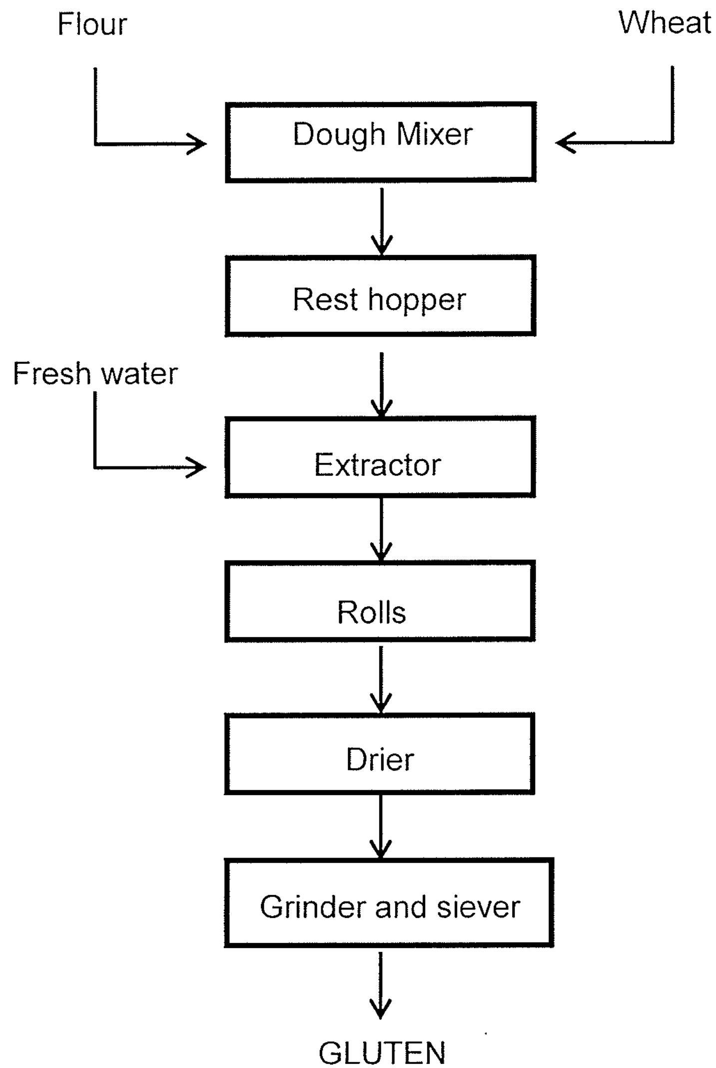


Figure 1. Martin (or dough) process for obtaining gluten (Adaptation from Wadhawan, 1988)

2.1.4 Uses of gluten

Gluten is an intrinsic component of the dough, as mentioned before, but it can also be considered as an additive when isolated. The largest use of wheat gluten is in bakery products as an ingredient for increasing the protein content and quality of the flours, strengthening the doughs and improving the products baked from frozen doughs (Magnuson, 1985; Eliasson & Larsson, 1993; Hoseney, 1994).

As for any other protein, gluten represents nowadays a great material for the coating and packaging industry since it is biodegradable, fairly low priced, renewable and edible. Gluten is characterised by its ability to create homogeneous, transparent, strong, and water-resistant films. Gluten may be used as a coating for different products or as an edible film, allowing the consumer or manufacturer to create the right texture and functional characteristics, regardless of the product for which it is mainly used. Uses include surface coatings on dough products and paper, sausage casings, nut coatings, encapsulation of additives, and windows in envelopes (Magnuson, 1985; Cuq *et al.*, 1998; Redl *et al.*, 1999). Other uses of gluten are included in Table 1 (Magnuson, 1985; Hoseney, 1994).

Table 1. Common uses of gluten in addition to the baking and food filming industry

Industry	Uses
Breakfast cereals	• Increase of protein content • Adhesive for other ingredients
Pet foods	• Increase of nutrient content • As a meat analogue • Improve texture (imitating meat texture)
Meat, fish and poultry	• As a binder and texturising agent • As a product analogue • As the basis for artificial products
Cheese	• As a replacement of sodium caseinate • As a product analogue

(From Magnuson, 1985; Hoseney, 1994)

2.1.5 Functionality of gluten

Wheat flour products owe their end-product quality mainly to the functional properties of the gluten proteins. Due to its functionality, gluten proteins make wheat the prime cereal for bread making. In the baking industry, the importance of the gluten proteins is related to their viscoelasticity and gas-holding capacity. Functionality of gluten relies on its physical and chemical properties (i.e., a good balance of gliadin and glutenin content, links or interaction between them, and interactions between the proteins and other dough components) (Wadhawan, 1988; Eliasson & Larsson, 1993; Gras *et al.*, 2001).

2.1.5.1 **Composition of Gluten.** As isolated from flour, gluten contains (on a dry basis) about 80% protein and about 8% lipids (both polar and non-polar bound to gluten during dough formation prior to gluten separation). The remainder in gluten composition is composed of ash and carbohydrates (the

latter consisting mainly of starch). Commercially produced gluten contains about 72 to almost 90% protein (Wadhawan, 1988; Eliasson & Larsson, 1993; Hoseney, 1994). The balance between gliadins and glutenins is related to baking performance (i.e., loaf volume) and the viscoelasticity of gluten doughs (Kasarda *et al.*, 1971; Eliasson, 1990; Eliasson & Larsson, 1993; Hoseney, 1994).

Gliadins (also called prolamins), with an average molecular weight of about 40,000, are single-chain proteins stabilised by hydrophobic interactions, disulphide bonds and hydrogen bonds. Gliadins are sometimes classified by their sulphur amino acids content into sulphur-rich prolamins (α, β and γ gliadins) and sulphur-poor prolamins (ω gliadins) (Eliasson & Larson, 1993; Lindsay & Skerritt, 1999). Gliadins have a higher hidrophobicity when compared to glutenins (Eliasson & Larson, 1993). Gliadins are extremely sticky when hydrated, have no or little resistance to extension, and seem to be the component that is mainly responsible for the dough's cohesiveness and viscous nature (Kasarda *et al.*, 1971; Huebner, 1977; Eliasson, 1990; Hoseney, 1994; Shewry & Halford, 2002).

Glutenins are divided into two subunits based on their molecular weigth, high molecular weight and low molecular weight glutenin subunits (HMG-GS and LMW-GS). Glutenins have a high content of glutamine (35 mol %) and a significant content of glycine (20 mol %) and proline (10 mol %). Glutenin subunits are able to form intra- and inter-chain disulphide bonds (Lindsay & Skerritt, 1999). Hydrated glutenins are rubbery but are prone to rupture and

seem to be the main component responsible for dough's resistance to extension and elasticity (Kasarda *et al.*, 1971; Eliasson, 1990; Eliasson & Larsson, 1993; Hoseney, 1994; Belton, 1999).

2.1.5.2 **Viscoelasticity of Gluten.** Gluten proteins are primarily responsible for the viscoelastic and cohesive properties of wheat flour doughs, which gives wheat flour dough its capability to retain gas during fermentation (Castell-Perez & Steffe, 1992; Eliasson & Larsson, 1993; Hussain *et al.*, 1998; Lindsay & Skerritt, 1999). It is generally thought that the main components responsible for dough elasticity are the glutenins, while the gliadins exhibit viscous behaviour. The correct balance between extensibility and elasticity of gluten will determine the end-use quality of the wheat flour (Veraverbeke & Delcour, 2002; Shewry *et al.*, 2002).

2.2 GLUTEN STRUCTURE

2.2.1 Gluten and its physicochemistry

2.2.1.1 **Amino Acid Composition.** Gluten has a high amount of proline, which accounts for about 14% of the total composition of amino acids. Practically all the acid groups (glutamic and aspartic acid) occur as their amides (glutamine and asparagine). Therefore, although it has been reported that glutamic acid accounts for about 35% of the total amino acid composition of gluten, it is fair to say that glutamine accounts for about 35% of the gluten composition. About 35% of the total amino acids have hydrophobic side-chains, therefore the amount of surface hydrophobicity is high. Sulphur-containing and basic amino acids are found in relatively low amounts (Table 2) (Wu & Dimler 1963[ab], Hoseney, 1994; Gil *et al.*, 1997; Belton, 1999).

Table 2. Amino acid composition of gliadin, glutenin and gluten (mol/10^5 g of protein)

Amino acid Side-group	Amino acid	Gliadin	Glutenin	Gluten
Acidic	Glutamic acid	317	278	290
	Aspartic acid	20	23	22
Basic	Lysine	5	12-13	9
	Arginine	15	20	20
	Histidine	14-15	13	15
	Tryptophan	5	8	6
Thiol	Cystine	5	5	7
	Methionine	12	12	12
Hydroxyl	Serine	38	50	40
	Threonine	18	26	21
Aliphatic	Alanine	25	34	30
	Valine	43	41	45
	Leucine	62	57	59
	Isoleucine	37	28	33
	Proline	148	114	137
	Phenylalanine	38	27	32
Ammonia		301	240	298

(Adaptation from Wu & Dimler, 1963[b])

2.2.1.2 **Physicochemical Characteristics.** The overall stability of the native structure of gluten, as with all other proteins, is the result of attractive and repulsive interactions between the protein polymers and the surrounding solvent. In general, conformational properties of gluten are determined by hydrogen bonds, hydrophobic interactions and disulphide bonds. The non-covalent interactions (i.e., hydrogen bonding, hydrophobic and electrostatic interactions) are highly dependent on the pH and ionic strength of the gluten (Pauling, 1945; Eliasson & Larsson, 1993; Hoseney, 1994). As found by Wu and Dimler (1963[ab]), the iso-ionic, or isoelectric, point was found to be 7.5 for gluten, 8.1 for gliadin and 7.1 for glutenin. Therefore, at a neutral pH, a positive net charge is expected for gluten, while at pH values above 7.5 a net negative charge will dominate. As the result of charged amino acids, there are more sites for the formation of salt bridges in the protein (Voet & Voet, 1990).

2.2.1.3 **Gluten Conformation Models.** As mentioned before, gluten is composed of water, lipids, carbohydrates and mainly proteins. Therefore a model describing gluten conformation should consider all of these components, their interaction within each other and their function. Although no complete models of gluten have been proposed due to its complexity and as-yet unknown characteristics, it has been described as a globular protein with a hydrophilic surface and a hydrophobic core (Eliasson, 1990). So far, models based on gluten behaviour (rheological responses) have been elucidated with the help of different techniques (i.e., empirical and fundamental rheology instruments, and microscopic techniques).

Galal and co-workers (1978) developed a model for gluten conformational changes based on enhancement (acid addition) or suppression (salt addition) of electrostatic repulsions between gluten polymers. In this model, the exposure of hydrophobic groups is proposed as the result of addition of acids. MacRitchie and Lafiandra (1997) suggested that polymers within gluten form entanglement points (small areas of the polymers interact with each other) and are separated by long regions that do not interact. Létang and co-workers (1999) proposed a development model for gluten proteins during dough mixing. At the beginning of the mixing, protein polymers are randomly orientated and thus the interactions between them are restricted. As mixing proceeds, protein polymers get aligned and thus interactions between them occur. As the result of overmixing, there is a breakage of disulphide bonds and thus the proteins get partially depolymerised. Belton (1999) proposed the loop and train model for explaining gluten viscoelasticity where the role of hydrogen bonding was highlighted as an important factor in the elastic behaviour of gluten. The basis of this model is the high level of glutamine residues within the proteins, which promotes the formation of intra- and inter-hydrogen bonds between gluten polymers when no hydration of the gluten takes place. As the result of hydration of the proteins, hydrogen bonds between the water molecules and the glutamine residues will occur and take the place of hydrogen bonding between glutamine residues. The higher the hydration level, the higher the amount of glutamine-water bonds. The sections of polymers where glutamine-glutamine bonds take place are called trains, whereas the sections of glutamine-water bonds are called loops. As the result of stress on the gluten polymers, the loops will be deformed (extended)

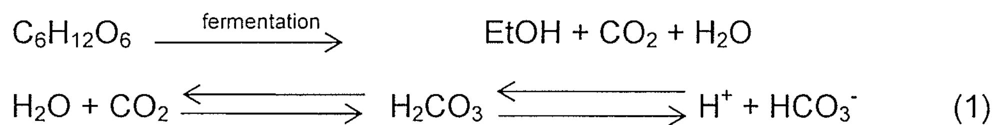
and further application of stress will lead to a flat conformation of the proteins that will allow polymers to slip over each other.

2.2.2 Effects of processing and ingredients on gluten structure

The main process to which wheat flour (and thus its constituent gluten proteins) is subjected to is the breadmaking process. Within this process, the gluten proteins are subjected to either mechanical forces or chemical reactions that will affect the conformation of the gluten polymers doughs and thus the rheological properties of the gluten dough. The highest level of stress is applied to the dough during mixing, making it deform beyond its rupture limit; dough deforms the least during fermentation, while subject to intermediate deformations during sheeting and molding; during the proofing and baking steps, the dough is subject to a range of deformations (Bushuk, 1990). A clear understanding of dough's rheological behaviour, the effect of each of the components, and the relationship between different factors, ingredients and responses would have great applications in the breadmaking industry, but such an understanding represents a big challenge for food scientists (Castell-Perez & Steffe, 1992; Mani *et al.*, 1992).

Moreover, in addition to the physical effects of the steps in the process, the conformation of the gluten proteins is also affected by the chemical components, either added or produced at each step. During mixing, salt is added to the formula as a common ingredient and air is incorporated into the dough (forming gas cells) (Hoseney, 1984). As fermentation starts, the

concentration of oxygen is constant. As fermentation takes place the yeast will consume the oxygen available and start producing carbon dioxide; the yeast will then produce the latter gas anaerobically. The carbon dioxide saturates first the aqueous phase of the dough, and then diffuses to the gas cells preformed during mixing. During fermentation, the amount of carbon dioxide increases and ends up saturating the whole dough system. When carbon dioxide interacts with water, carbonic acid is formed (Eq. 1). The bicarbonate ions diffuse to the gas cell nuclei where gaseous CO_2 is then formed from the bicarbonate ions. As a result the gas cells expand as fermentation proceeds (Elmehdi, 2001). When the dough is heated (baked in the oven) a number of changes take place as a result of the temperature changes, particularly to the gluten and starch polymers (Hoseney, 1984; Hoseney, 1992; Serna, 2001).



$$C_6H_{12}O_6 \xrightarrow{\text{fermentation}} EtOH + CO_2 + H_2O$$

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad (1)$$

2.2.2.1 **Presence of Air.** Wheat flour dough's ability to retain gas on expansion makes it so unique for bead production. The importance and role of gluten proteins on gas retention has been extensively studied (Hoseney, 1984; Gan *et al.*, 1995). Although air incorporation during mixing is desirable in order to preform the gas cells, it has been noticed that the presence of air (i.e., oxygen) affects gluten dough's development and thus the physical and mechanical properties of the dough (Baker & Mize, 1937; Smith & Mullen, 1970). When mixing under vacuum, doughs were denser and showed higher resistance to break-down as a result of overmixing (Baker & Mize, 1937).

In the packaging industry, gas-barrier properties are crucial characteristics of protein-based films. The control of gas exchange between the product and the environment is important for the preservation of the freshness of the food as well as for controlling oxidation reactions in the product. A parameter usually reported as important for the selection of a film is its oxygen and carbon dioxide permeability. Gluten has been reported as having higher oxygen permeability than other packaging materials (low-density polyethylene and polyamide 6) (Cuq *et al.*, 1998). Therefore, the study of gluten doughs when exposed to different types of gas (oxygen, carbon dioxide and nitrogen) could indicate the application of the gluten films in the packaging industry.

2.2.2.2 **Presence of Acids.** Wheat flour dough experiences significant lowering of its pH as the result of the fermentation process where carbonic acid is produced (Eq. 1). For the case of sourdoughs, where additional acidification occurs from organic acids (i.e., lactic and acetic acids), the pH can reach values of about 4 - 4.3 (Clarke *et al.*, 2002). The effect of pH on the functional properties of gluten doughs has been extensively studied by different researchers for over 40 years (Bennett & Ewart, 1962; Galal *et al.*, 1978; Gennadios *et al.*, 1993; Sekhon *et al.*, 1995; Shiau & Yeh, 2001; Takeda *et al.*, 2001; Larsson, 2002). In general, the resultant dough after acid addition is weaker, less resistant to mixing and less elastic.

Gluten proteins also experience a lowering in their pH during the wet process for film formation. In the wet process, often followed for obtaining gluten films, pH adjustment (by addition of acids and bases) and ionic strength control (by electrolyte addition) takes place. The response of the gluten proteins

to changes in pH will determine the functional characteristics of the final packaging product (Gontard *et al.*, 1992; Cuq *et al.*, 1998).

The high buffering capacity and low solubility characteristics of gluten are well known and are related to its chemical structure (Bull, 1951; Eliasson & Larsson, 1993; MacRitchie, 1987). The effect of pH on gluten structure is related to the isoelectric point of its constituent polymers. Moving pH away from the isoelectric point creates a net charge on the protein, producing electrostatic repulsions and leading to swelling of the gluten. Electrostatic repulsions will thus change the conformation of gluten and will be reflected in a change in its mechanical properties (Bull, 1951; Hoseney & Brown, 1983; Eliasson & Larsson, 1993).

2.2.2.3 **Presence of Salts.** Sodium chloride is a common additive in the breadmaking industry since it improves the flavour and loaf volume (by influencing gas retention) of the final product (Eliasson & Larsson, 1993). Due to the effect of salts on protein structure, different salts (anions and cations) have been added to gluten films in order to improve or modify the film's functional properties (Balla *et al.*, 1998). Hlynka (1962) suggested that salt reduces the amount of "bound" water and thus more "free" water will be available as the result of a modified gluten structure.

The effects of different salts on flour doughs depend mainly on the salt's concentration (Preston, 1981). At low salt concentrations (0.05-0.15 M), non-specific charge shielding of the protein's polar groups happens (electrostatic effect). Electrostatic effects are called direct interactions, since the ions interact directly with the protein chemical groups (amino acid side-residues, peptide

bond, amino and carboxyl group) neutralising their charge. These interactions depend primarily on the type of ion (cation or anion) and its valence (Von Hippel & Schleich, 1969; Balla *et al.*, 1998). At higher salt concentrations (>0.5 M) specific-ion interactions dominate according to the place the ions occupy in the Hofmeister or lyotropic series. Lyotropic effects are called indirect interactions, since the ions affect bonding (mainly hydrophobic interactions) within the protein by interacting with the solvent molecules (Von Hippel & Schleich, 1969; Kinsella & Hale, 1984; Butow *et al.*, 2002). Non-chaotropic ions induce order in the water structure and thus promote the formation of hydrophobic interactions between gluten polymers (Bull, 1951; Hatefi & Hanstein, 1969; Damodaran & Kinsella, 1982). In general, the addition of high concentrations of salt decreases water absorption, increases strength and mixing time for dough development (as assessed by the mixograph) (He *et al.*, 1992; Eliasson & Larsson, 1993).

Due to the important effects of different salts on the properties of wheat and gluten doughs, different studies have been performed where the influence of salts alone and/or in combination with acids has been studied (Tanaka *et al.*, 1967; Holmes & Hoseney, 1987; Kim & Bushuk, 1995; Sekhon *et al.*, 1995; Wehrle *et al.*, 1997). In general, the results showed a synergistic effect of the salts and acids so that stronger doughs with longer mixing times were obtained.

2.3 RHEOLOGY OF GLUTEN

Before the formal introduction of the term "rheology" by Eugene C. Bingham (*circa* 1928), the sense of rheology had been appreciated for centuries. The origins of rheological concepts, such as the "thickness" of a material, as well as the flow of liquids, go back into the Sumerian, Chinese, Egyptian, Indian, Greek and Jewish cultures. Confucius (551-479 BC) and Heraclitus (540-475 BC) thought and talked about flow in philosophical contexts (Tanner & Walters, 1998). Nowadays, rheology is the science devoted to the study of deformation and flow of matter (Steffe, 1996). Rheological properties of a system are expressed in terms of force, deformation and time or their derivatives (Mohsenin, 1986).

Stress is a measure of internal forces or force components of a material when external forces are applied, and it is independent of the size and shape of the specimen, as given by Eq. 2,

$$\text{stress} = F/A, \qquad (2)$$

where F is the force applied [N] and A is the area of the face of the specimen to which force is applied [m^2]. Strain is a measure of the deformation of the material, the displacement between its particles normalised according to the specimen's original size or shape (Eq. 3),

$$\text{strain} = \Delta l/L, \qquad (3)$$

where Δl is the change in length of the specimen [m] and L is the original length of the specimen [m]. When stress is applied to an elastic material it deforms;

when removed it recovers the original dimensions of the specimen (Menjivar, 1990; Mohsenin, 1986).

Viscosity of a material refers to its resistance to flow as indicated by the ratio of shear stress (stress applied tangential to the material to be tested) to shear rate (the velocity difference *per* unit thickness of the material) in the fluid. While the stress is applied, a viscous material will deform; after the cessation of the stress, there will be no elastic recovery of the deformation. The distinction between an ideal viscous fluid and an ideal elastic solid is that the stress is proportional to the rate of the strain, rather than to the strain (Mohsenin, 1986; Tung, date unknown).

The relevance of rheology lies in its applications for both industry and cereal science. Rheological studies allow researchers to probe molecular properties of doughs and films (conformation and interactions) and translate functional properties to a physical property (mechanical behaviour). Applications of rheology include studies of the raw materials (product formulation and handling), the process (its development, optimisation, and quality control), and end-product (assessments and predictions of the textural properties) (Rha & Pradipasena, 1986; Menjivar, 1990; Eliasson & Larsson, 1993; Cuq *et al.*, 1998; Dobraszczyk & Morgenstern, 2003).

On the basis of studies of the rheological response of wheat and gluten doughs, they are considered as non-linear viscoelastic materials (Faubion & Hoseney, 1990). The viscoelasticity of gluten arises from protein interactions between the protein molecules (hydrogen bonds and disulphide cross-links) (Bloksma, 1975). Dough rheology can be assessed by either empirical or

fundamental rheological instruments. Fundamental instruments differ from empirical ones in that the strain and stress are known and are well characterised (i.e., in fundamental units) in the whole of the test piece (Rha & Pradipasena, 1986; Eliasson & Larsson, 1993). Although there has been extensive work using both empirical and fundamental rheological studies of doughs, the definition of a constitutive model for dough still represents a major challenge (Bagley *et al.*, 1998).

2.3.1 Empirical rheology

In the cereal chemistry literature, empirical rheological instruments have been widely used for several decades. Empirical instruments for studying dough rheology include two mixers (i.e., mixograph and farinograph) and two load-extension instruments (i.e. extensigraph and alveograph). The mixograph and farinograph determine resistance to mixing, with the former instrument assessing optimal mixing time, and the latter one assessing optimal water absorption. The extensigraph and the alveograph determine dough's resistance to extension and flour strength; the former instrument subjects the dough to uni-axial extension, while the latter applies a biaxial extension (Weipert, 1992; Hoseney, 1994; MacRitchie, 1999; Serna, 2001). Although empirical instruments have the advantages of imitating the breadmaking process (being very useful for practical quality control), are relatively inexpensive, fast, and rugged, they have the limitation that they do not define the rheological properties of doughs in fundamental units (Bushuk, 1990; Menjivar, 1990).

The mixograph determines the resistance of a dough to mixing. Mixograph curves represent the torque (%) applied to the dough as a function of time (min) of the test. Mixograms show a maximum torque after a specific period of mixing (peak time), which indicates the time when the dough has the strongest properties and this can be used as an indicator of flour quality (i.e., its optimal development has been reached) (MacRitchie, 1987; Serna, 2001). Several mixograph studies have been conducted over the years with the aim of correlating different factors (type of cultivars, mixing time, water absorption, absence of oxygen, addition of chemicals, gluten components, mixing rate) to gluten quality (Weak *et al.*, 1977; Holmes & Hoseney, 1987; Khan *et al.*, 1989; Anderssen *et al.*, 1998; Hussain *et al.*, 1998; Nierle & Kersting, 1998).

2.3.2 Fundamental rheology

Fundamental rheology includes different types of tests, which are mentioned as follows (Cumming & Tung, 1975; Eliasson & Larsson, 1993; Gras *et al.*, 2001):

- Viscosity measurements that relate shear rate and shear stress.
- Creep measurements that consist in the sudden application of a stress, which is kept constant while measuring the resultant strain as a function of time.
- Stress-relaxation measurements consist in the application of a strain while measuring the resultant stress.

- Dynamic measurements, where a small amplitude stress or strain is applied in an oscillatory manner. A phase shift is measured for the specimen, a value of 0° is expected for purely elastic materials, and a phase shift of 90° for purely visocus ones.
- Surface rheological behaviour that characterises the bulk behaviour of materials.

Fundamental rheological studies have been done for over the last four decades and have many advantages over measurements made with empirical rheological instruments. However, empirical rheology is still more widely used compared to the former. The advantages of fundamental rheology assessments include: the results are independent of size and shape of the material being tested, small samples can be used, the applied stress (or strain) is controlled, and the results are obtained in absolute units, as well as being described by defined rheological parameters (stress, strain, strain rate, modulus or viscosity). The disadvantages of fundamental techniques include: high cost, the tests are time consuming and of limited use in industrial environments, the instruments require high technical skills to operate, and the results are difficult to interpret (Weipert, 1990; Weipert, 1992; Dobraszczyk & Morgenstern, 2003).

Commonly, rheological techniques are categorised according to the magnitude of the imposed strain into either low or high strain. Moreover, different instruments have been designed in order to analyse the material at low or high frequencies. In the low frequency range, tests can be performed in stress/strain controlled rheometers, while for measurements in the high

frequency range, wave propagation methods such as ultrasound can be used (Létang *et al.*, 1999).

2.3.2.1 **Oscillatory Tests.** Oscillatory shear tests at low strain (or amplitude) provide researchers with rheological information that can be related to the native structure of the material. The deformations are kept so small that the instruments stretch but do not disrupt bonds and entanglements within the sample being tested, thus providing information on the native undisturbed structure of the material (Gras *et al.*, 2001). The two parameters for characterising flour doughs that are obtained from oscillatory tests are the shear moduli, G' (elastic or storage modulus) and G" (viscous or loss modulus). G' is related to the elasticity of the material, while G" is related to its viscosity as given by Eqs. 4 & 5,

$$G' = (\sigma_0 / \gamma_0) \cos \delta, \tag{4}$$

where σ_0 is the stress amplitude [Pa], γ_0 is the strain amplitude, and δ is the loss angle,

$$G'' = (\sigma_0 / \gamma_0) \sin \delta = \omega\eta', \tag{5}$$

where ω is the oscillatory frequency [rad/s] and η' is the dynamic viscosity [Pa.s]. A function that involves both moduli is called tan δ as the ratio between G" and G' given by Eq. 6,

$$\tan \delta = G'' / G'. \tag{6}$$

Tan δ defines the relative importance of the viscous and elastic components of the strain energy within a material at a given frequency (Dreese *et al.*, 1988[c]; Weipert, 1990; Tung, date unknown).

A considerable literature exists regarding oscillatory tests on wheat flour doughs (Bohlin & Carlson, 1980; Edwards *et al.*, 1999; Zhao *et al.*, 1999; Larsson *et al.*, 2000; Autio *et al.*, 2001; Shiau & Yeh, 2001; Larsson, 2002). Furthermore, extensive rheological research has been done on isolated wheat gluten doughs (Table 3). Results showed that all the studied factors in Table 3 affect the rheological properties of the doughs.

Table 3. Studies done on gluten doughs by oscillatory tests

Aim of the study	Researchers
Effects of temperature	Attenburrow *et al.*, (1990), and Dreese *et al.* (1988[ac])
Importance of moisture content	Cumming & Tung (1975), Khatkar *et al.* (1995), and Georgopoulos *et al.* (2004)
Effect of processing ingredients	Dreese *et al.* (1988[b])
Importance of wheat variety	Khatkar *et al.* (1995); Janssen *et al.* (1996[b]); Uthayakumaran *et al.* (2002)
Effect of mixing time	Janssen *et al.* (1996[a])

As reported by Létang *et al.* (1999), gluten rheology is dependent on the frequency at which the experiment is performed. In the low-frequency range ($10^{-2} \leq f \leq 10^{2}$ Hz), dynamic tests can be conducted with a controlled stress/strain rheometer. These instruments directly measure stress (σ) and strain (γ), which vary sinusoidally with time (Létang *et al.*, 1999).

2.3.2.2 **Ultrasound.** Low-amplitude ultrasounic waves can be used for determining food composition, structure, flow rate, physical state, and molecular properties of even optically opaque materials. This way ultrasonic waves promote a rapid, precise, relatively inexpensive and non-destructive analytical technique (Blitz, 1963; Samari, 1994; McClements, 1997; Létang *et al.*, 2001). Due to its nondestructive nature, ultrasound has found wide application over

about the last 15 years in studies of foods mainly in on-line process analysis, even though it has not been used much to study flour or gluten doughs. The physical principle of ultrasound is the propagation of a high-frequency sound wave (20 kHz to over 100 MHz) through the material to be tested (Samari, 1994; McClements, 1997). Typically, ultrasound equipment consists of a pulser, a receiver, an amplifier, the transducer(s) and an oscilloscope (Samari, 1994).

Three main parameters can be obtained from ultrasonic measurements: the ultrasonic velocity, the attenuation coefficient, and the acoustic impedance (McClements, 1997; Verdier & Piau, 1997). The first two are the basis for most ultrasonic techniques when evaluating the material properties of food systems, and both are frequency dependent characteristics. Ultrasound velocity is a measurement of the distance travelled by the ultrasonic wave *per* unit time through the sample, and it is related to the wavelength and the frequency of the wave ($v=\lambda f$). The velocity of ultrasonic waves is dependent on the density and elastic properties of the materials and thus it can be correlated to the rheological properties of the specimen, as given by Eq. 7

$$v = (\beta/\rho)^{1/2}, \qquad (7)$$

where v is the longitudinal phase velocity of ultrasonic waves [m/s], β is the longitudinal modulus of the sample [Pa], and ρ is the density of the sample [kg/m^3] (Blitz, 1963; Samari, 1994; McClements, 1997; Elmehdi *et al.*, 2003). The attenuation coefficient indicates how rapidly the amplitude of a wave decreases as it travels through a material, as the result of either absorption of

the ultrasonic energy, or the scattering of the beam by heterogeneities in the sample. The attenuation coefficient is given by Eq. 8 as follows,

$$A(L) = A_0 \exp[- \alpha^* L/2)], \qquad (8)$$

where A(L) is the signal amplitude [Pa] at after the wave has traveled a distance L, A_0 is the signal amplitude at the edge of the sample [Pa], α is the attenuation coefficient (intensity defined) [m^{-1}], and L is the sample thickness [m] (Samari, 1994; McClements, 1997; Elmehdi *et al.*, 2003).

Ultrasound has either been used as a method for characterising gluten structure or the effects of different factors on wheat flour doughs, such as fermentation, temperature or mixing time (Létang *et al.*, 2001; Singh & MacRitchie, 2001). Both ultrasonic velocity and attenuation coefficient of the flour dough have been proved to be adequate for the characterisation of dough systems. Elmehdi and co-workers (2003) studied the effect of fermentation on flour doughs. They found that fermentation leads to structural changes of the protein and bigger gas bubbles that are observed by a decrease of the ultrasonic velocity and an increase of the attenuation coefficient. The effect of mixing on dough consistency and structure has played an important role for determining the functionality of the final product. Létang *et al.* (2001) and Ross *et al.* (2004) assessed the effects of mixing time on flour dough by ultrasound. Their work showed that differences in dough development lead to differences in ultrasonic velocity and attenuation coefficient, and thus ultrasound can be used for the prediction of dough development. The results were correlated to hydration states of the dough, where bound and free water determined the

values of the ultrasonic parameters. Elmehdi and co-workers (2004) used ultrasonic measurements to show the importance of air bubbles included during mixing to the rheology of the dough. Gluten quality has been assessed as well by ultrasound, and it was reported that gluten from stronger wheats gives high values for the ultrasonic velocity (Elmehdi & Kovacs, 2003).

2.4 SPECTROSCOPY OF GLUTEN

Spectroscopy is the study of the interaction of radiation with matter. Nuclear magnetic resonance (NMR) is considered by some the richest spectroscopy technique (Hornak, 2003), and thus it was used for probing gluten molecular structure in this thesis.

2.4.1 Basic concepts of NMR

2.4.1.1 **Spin and Magnetic Moment.** NMR is based on examining the properties of nuclear spin. Nuclear spin is a fundamental property of the atomic particles in a nucleus, i.e., the neutrons and the protons. The spin behaves as a magnetic vector, having direction and magnitude (the nuclear spin values of hydrogen and deuterium are equal to ½ and 1, respectively). When the nuclear spin value is different from zero, the nucleus of that atom is magnetically active (i.e., it acts as a magnet when exposed to a magnetic field) (Hornak, 2003).

When exposed to an external magnetic field of strength $\mathbf{B}_0$, the nuclear spins align themselves into one of two energy levels along the direction of $\mathbf{B}_0$; an upper, or higher, state of the spins will result in an antiparallel alignment of the nuclear magnetic moment of the nuclei to the direction of $\mathbf{B}_0$, and a lower energy state will result in a parallel alignment to $\mathbf{B}_0$. The difference between the population of spins in the parallel and antiparallel configurations corresponds to a bulk or net equilibrium magnetisation vector of nuclear spins, $\mathbf{M}_0$. As a result

of the magnetic field $\mathbf{B}_0$, M_0 will precess about B_0 at a frequency called the Larmor or resonant frequency given by Eq. 9

$$\nu = \gamma \mathbf{B}_0 / 2\pi \quad \text{or} \quad \omega_0 = \gamma \mathbf{B}_0, \tag{9}$$

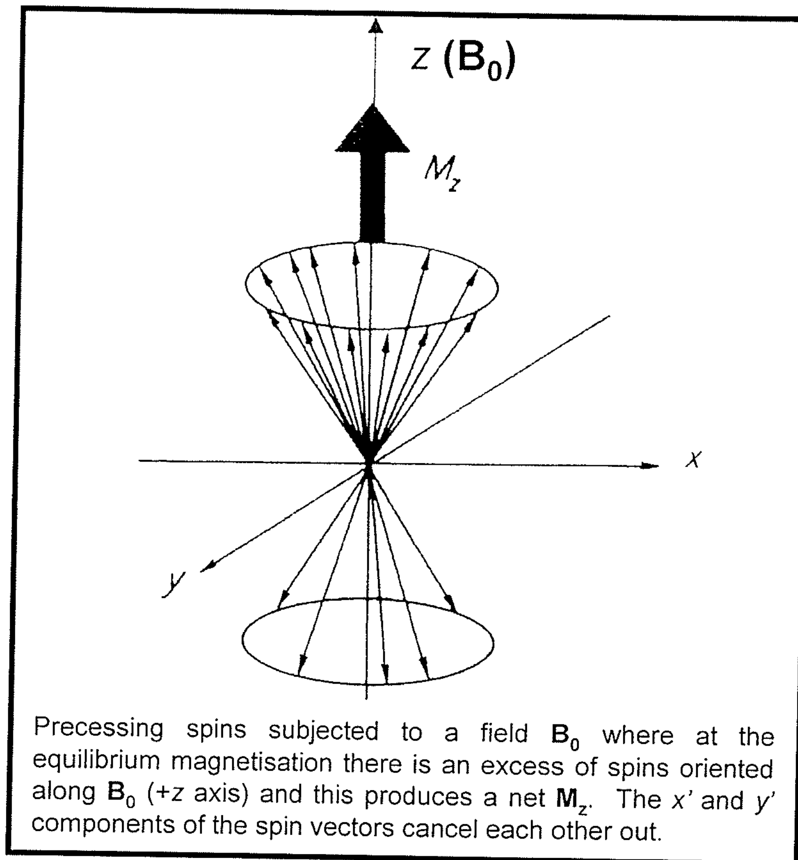
where ν is the resonant or Larmor frequency [Hz], ω is the resonant or Larmor frequency [rad/s], γ is the gyromagnetic ratio [rad/Ts], and $\mathbf{B}_0$ is the strength of the external magnetic field [T] ($\nu_{hydrogen} = 42.58 \times 10^6$ Hz ; $\nu_{deuterium} = 6.54 \times 10^6$ Hz) (Campbell & Dwek, 1984; Sanders & Hunter, 1987).

Generally, the field $\mathbf{B}_0$ is designated as acting along the *z* axis and therefore this axis is called the equilibrium axis. Due to the precession of the spins about $\mathbf{B}_0$, the components of the vectors of the spins acting in the *x* and *y* directions (*x*' and *y*' in rotating frame axes) are randomly aligned in this plane. As a result of the spins cancelling each other out, there is no net magnetic resultant $\mathbf{M}_{xy}$. However, there is a net resultant magnetic moment $\mathbf{M}_z$ since the majority of the spins are aligned along the +*z* direction and do not cancel out completely (Fig. 2) (Knowles *et al.*, 1976).

To obtain a transition between the energy states of nuclear spins, an energy amount that matches the difference between the two states has to be applied. This energy is obtained by the application of a pulse of electromagnetic radiation at the resonant frequency (ν), which is related to energy (E) through Planck's constant ($h = 6.62 \times 10^{-34}$ Js) as in Eq. 10,

$$\Delta E = h\nu \tag{10}$$

In NMR experiments, the resonant frequency of the radiation is in the radio frequency range (RF) (Knowles *et al.*, 1976; Hornak, 2003).



Precessing spins subjected to a field $\mathbf{B}_0$ where at the equilibrium magnetisation there is an excess of spins oriented along $\mathbf{B}_0$ (+*z* axis) and this produces a net $\mathbf{M}_z$. The *x'* and *y'* components of the spin vectors cancel each other out.

Figure 2. Depiction of equilibrium magnetisation moment, M_z (Knowles *et al.*, 1976)

In general, the RF pulse is applied perpendicularly (on the *x* axis) to the static magnetic field $\mathbf{B}_0$ for sufficient duration to induce the necessary net magnetisation in the *xy* plane (Fig. 3). The RF radiation generates another magnetic field, called the $\mathbf{B}_1$ field that is transverse to the equilibrium magnetisation (on the *y* axis). When the nuclear spins absorb energy from $\mathbf{B}_1$, the net nuclear magnetisation is rotated around $\mathbf{B}_1$ from the +*z* axis onto the +*y* axis (therefore onto the *xy* plane). As a result, net magnetisation on the *z* axis is diminished, and there is an increase of net magnetisation on the *xy* plane, $\mathbf{M}_{xy}$. The precessing $\mathbf{M}_{xy}$ induces an oscillating current in the RF coil (which is now

acting as a receiver), and it is this induced current, which (after suitable amplification and analysis) that is the NMR signal. The rotation of $\mathbf{M_0}$ thus occurs at an angle of 90° from the equilibrium axis, therefore it is said that a 90° or a $\pi/2$ pulse has been applied (Fig. 4) (Sanders & Hunter, 1987; Gladden & Alexander, 1996).

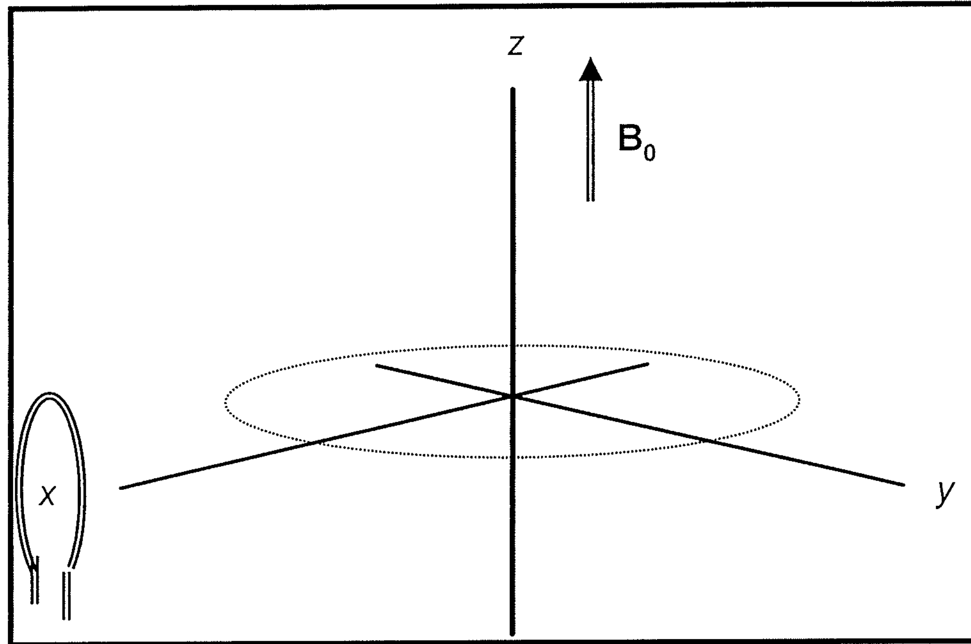
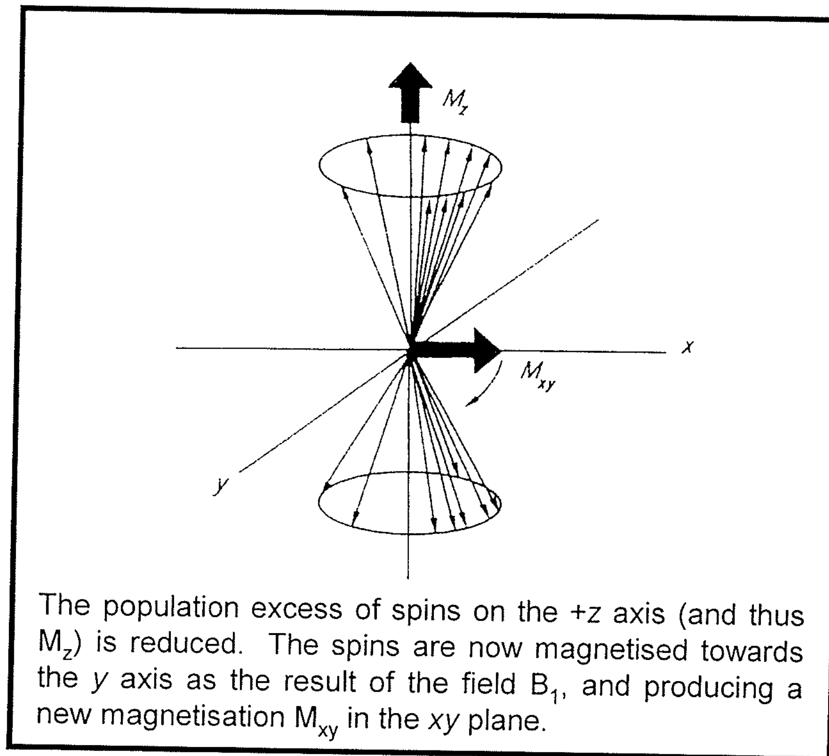


Figure 3. The RF coil placed around the *x* axis (Hornak, 2003)

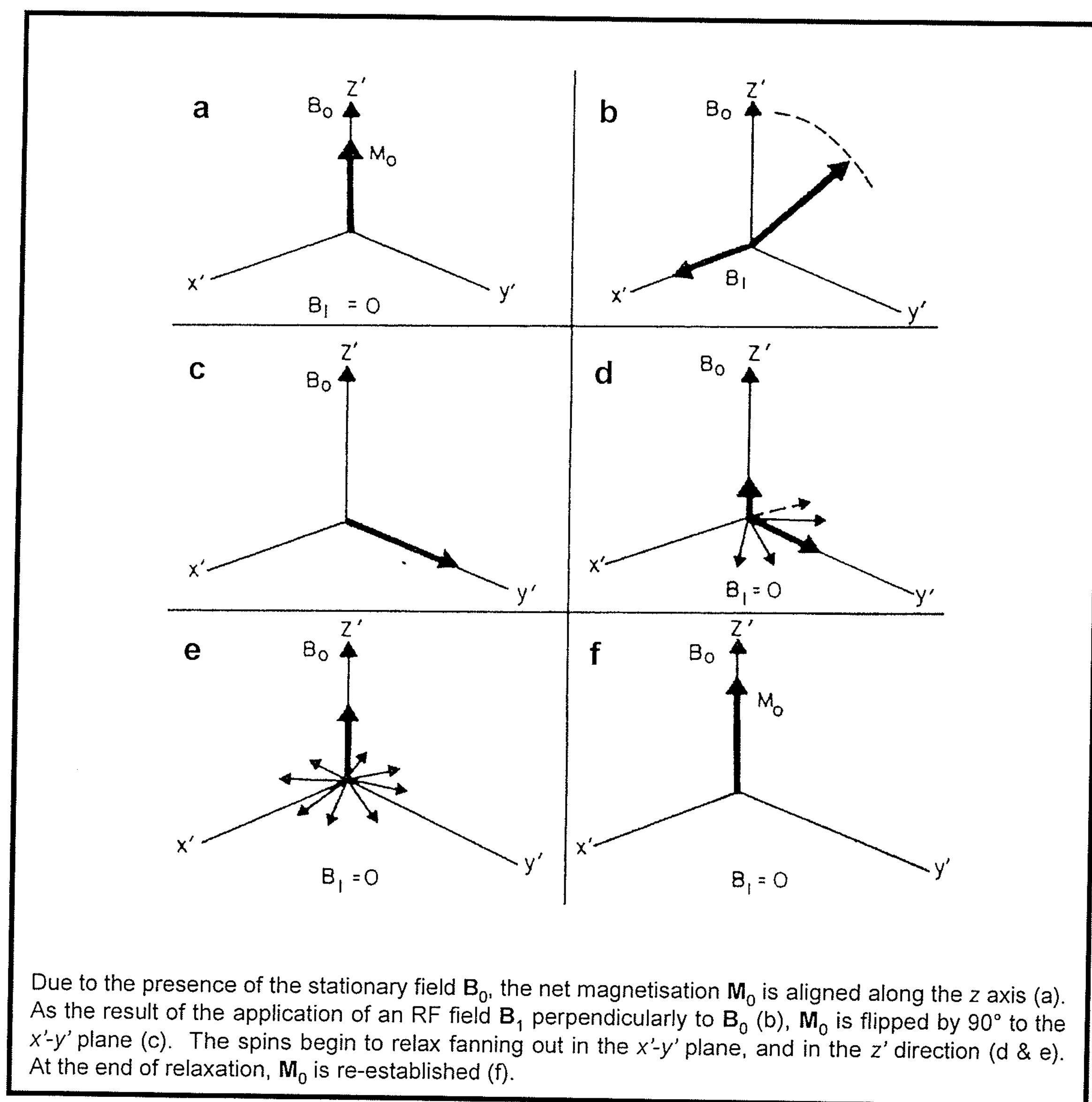


The population excess of spins on the +z axis (and thus M_z) is reduced. The spins are now magnetised towards the *y* axis as the result of the field B_1, and producing a new magnetisation M_{xy} in the *xy* plane.

Figure 4. Production of the magnetisation moment, M_{xy} (Knowles *et al.*, 1976)

2.4.1.2 **Relaxation Times (T_1 & T_2).** Following the cessation of the perturbation by the RF pulse, the $\mathbf{B}_1$ magnetisation ceases and the nuclear spins start to go back to their equilibrium state; this phenomenon is known as relaxation of the spins. The relaxation process takes time, and two parameters are associated with it: T_1 and T_2 relaxation times. T_1 is a measurement of the time for $\mathbf{M}_0$ to develop on the equilibrium axis, *z*. T_2 (spin-spin relaxation time) is defined as the time constant for the decay of the magnetisation moment $\mathbf{M}_{xy}$, as the result of fanning out of the spins. Individual spins will precess at slightly unequal rates over time after cessation of the 90° pulse, and so the induced magnetisation, $\mathbf{M}_{xy}$, will decay. During the time T_2, energy is transferred between spins (Fig. 5). Relaxation of spins will depend on the physical and

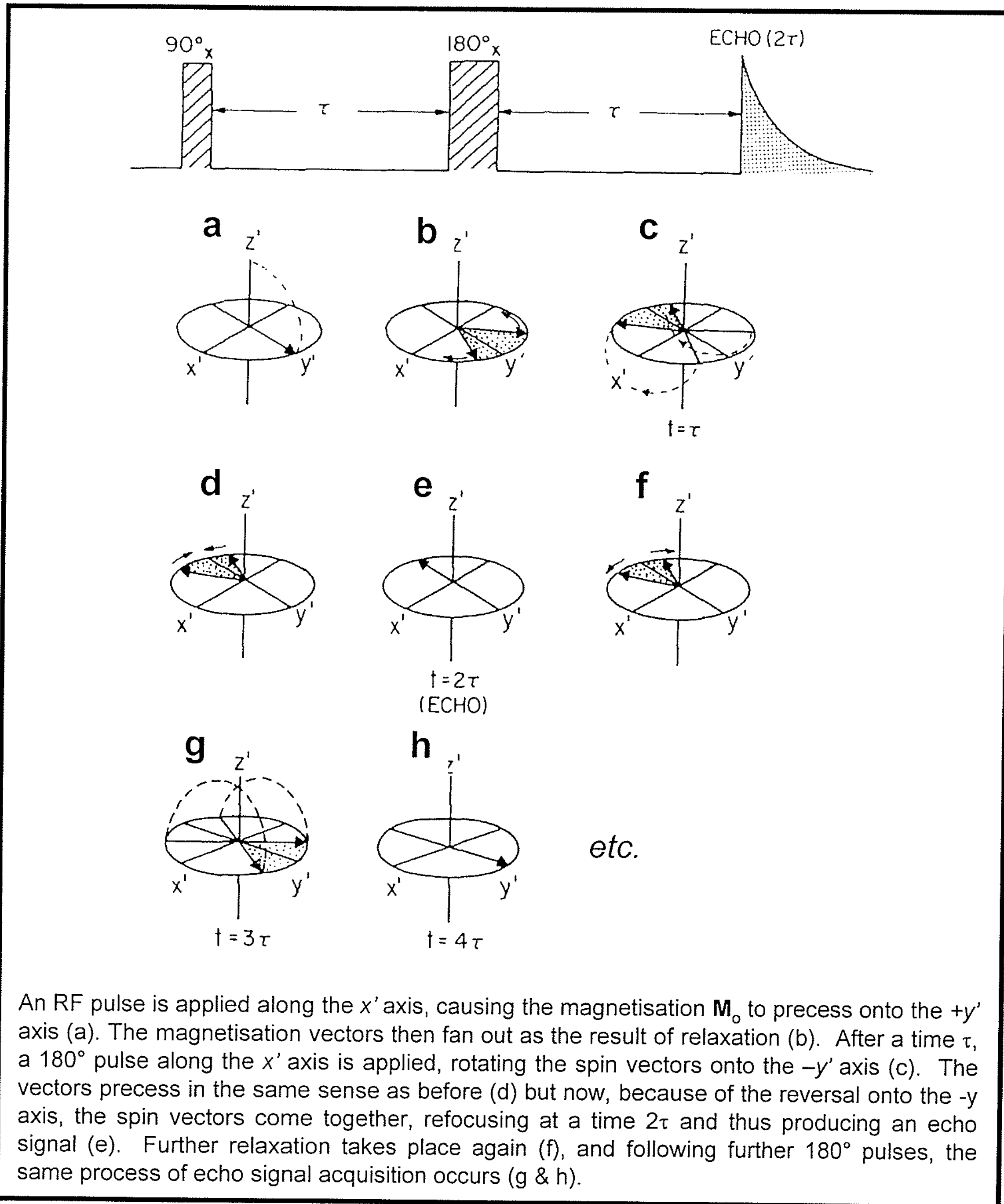
chemical environment of the nucleus under study and can be correlated to the population and rotational diffusion of the atoms in the sample (Callaghan & Gil, 1999; Gran, *et al.*, 1999; Godefroy & Callaghan, 2003; Roudaut *et al.*, 1998).



Due to the presence of the stationary field $\mathbf{B}_0$, the net magnetisation $\mathbf{M}_0$ is aligned along the *z* axis (a). As the result of the application of an RF field $\mathbf{B}_1$ perpendicularly to $\mathbf{B}_0$ (b), $\mathbf{M}_0$ is flipped by 90° to the *x'-y'* plane (c). The spins begin to relax fanning out in the *x'-y'* plane, and in the *z'* direction (d & e). At the end of relaxation, $\mathbf{M}_0$ is re-established (f).

Figure 5. Description of the pulsed NMR experiment (Bovey *et al.*, 1989)

2.4.1.3 **The Spin-Echo Sequence.** The spin-echo sequence is based on the spin-echo-sequence concept proposed by Hahn and modified by Carr and Purcell (Stejskal & Tanner, 1965; Antalek, 2002). The spin-echo-sequence consists of the application of a 90° pulse along the *x'* axis, followed by a 180° pulse also along the *x'* axis after a time interval, τ. As described before, the 90° pulse will flip the magnetisation moment, $\mathbf{M}_0$ onto the +*y'* axis. After the cessation of the RF pulse, the magnetisation vectors will start fanning out as the result of the spin-spin relaxation process. This dephasing of spins continues until a 180° pulse is applied, after a time τ. The 180° pulse rotates the magnetic vectors onto the –*y'* axis. The magnetisation vectors are still fanning out, but because of the 180° pulse, they now they start coming together instead of moving away from each other. Therefore, the magnetisation vectors will partially rephase, producing a so-called echo at a time 2τ. The amplitude of the signal and its decay after the application of further 180° pulses will be acquired as in the Carr-Purcell method (Fig. 6).



An RF pulse is applied along the *x'* axis, causing the magnetisation $\mathbf{M}_o$ to precess onto the +*y'* axis (a). The magnetisation vectors then fan out as the result of relaxation (b). After a time τ, a 180° pulse along the *x'* axis is applied, rotating the spin vectors onto the –*y'* axis (c). The vectors precess in the same sense as before (d) but now, because of the reversal onto the -y axis, the spin vectors come together, refocusing at a time 2τ and thus producing an echo signal (e). Further relaxation takes place again (f), and following further 180° pulses, the same process of echo signal acquisition occurs (g & h).

Figure 6. The spin echo experiment (Bovey *et al.*, 1989)

2.4.1.4 **Pulsed-Gradient-Spin-Echo Sequence (PGSE).** A major advantage of NMR spectroscopy is its ability to study the molecular self diffusion coefficient (D), i.e., the motion of molecules due to Brownian motion. Dynamic studies in NMR can be done when gradient coils are present in the instrument that enables researchers to apply the pulsed-gradient-spin-echo (PGSE) sequence.

The PGSE sequence is performed with the creation of a gradient magnetic field (**g**) of intensity G. The field **g** is created in the sample between two gradient coils that are placed around the equilibrium axis (i.e., the *z* axis). In the bottom coil, current flows in the opposite direction to that in the top coil. As a result the **g** field at the top end of the gradient will add to $\mathbf{B}_0$, and the **g** field at the opposite extreme of the gradient will subtract from $\mathbf{B}_0$ (Fig. 7) (Antalek, 2002; Hornak, 2003).

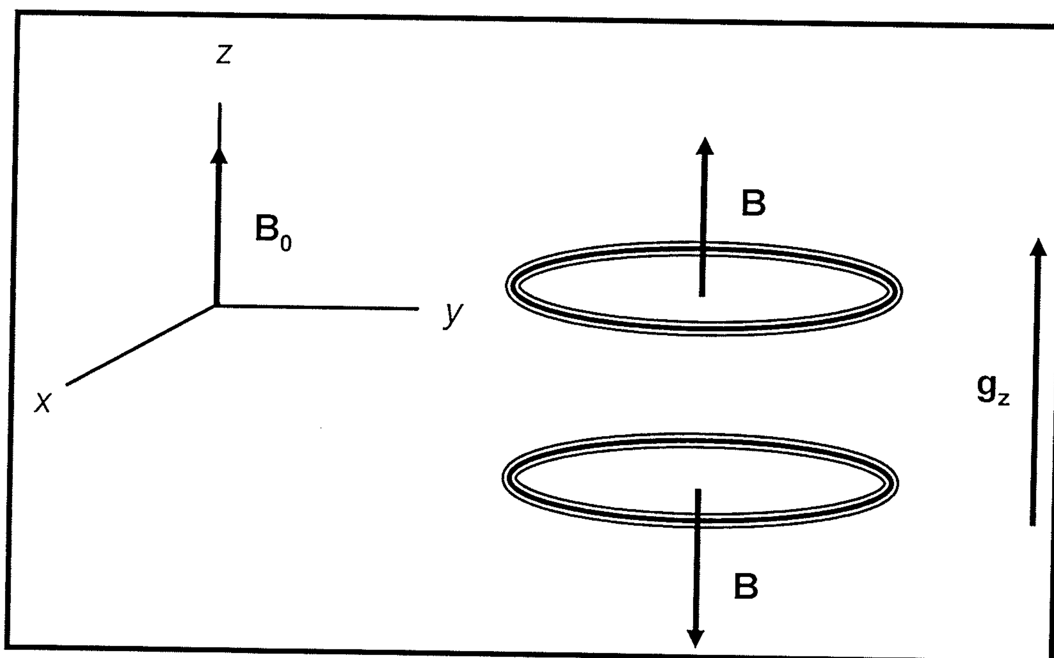


Figure 7. Gradient magnetic field (Hornak, 2003)

Diffusion measurements rely on the effect of the field **g** on the Larmor frequency (ω_z) of the nuclear spins with respect to its position along the *z* axis, as given by Eq. 11,

$$\omega_z = \gamma \mathbf{B}_0 + \gamma G_z z, \quad (11)$$

where ω_z is the Larmor frequency of the spins along the *z* axis [rad/s], γ is the gyromagnetic ratio [rad/Ts], $\mathbf{B}_0$ is the strength of the permanent magnetic field [T], G_z is the strength of the gradient field **g** [T/m], and *z* is the position of the nuclear spin of interest [m]. The PGSE sequence consists then on the application of gradient pulses with the same characteristics: field strength (G) and duration (δ). The gradient pulses are separated by a time (Δ) and placed symmetrically about the 180° pulses (Price, 1997; Antalek 2002).

When the first gradient pulse is applied at a time t_1 (i.e., right after the 90° RF pulse during the first τ period) individual nuclear spins (*i*) experience a spatially dependent phase angle shift (φ_z) given by Eq. 12,

$$\varphi_z = \delta \gamma G_z z, \quad (12)$$

where φ_z is the induced position-dependent phase angle of a given nuclear spin at a specific location (along the z direction) [rad], δ is the duration of the applied gradient pulse [s], γ is the gyromagnetic ratio [rad/Ts], G_z is the strength of the gradient field **g** [T/m], and *z* is the position of the given spin along the *z* direction [m]. An interpretation of this would be that the position of the spin within the sample is labelled or recorded indirectly by the adjustment of its Larmor frequency following the application of the first gradient pulse. The second gradient pulse is then applied at a time $t_{1+\Delta}$, producing again a phase angle shift

equal to that produced by the first gradient pulse. However, because this second gradient pulse occurs after the application of the 180° RF pulse, this new phase shift is subtracted from the old one. Because of the spatially dependent nature of the phase shift (Eq. 12), cancellation of the effect of the first gradient by the second gradient only occurs if the nuclei with their encoded spins have not diffused along the *z* axis (i.e., $\varphi_z=0$ for all those spins that have not diffused). However, if the nuclei have diffused away from their original position, the shift in the phase angle of the spins following the application of the second gradient will be proportional to the translational diffusion of the nucleus along the *z* axis in the time Δ. The change in the position of the spins due to diffusion of the molecules containing that nucleus will not allow the effect of the first gradient to be cancelled out by the second one. As the result, the acquired echo signal associated with diffusing molecules will lose intensity (i.e., it will be attenuated); the greater the displacement due to diffusion, the larger the attenuation of the echo signal. It is important to keep in mind that the attenuation of the signal due to diffusion and the attenuation due to relaxation of the spins are independent of each other (Stejskal & Tanner, 1963; Price, 1997; Antalek, 2002).

When the combination of the RF and gradient pulses are acquired, a "space" spectrum can be obtained. The resultant spectra will then show which nuclear spins have undergone diffusion as evidenced by substantial decay in the signal intensity at specific frequencies. For the first spectrum, where no gradient has been applied, the spectrum will show all the spins present in the sample; for

the spectrum at the maximum gradient, the spectrum will only show those spins that have not diffused at the end of the pulse sequence (Zhang & Hirasaki, 2003).

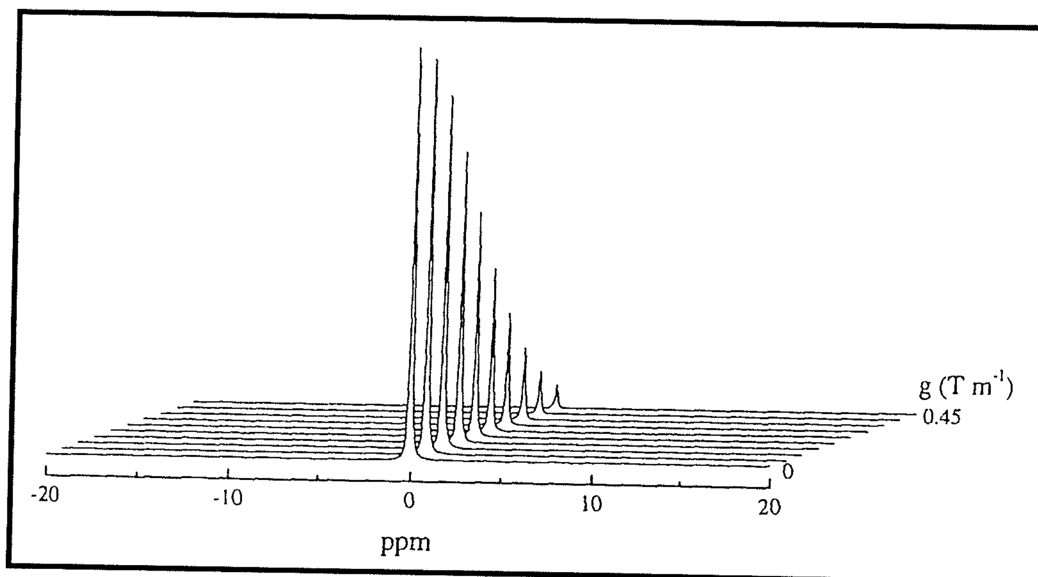


Figure 8. Diffusion of the molecules as measured by PGSE sequence, as the intensity of the gradient increases, the echo intensity decreases as the result of molecular diffusion. ^{13}C-PFG NMR spectra of $^{13}CCl_4$. (Price, 1997)

2.4.2 NMR studies of gluten proteins

NMR studies have been conducted on gluten proteins for about 15 years. In general, researchers have focussed their efforts on the elucidation of gluten structure as the result of imposition of different factors that are expected to affect gluten conformation. The factors that have mainly been studied are hydration, temperature and various steps of the breadmaking process.

Experiments performed on hydration of gluten proteins have identified gluten conformation that is consistent with the loop and train model proposed by Belton (1999). At low hydration values (under 35%), gluten proteins tend to form

intra- and inter-hydrogen bonds where the glutamine residues play an important role. At higher hydration values, the number of glutamine-glutamine bonds is reduced while more water-glutamine bonds are encountered (Umbach *et al.*, 1992; Belton *et al.*, 1995; Gil *et al.*, 1997; Callaghan & Gil, 1999; Gil *et al.*, 2002; Calucci *et al.*, 2003). Gil (1995) proposed a representative model for the hydration process showing the changes in the interpolymer links. Furthermore, high hydration leads to a more mobile (less rigid) gluten structure that is explained by polymer conformational changes (i.e., the formation of looser β-turns within the polymers). The importance of hydrophobic interactions and disulphide bonds for the structural stability of the gluten polymers was also highlighted in these NMR studies (Belton *et al.*, 1995; Gil *et al.*, 1997).

The effects of different processing conditions on gluten proteins as assessed by NMR have also been performed. After long kneading, the gluten polymers are broken and water is released; whereas molding leads to a rigid gluten structure with low mobility of the water (Esselink *et al.*, 2003[b]). Freezing has been shown to damage both flour and gluten doughs as a result of ice crystal formation; a model of water distribution during frozen storage was proposed to account for this (Esselink *et al.*, 2003[a]). This model accounted for three types of water in the gluten doughs: rigid water (tightly bound to the starch), bound water (bound to the proteins), and free water.

3. MANUSCRIPT I

Effect of mixing conditions and fermentation process products on the mechanical properties of wheat gluten assessed by rheological measurements

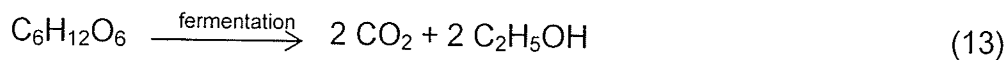
ABSTRACT

Gluten proteins provide wheat dough with unique viscoelastic characteristics, therefore they are considered as the key functional component of wheat. Gluten molecular structure and interaction of molecules within the protein matrix are of critical importance in determining dough properties and thus the quality of the final product. Variations in pH and oxygen content of the dough are two important processing conditions that affect gluten structure and bonding between components, and thus have an effect on the mechanical properties of dough. This part of my thesis studied the effect of acid conditions and dissolved oxygen on rheological properties -loss modulus (G') and tan δ- of a dough made from commercial vital wheat gluten. Acetic and hydrochloric acid solutions were used to drop the pH of the gluten in five decrements from 6.3 to 4.3. Gluten doughs at all pH values were mixed under atmospheric pressure. The dough with the lowest pH value (4.3) was mixed under atmospheric pressure and vacuum conditions (1 and 0.1 atm respectively) in order to manipulate the oxygen content in the dough. G' and tan δ of the gluten dough

were determined within the linear viscoelastic region using small stress oscillatory shear testing (from 0.5 to 10 Pa). Incorporation of acids had a significant effect on mixing and rheological parameters. Optimal mixing time (according to Mixograph results) dropped from 10.5 to 3.2 min as pH dropped when using acetic acid, and from 10.5 to 6 min using hydrochloric acid. Dough elasticity was strongly affected by the first drop in pH (from 6.3 to 5.8), where G' values were reduced from 1900 to 1500 Pa. Another significant decrease in gluten elasticity was observed at a pH value of 4.3 when acetic acid was used (1000 Pa from 1500 Pa at pH 4.8). No significant differences ($P \leq 0.01$) in tan δ (with an average value of 0.5) were observed due to changes in pH. Gluten at the lowest pH value (4.3) mixed under vacuum conditions was less elastic when hydrochloric acid was added compared to acetic acid (7300 *vs.* 7600 Pa). Tan δ, with a value of 0.58, was not affected by the type of acid when mixing under vacuum. The results suggested that the presence of acids and mixing conditions affected gluten structure. When pH changed, amino acid side-chains in the gluten polymer, such as histidine and glutamic acid, changed their electrostatic charge, increasing the net positive charge among protein polymers. Therefore electrostatic repulsions are thought to occur within the gluten matrix as pH drops, causing breakage of intermolecular hydrogen bonds and conformational changes of the gluten structure, making the gluten dough less elastic. Mixing under vacuum was thought to have an effect on hydrophobic interactions, and these were responsible for the change in the mechanical properties of the gluten dough.

3.1 INTRODUCTION

Wheat-based food products have played an important role throughout human history and nowadays are consumed world-widely. Bread is by far the most accepted fermented wheat product, therefore bread's quality, and factors that may affect it, have been extensively studied for the past 70 years. It has been proved that mixing conditions, such as: time, water absorption and presence/absence of air represent important factors in bread-making quality (Baker & Mize, 1937; Smith & Mullen, 1970; Bohlin & Carlson, 1980; Larsson & Eliasson, 1996; Campos *et al.*, 1997; Létang, *et al.*, 1999). As a fermented product, two main chemical reactions (Eqs. 13 & 14) occur in wheat dough that will affect, and in some cases determine, the characteristics of the final product (Miller *et al.*, 1994).



$$C_6H_{12}O_6 \xrightarrow{\text{fermentation}} 2\,CO_2 + 2\,C_2H_5OH \qquad (13)$$

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \qquad (14)$$

Carbon dioxide (from 1) dissolves in the aqueous dough phase forming carbonic acid (2) which dissociates into hydrogen and bicarbonate ions lowering the pH of the system.

Several studies (Shellenberg, 1971; Funt Bar-David & Lerchenthal, 1975; Wadhawan, 1988; Eliasson & Larsson, 1993; Gras *et al.*, 2001) have referred to wheat flour storage proteins, known collectively as gluten, as the primary agent for the superior viscoelastic characteristics of wheat flour doughs, and thus they are one of the most important components affecting bread quality performance.

Despite its importance, not much attention has been focused on the physicochemical and structural changes that wheat gluten doughs experience during the bread-making process (compared to wheat flour doughs). In addition, relatively little is known about the factors affecting the quality of commercial vital wheat gluten. This lack of knowledge is caused, in part, by the lack of adequate methods for evaluating gluten properties; most of the work has been conducted with empirical instruments, such as: the mixograph, farinograph, alveograph or the extensograph (Dreese *et al.*, 1988[c]; Weipert, 1992; Hoseney, 1994; MacRitchie, 1999; Serna, 2001). Even though these instruments have proved to be helpful for description of the physical characteristics of the dough, they have not been successful in elucidating the chemical and the physical changes that take place during the breadmaking process (Funt Bar-david & Lerchenthal, 1975). Another disadvantage of empirical instruments is their limitation in not defining the results in fundamental international system units (Bushuk, 1990; Menjivar, 1990). Therefore, the need for objective methods capable of a finer description of dough systems has arisen (Eliasson & Larsson, 1993).

Gras *et al.* (2001) reported insights into the physico-chemical changes of wheat gluten that take place during dough formation that can be obtained with fundamental rheological measurements. Furthermore, that study showed that oscillatory shear tests can provide information about dough systems, delicately-structured systems that would be otherwise destroyed in steady shear tests.

Over the years, some researchers have studied wheat gluten doughs as isolated systems and studied the effect of some mixing and fermentation factors such as temperature (Attenburrow *et al.*, 1990; Dreese *et al.*, 1988[ac]), moisture

content (Cumming & Tung, 1975; Khatkar *et al.*, 1995; Georgopoulos *et al.* 2004), oxidants (Dreese *et al.*, 1988[c]), mixing time (Janssen *et al.*, 1996[a]), and direct impact of acid incorporation (Clarke *et al.*, 2004) on the mechanical properties of wheat gluten doughs. Therefore, in this study, fundamental rheological properties were used for the description and understanding of wheat gluten doughs under different conditions that would mimic the pH changes occurring during fermentation. The main hypothesis that was investigated is as follows: a change in the structure of gluten would arise due to electrostatic repulsions as a result of acid addition, which would lead to a weaker, less elastic gluten structure.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Commercial wheat gluten (spring wheat premium quality) purchased from Premier Cereals, Inc. (Winnipeg, MB) was used. The water and protein composition (N x 5.7), as analysed by AACC methods (44-15A & 46-13, AACC 1983) were: 6.0 ± 0.1% and 70 ± 1.7%, respectively. Acetic and hydrochloric acid were purchased as reagent grade from Fisher Scientific (Nepean, ON). Distilled water was used for dilutions.

3.2.2 Methods

3.2.2.1 **Gluten Dough Preparation.** Commercial wheat gluten flour was rehydrated to two moisture contents on a flour weight basis as follows:

- To 210% moisture (10 g/21 mL), when mixed with the 10-g Mixograph.
- To 110% moisture (100 g/110 mL), when mixed with the GRL200 Mixer.

The difference in dough moisture between the mixers used was due to mechanical differences between the mixers. The 10-g mixograph is a more delicate instrument than the GRL200, so more water was needed for mixing the dough in order to make it weaker so as to avoid breakage of the mixer pins of the instrument.

The 10-g Mixograph (witn a spring setting of 10) was used for gluten dough mixing at atmospheric pressure (1 atm). The temperature was maintained at 25° C during mixing, with a water circulating system, and the speed was kept constant

at 88±2 rpm (Micromixer, TMCO, Lincoln, NE). Mixograh plots were analysed using the Mixsmart® Computerized Data Analysis program (V 3.4, TMCO, Lincoln, NE).

The GRL 200 Mixer (Muzeen & Blythe Ltd., Winnipeg, MB) was used for the vacuum mixing experiments (0.1 atm). Temperature was regulated with a water circulating system maintained between 10 and 14 °C in order to keep the temperature of the dough at 25 °C until the end of mixing.

The three main treatments which were used with this mixing process are described in the following three sections.

3.2.2.2 **Adjusting Gluten Dough pH.** To bring the pH of the dough down without yeast addition, two types of acid were used: acetic (HAc) and hydrochloric acid. In this way, the effect of two simple acids (one organic and one inorganic) on gluten structure was tested.

The pH was brought down from 6.3 to 4.3 in 5 steps, since 6.3 was the measured pH when gluten flour was mixed with water and approximately 4.3 is the lowest pH of wheat dough acquired after fermentation (Clarke *et al.*, 2002). The necessary concentrations of acids in the mixing water needed to decrease the pH of the dough were determined by trial and error pre-experiments (Table 4).

Table 4. Acid solutions used for bringing down the pH of gluten dough

Desired pH of dough	HAc Concentration (mM)	HCl Concentration (mM)
6.3 (control)	0	0
5.8	3.5	32.9
5.3	8.8	115.6
4.8	17.5	165.3
4.3	177.1	265.4

HAc Acetic acid
HCl Hydrochloric acid

For pH determinations of doughs, a Fisher Scientific pH Meter, model 915 (Pittsburgh, PE), with a silver/silver chloride pH electrode, was used in temperature compensation mode. The traditionally followed official method described by AACC (2-52, AACC 1983) was not followed since it specifically states that it is not applicable to dough. Instead, to determine the pH of the gluten dough, the electrode was inserted directly into the dough (Salovaara, 1982; Larsson, 2002). This method was later validated by comparing the pH readings with the results using a SENTRON Hot-Line probe (LanceFET; Roden, The Netherlands), which is designed for using on similar type of food products. Although the method used in this thesis is not officially accepted, it does give a direct reading of the concentration of free hydronium ions which interact with the pH electrode, and therefore it measures their free concentration within the hydrated gluten matrix in its native state. The pH of each mixed gluten dough was measured immediately after mixing by taking five pH readings on each dough.

3.2.2.3 **Mixing Times for the Development of Gluten Doughs.** When doughs were mixed based on full development of the gluten using the selected

treatment, the term optimal mixing time (OMT) was used; this meant mixing for 10% more in time past the time required to reach the maximum torque or peak (Table 5). Doughs were mixed at least in triplicate

A fixed mixing time (FMT) was used when mixing as well. The FMT for acetic and hydrochloric acid doughs (6.85 and 8.25 min, respectively) was obtained from the following formula,

$$FMT = (OMT_1 + OMT_2)/2, \qquad (15)$$

where OMT_1 is the OMT when the dough pH was 6.3 (this being the longest mixing time, 10.5 min), OMT_2 is the OMT when the pH of dough was 4.3 (this being the shortest mixing time for both acetic and hydrochloric acid, 3.2 and 6 min, respectively).

Table 5. Optimal Mixing Time (OMT) of gluten dough samples mixed with a 10g Mixograph

PH of dough	Type of acid	OMT (min)
6.3 (control)	-	10.5 ± 0.4
5.8	HAc	9.1 ± 0.4
	HCl	8.6 ± 0.4
5.3	HAc	9.0 ± 0.6
	HCl	7.8 ± 0.1
4.8	HAc	8.0 ± 0.6
	HCl	6.8 ± 0.3
4.3	HAc	3.2 ± 0.3
	HCl	6.0 ± 0.4

HAc Acetic acid
HCl Hydrochloric acid
OMT Optimal mixing time

3.2.2.4 **Mixing Gluten Dough Under Different Headspace Conditions.** When mixing under vacuum, it was strictly necessary to completely seal the mixing system, in order to keep the vacuum conditions as stable as possible, but due to limitations of the system, it was not possible to mix for longer than 6 min.

Thus, only the doughs with the lowest pH value (4.3) were mixed under vacuum conditions, since their OMT was the shortest in the pH series. The mixing times for each gluten dough were: 3.2 min for the one treated with acetic acid, and 6 min for the one treated with hydrochloric acid. The necessary concentrations of acids in the mixing water needed to decrease the pH of the dough were determined by trial and error pre-experiments

3.2.2.5 **Preparation of Gluten Dough Samples for Rheological Measurements.** Immediately after mixing, each gluten dough was removed from the mixing bowl and placed between Glutork2020 plates (Glutomatic System, Perten Instruments AB, Huddinge, Sweden) for 90 min at room temperature. Afterwards, the pressed gluten sheet was stored on a teflon coated sheet (Non-stick Good Cook™, Premium Bakeware Baking Pan) for 10 more minutes. The aim of the total resting time (100 min) was to allow dissipation of stored energy resulting from handling (Georgopoulos *et al.*, 2004). In order to prevent dough moisture loss, the Glutork2020 and the pressed gluten sheet were kept in a covered container (Tupperware) adjacent to trays of water. The Glutork2020 not only allowed the gluten to relax stresses from the mixing, but also shaped the dough into a thin sheet (average thickness of 3 mm). After resting, dough samples were cut from the sheet with a metal cork borer of 12 mm diameter, and the resultant disks were then placed on the rheometer. For each dough at least four gluten disks were cut for mechanical determinations on the rheometer; these disks are referred to as subsamples later on.

3.2.3 Dynamic rheological measurements at small deformations

Dynamic shear measurements were performed on an AR 1000 Rheometer (Thermal Analysis and Rheology, TA Instruments, V4.0.1, Waters Corporation, New Castle, DE). Data were analysed with the AR1000 software package (TA Data Analysis, V4.0.23, TA instruments, Ltd., 1994-2002). The geometry used was parallel plates of 12-mm diameter. A metallic solvent trap, surrounding and covering both the geometry and the sample, was used once the subsample was loaded in order to prevent dehydration of the gluten.

Since the thickness of the gluten subsamples varied across the sheet of dough, the rheometer was not set up based on the gap distance between the lower and upper plate but on a normal force basis. When the subsamples were placed on the lower plate of the rheometer, a normal force was registered by the AR1000 system due to the weight of the subsamples; this normal force was called the base line normal force (BLNF). Then the normal force of the system was reset to zero and the upper plate was brought down to a point where the measured normal force was 100% of the value of the BLNF. This application of stress prevented the subsample from slipping (as shown by pre-experimental work) and ensured that the subsample surface was touching both rheometer plates. Since the placement of subsample in between the rheometer plates would stress the gluten sample, 5 min were then allowed for the relaxation of the subsamples prior to testing.

From preliminary tests, the linear viscoelastic region for these doughs was found in the stress regime from 0.5 to 10 Pa; therefore, oscillation measurements

were performed within this stress regime at an angular frequency of 1 Hz. The temperature was kept constant throughout testing and in all the experimental runs at 20 °C (as set up in the instrument). Readings were performed as a stress sweep in a log mode with detection of 20 points/decade, so that 21 measuring points were recorded. The final results were based on using at least 11 of these points taken from the linear viscoelastic region as the result of discarding five of the initial and last points.

3.2.4 Rheological parameters reported

Two dynamic shear rheological parameters were determined for the various gluten disks. These were the dynamic shear storage modulus (G'), and the loss tangent (tan δ). The G' parameter represents the non-dissipative component of mechanical properties and tan δ is the ratio of G"/G', where G" represents the dissipation component of mechanical properties, or in other words, G" is the shear loss modulus (Dreese *et al.*, 1988[a]; Weipert, 1990).

3.2.5 Statistical analysis

Statistical analysis was conducted with the Statistical Analysis System (SAS system for windows V8, SAS Institute Inc., Cary, NC).

Data were evaluated by non-parametric analysis of variance (ANOVA) called the Kruskal-Wallis test (Hollander & Wolfe, 1973; Neter *et al.*, 1990). This method was used after consulting with the Statistical Advisory Service and Hsing

Chang, a Statistics graduate student from the Department of Statistics at the University of Manitoba. A criterion of $P \leq 0.01$ was followed to determine significant differences among treatments using Scheffe's multiple-comparison procedure.

The treatments analysed were:

- Effect of acid concentration on pH at OMT and FMT.
- Effect of pH on G' and tan δ of gluten dough at OMT and FMT.
- Effect of mixing time on pH, G' and tan δ, for the same acid and same concentration.
- Effect of type of acid on G' and tan δ for OMT and FMT and the same pH.
- Effect of pressure (1 and 0.1 atm) during mixing on pH, G' and tan δ, for the same acid and same concentration.

For analysing the pH data, the results were obtained from triplicate measurements for each treatment (concentration, type of acid, and OMT/FMT). For the analysis of mechanical properties the selected points in the linear viscoelastic region were analysed. This means that for each treatment a triplicate of doughs was prepared, and from each dough 4 disks were obtained (referred to as subsamples), and for each subsample at least 11 points calculated by the AR1000 software package (TA Data Analysis. V4.0.23. TA instruments. Ltd. 1994-2002) within the linear viscoelastic region were considered as raw data. The subsamples were tested in order to obtain more representative data from the doughs, since the pressing by the Glutork2020 plates caused a variable thickness in the subsamples' surface.

3.3 RESULTS

3.3.1 Effect of mixing time on the pH of gluten doughs

The pH values of the doughs were measured when the gluten was mixed to either an Optimal Mixing Time (OMT) or to a Fixed Mixing Time (FMT, defined previously in 3.2.2.3). Different values of dough pH were obtained with different mixing times, regardless of the type of acid (Tables 6 & 7). As a result of the use of a FMT, some doughs were overmixed (dough with highest addition of acetic acid and the three most acidic of the hydrochloric acid doughs), while some others were undermixed (control, most acetic acid doughs and dough with the lowest addition of hydrochloric acid).

Table 6. Effect of mixing time on gluten dough pH made with acetic acid

	Acid concentration (mM)	Mixing time (min)	Dough pH
Control	0	10.5	6.3 ± 0.03
		FMT	5.6 ± 0.05
HAc	3.5	9.1	5.9 ± 0.09
		FMT	5.7 ± 0.05
	8.8	8.9	5.3 ± 0.07
		FMT	5.8 ± 0.11
	17.5	8.0	4.8 ± 0.07
		FMT	5.7 ± 0.05
	177.1	3.2	4.4 ± 0.04
		FMT	4.7 ± 0.04

HAc Acetic acid
FMT Fixed mixing time for acetic acid doughs = 6.85 min.
Each pH value is the mean ± SD, n= 3

Table 7. Effect of mixing time on gluten dough pH made with hydrochloric acid

	Acid concentration (mM)	Mixing time (min)	Dough pH
Control	0	10.5	6.3 ± 0.03
		FMT	5.2 ± 0.04
HCl	32.9	8.6	5.8 ± 0.02
		FMT	4.7 ± 0.07
	115.6	7.8	5.3 ± 0.04
		FMT	4.7 ± 0.06
	165.3	6.8	4.8 ± 0.03
		FMT	4.5 ± 0.06
	265.4	6.0	4.3 ± 0.02
		FMT	4.1 ± 0.04

HCl Hydrochloric acid
FMT Fixed mixing time for hydrochloric acid doughs = 8.25 min
Each pH value is the mean ± SD, n= 3

During mixing, glutenin polymers go through an orientation process where alignment and thus, formation of links between the polymers occur. When dough is undermixed, the optimal alignment and hydration of glutenin polymers has not yet occurred; on the other hand, when overmixed, breakage of disuphide bonds takes place leading to conformational changes in the gluten proteins (Létang *et al.*, 1999). Therefore due to the effect of either under or over mixing, conformational changes related to bonding between, and within, the protein polymers occurs. These conformational changes appear to influence the liberation and/or sorption of hydronium ions, giving (in general) as a result different pH values of the doughs when using the same concentration and type of acid, but different mixing times (Hoseney & Brown, 1983; Létang, *et al.*, 1999).

3.3.2 Effects of acids on mixing time of gluten doughs

Addition of acetic and hydrochloric acid at different concentrations affected the shape of mixograms and changed the mixing time for optimal development of the gluten (Fig. 9 & Table 8).

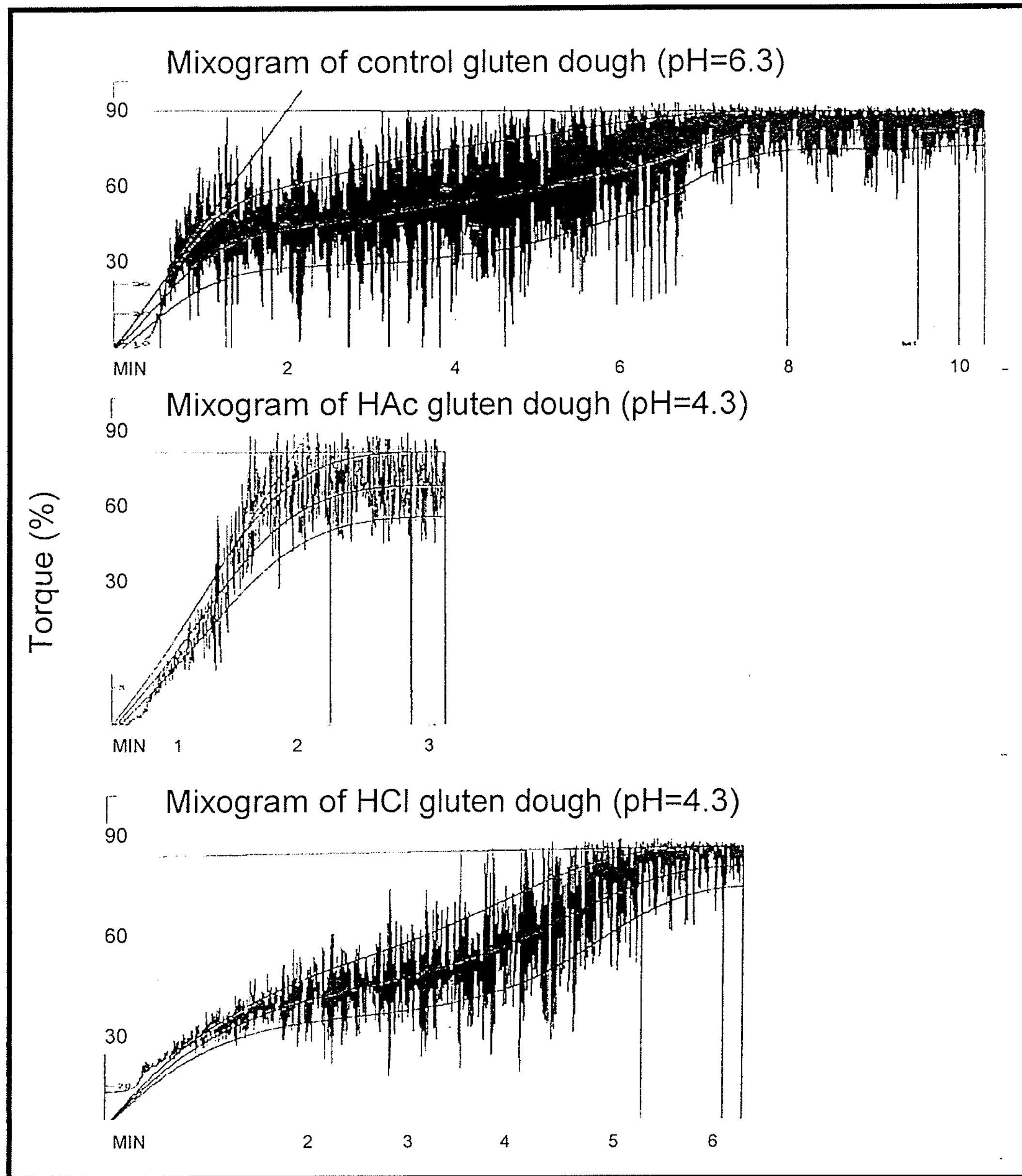


Figure 9. Effect of acetic and hydrochloric acid on the mixograms of gluten dough with a pH of 4.3

Table 8. Mixing times of gluten doughs when acid was added to the formula

	Acid concentration (mM)	OMT (min)
Control	0	10.5 ± 0.4
HAc	3.5	9.1 ± 0.4
	8.8	9.0 ± 0.6
	17.5	8.0 ± 0.6
	177.1	3.2 ± 0.3
HCl	33.0	8.6 ± 0.4
	115.6	7.8 ± 0.1
	165.3	6.8 ± 0.3
	265.4	6.0 ± 0.4

OMT Optimal mixing time
HAc Acetic acid
HCl Hydrochloric acid
Each value of OMT is the mean ± SD, n= 3

Mixograph curves (Fig. 9), showed weakening of the doughs when acid was added to the flour; the curves were thinner, showed a shorter tail and an earlier peak time was recorded, so that the optimal mixing time was affected (Table 8), decreasing from 10.5 to 3.2 minutes with addition of acetic acid and from 10.5 to 6 minutes when hydrochloric was added. The decrease in mixing time for gluten doughs when lowering pH agreed with results obtained by Holmes & Hoseney (1987) and Wherle *et al.* (1997) on wheat flour doughs.

Previous experiments (Tanaka *et al.*, 1967; Smith & Mullen, 1970; Weak *et al.*, 1977; Galal *et al.*,1978; Hoseney & Brown, 1983) have noticed the weakening effect of low pH on flour dough structure as assessed by empirical rheological instruments, suggesting possible changes of the protein conformation and bonding within gluten proteins. These physicochemical changes of gluten structure and their resulting effect on gluten's mechanical

properties would be better described by fundamental rheological measurements instead of by empirical ones (Bagley *et al.*, 1998).

3.3.3 Effects of pH on mechanical properties of gluten doughs

Rheological properties of doughs mixed to a fixed time were determined and are shown in Table 9. As seen in Table 9, significant differences ($P \leq 0.01$) and no clear trend were found in both G' and tan δ when over- and under-mixing the doughs. Changes in rheological properties of wheat doughs due to differences in mixing times when acids were added to the formula were also noticed by Wehrle *et al.* (1997), which the authors attributed to structural changes in the dough. The difference in molecular orientation of the proteins with different mixing times and the presence of an excess of positive charges in the gluten, was surmised to cause different alignments and thus variation in the bonding within polymers. Therefore it is expected that each of the resulting gluten structure after acid addition will show a different deformation response following the application of an external force, leading to measurement of different viscoelastic properties (Bennett & Ewart, 1962).

Table 9. Viscoelastic parameters of acid gluten doughs mixed with a 10g Mixograph to a fixed time (6.85 and 8.25 min for HAc and HCl doughs, respectively)

	Acid concentration (mM)	G' (Pa)	Tan δ
CD1	0	1800 ± 190 a	0.52 ± 0.025 c
CD2	0	1840 ± 100 a	0.51 ± 0.022 d
HAc doughs	3.5	1860 ± 250 a	0.50 ± 0.022 d
	8.8	1360 ± 100 d	0.50 ± 0.015 de
	17.5	1490 ± 140 c	0.50 ± 0.016 e
	177.1	1100 ± 110 e	0.56 ± 0.029 a
HCl doughs	32.9	1600 ± 200 b	0.51 ± 0.017 d
	115.6	1580 ± 150 b	0.50 ± 0.017 d
	165.3	1390 ± 160 d	0.51 ± 0.018 c
	265.4	1500 ± 110 c	0.53 ± 0.018 b

CD1 Control dough mixed for 6.85 min (fixed time for HAc doughs)
CD2 Control dough mixed for 8.25 min (fixed time for HCl doughs)
HAc Acetic acid
HCl Hydrochloric acid
Each value is the mean ± SD, n= 132 (at least)
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

Typical results of stress-sweep (0.5 to 10 Pa) measurements (G' and tan δ) for control and acidified gluten doughs, when mixed to optimal development, are shown in Fig. 10. Differences in G' and tan δ as a function of acid concentration for optimally developed gluten doughs are given in Table 10.

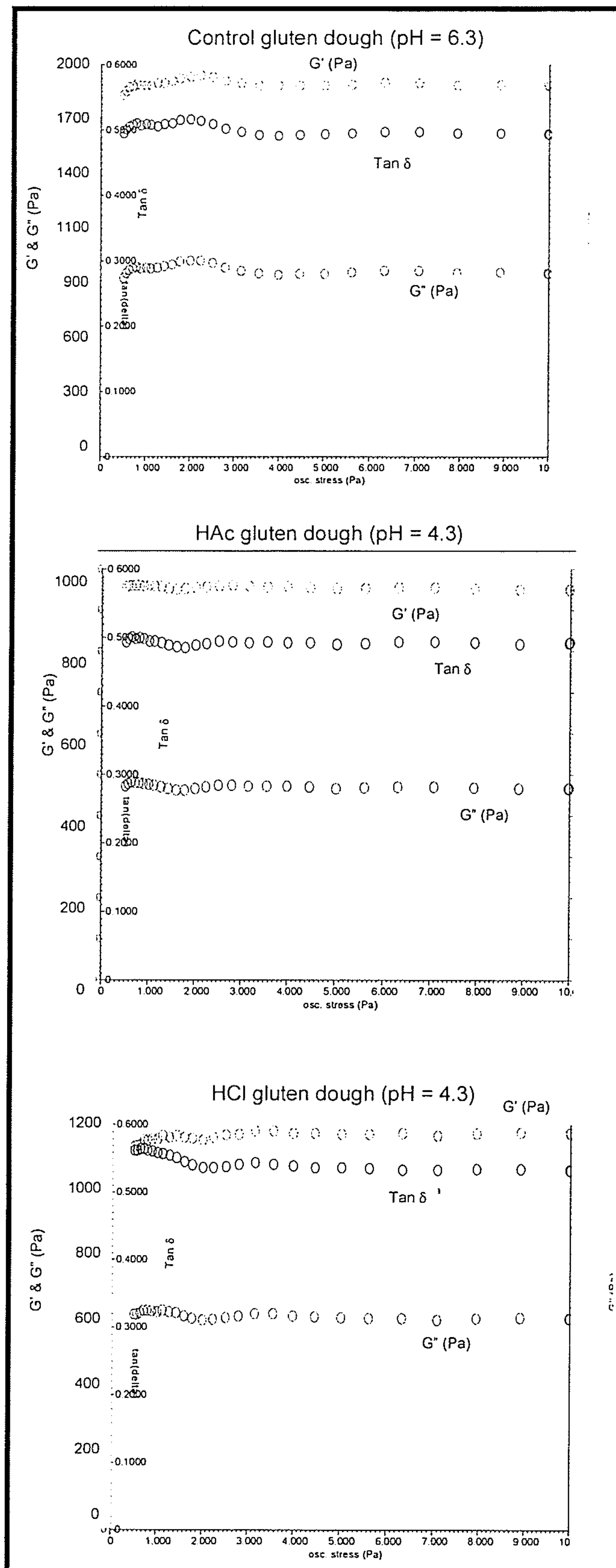


Figure 10. Stress-sweep plots of control and acidified gluten doughs

Table 10. Viscoelastic parameters of optimally mixed acidified gluten doughs

	Acid concentration (mM)	G' (Pa)	Tan δ
Control dough	0	1940 ± 130 a	0.50 ± 0.024 a
HAc doughs	3.5	1460 ± 160 b	0.51 ± 0.017 a
	8.8	1470 ± 110 b	0.50 ± 0.032 a
	17.5	1500 ± 120 b	0.50 ± 0.020 a
	177.1	1090 ± 100 c	0.52 ± 0.022 a
HCl doughs	32.9	1500 ± 100 b	0.50 ± 0.019 a
	115.6	1470 ± 120 b	0.50 ± 0.014 a
	165.3	1450 ± 120 b	0.51 ± 0.018 a
	265.4	1420 ± 90 b	0.51 ± 0.013 a

HAc Acetic acid
HCl Hydrochloric acid
Each value is the mean ± SD, n= 132 (at least)
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

Both acids made the protein matrix less stiff (elastic) in comparison with the control dough (pH of 6.3), bringing down the G' of the dough from around 1900 Pa to around 1500 Pa. There was no effect of pH on viscous dissipation of strain energy in the dough, since tan δ did not show a significant difference ($P \leq 0.01$) at different pH values. At pH values lower than 7.5, the isoelectric point of the gluten proteins (Wu & Dimler, 1963[ab]), electrostatic repulsions between and within protein polymers occur due to an excess of positive charges in the dough. As a result of this electrostatic repulsion, breakage of hydrogen bonds and hydrophobic interactions, as well as conformational changes in the protein polymers are expected, giving as a result, a gluten dough with different viscoelastic properties.

3.3.4 Effects of pH on mechanical properties of gluten doughs when mixed under vacuum

It has been reported that mixing under different headspace conditions, such as in vacuo or in the absence of oxygen, affects the mechanical characteristics of doughs (Baker & Mize, 1937; Bakhoum & Ponte, 1982; Smith & Mullen, 1970; Hoseney, 1984; Elmehdi, 2001). In order to evaluate whether there was an interaction between headspace conditions and gluten acidity, it was decided to mix acidic gluten doughs under vacuum. For assessing the effect of mixing under vacuum on the rheological properties of the gluten doughs, another mixer was used (as described in 3.2.2.1) and experiments were carried out under both atmospheric and vacuum conditions (Tables 11 and 12, respectively).

Table 11. Viscoelastic parameters of acidified gluten doughs optimally mixed with the GRL200 mixer under atmospheric conditions

	Acid concentration (mM)	G' (Pa)	Tan δ
Control dough*	0	7630 ± 940 b	0.55 ± 0.02 b
HAc dough**	177	8020 ± 780 a	0.57 ± 0.02 a
HCl dough**	265	6770 ± 770 c	0.53 ± 0.01 c

HAc Acetic acid
HCl Hydrochloric acid
* pH value of control dough = 6.3
** pH value of HAc and HCl doughs = 4.3
Each value is the mean ± SD, n= 132 (at least)
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

When mixing with the GRL200 mixer, the control dough, with a pH of 6.3, was less elastic than the acetic acid dough with a pH of 4.3 (G' values of 7630 *vs.* 8020 Pa, respectively), but more elastic than the hydrochloric acid dough,

also at a pH of 4.3 (7630 *vs.* 6770 Pa, respectively). When mixing, hydration of the gluten plays an important role in formation of bonds and hydrophobic interactions, within and between protein polymers during dough formation, and it thus affects the viscoelasticity of the gluten (Kinsella & Hale, 1984). Results when mixing under vacuum are shown in Table 12.

Table 12. Viscoelastic parameters of acidified gluten doughs (with a pH of 4.3) optimally mixed with GRL200 (vacuum conditions)

	Acid concentration (mM)	G' (Pa)	Tan δ
HAc doughs	177	7610 ± 590 a	0.58 ± 0.02 a
HCl doughs	265	7310 ± 540 b	0.58 ± 0.01 a

HAc Acetic acid
HCl Hydrochloric acid
Each value is the mean ± SD, n= 132 (at least)
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

Gluten at the lowest pH value (4.3), mixed under vacuum conditions resulted in a less elastic structure when adding hydrochloric acid rather than acetic acid (7600 *vs.* 7300 Pa). With a value of 0.58, tan δ was not affected by the type of acid when mixing under vacuum. The results suggested that the presence of acids and mixer headspace conditions affect gluten structure, which agreed with Baker & Mize (1937). To determine whether changes in dough mechanical properties were attributable to greater moisture loss in the doughs mixed under vacuum, moisture content of the samples was determined; no significant differences between atmospheric and vacuum acid doughs ($P \leq 0.01$) were found. Mixing under vacuum was therefore thought to primarily have an effect on hydrophobic interactions.

3.3.5 Effects of different acids on properties of gluten doughs

Some differences were noticed in the mechanical properties of the gluten mixed with the Mixograph due to the type of acid, when the pH of the doughs was 4.3 and mixed to optimal development. These differences are shown in Table 13.

Table 13. Effect of type of acid on the mechanical properties of optimally developed gluten doughs with a pH of 4.3

Acid	Acid concentration (mM)	Mixing time (min)	G' (Pa)	Tan δ
HAc	177	3.2 a	1090 $\pm$ 100 b	0.52 $\pm$ 0.022 a
HCl	265	6.0 b	1420 $\pm$ 90 a	0.51 $\pm$ 0.013 a

HAc Acetic acid
HCl Hydrochloric acid
Each value of mixing time is the mean, n= 3
Each value of G' and tan δ is the mean $\pm$ SD, n= 132 (at least)
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

Gluten doughs with the same pH value (of 4.3) but with addition of different type of acids to the formula, were significantly ($P \leq 0.01$) weaker (lower mixing time) and less elastic (lower G' value) with acetic acid. These results inferred that the presence of hydronium ions alone was not the only important factor detected through changes in the rheological properties of the resultant gluten doughs, but also that the type of anion was crucial in affecting gluten electrostatics and thus non-covalent bonding and hydrophobic interactions within and between polymers (Preston 1981, 1984, 1989).

Aside from differences in mechanical properties, the necessary concentration of acetic and hydrochloric acid required to bring down the pH of the gluten doughs was significantly different within acids (Table 14). This difference was interesting considering that both acetic and hydrochloric acids

were basically totally dissociated and sorbed by the gluten matrix despite the large differences in their dissociation constants (see Appendix 1 for details).

Table 14. Hydrogen ions (H^+) sorbed into the gluten matrix during mixing

	Acid concentration (mM)	Concentration of sorbed H^+ (M)	% of H^+ that are sorbed
HAc	3.5	2.42×10^{-4}	99.8
	8.8	3.94×10^{-4}	99.2
	17.5	5.52×10^{-4}	98.2
	177.1	1.76×10^{-3}	98.3
HCl	32.9	3.30×10^{-2}	≈100
	115.6	1.16×10^{-1}	≈100
	165.3	1.65×10^{-1}	≈100
	265.4	2.65×10^{-1}	≈100

HAc Acetic acid
HCl Hydrochloric acid
Acid concentration and H^+ concentration is equivalent since both acids are monoprotic and are essentially fully dissociated

The concentration of hydrochloric acid solutions necessary for the decrements in pH increased steadily (from 33 to 265 mM) throughout the pH range that was selected (5.8 to 4.3). Meanwhile, the concentration of acetic acid solutions increased steadily up to a pH of 4.8 of the dough, and then increased abruptly (from 18 to 177 mM) for the next pH value, of 4.3 As well, there is a one order of magnitude difference between the concentration of the different types of acid solutions necessary to achieve all acidic series except at the pH of 4.3, when the concentrations of acetic acid and hydrochloric acid were 177 and 265 mM, respectively. Based on the data shown in Table14, both acetic and hydrochloric acid are essentially 100% dissociated. Therefore, no differences in concentrations should be expected. This suggests that differences in the anionic species influence the mechanism by which the dough incorporates the acids within the protein matrix during mixing.

3.4 DISCUSSION OF RESULTS

During dough mixing, gluten proteins are hydrated and form a three-dimensional network, which is responsible for the unique viscoelastic properties of dough (Li *et al.*, 2003). The process followed by the gluten polymers is to align and develop links and interactions in between themselves (Fig. 11) (Létang *et al.*, 1999).

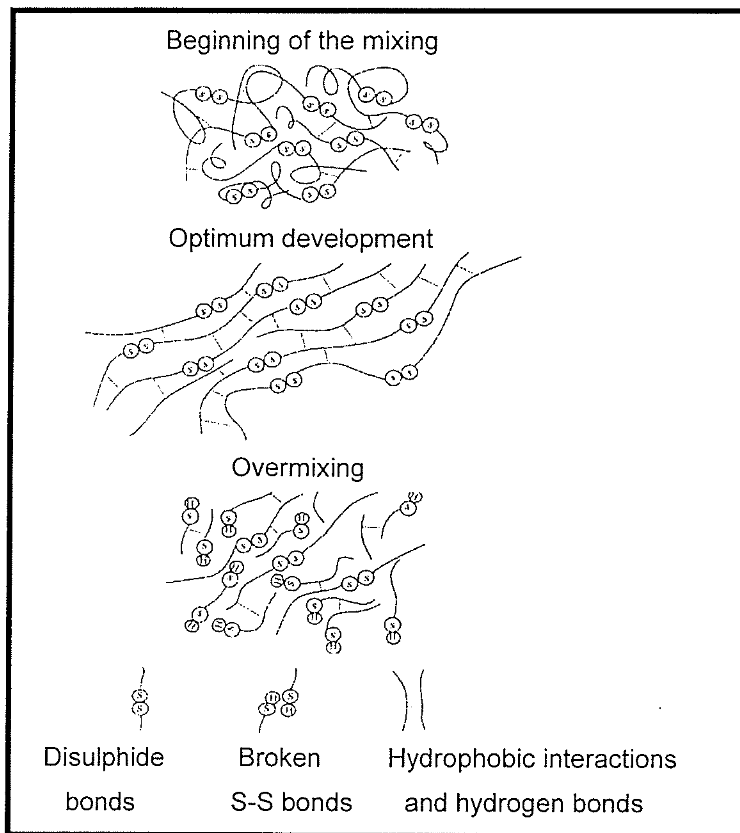


Figure 11. Molecular interpretation of gluten development during mixing (Létang *et al.*, 1999)

As proposed by Létang *et al.* (1999), undermixing will lead to a gluten system where only some interactions can exist due to a random orientation of the gluten polymers. The incomplete alignment exposes the side-chains of different amino acids so that they are free to interact with water molecules. As a result of the protein being in an aqueous environment, polar amino acid side-chains (including acid and basic groups) are the ones expected to be exposed (Pauling, 1945). Exposed acidic amino acids evidently contribute to the fall in pH of the gluten doughs (Tables 6 & 7).

At optimal development, gluten protein polymers are aligned and thus interactions will occur between them (i.e., hydrogen bonds and hydrophobic interactions) (Létang *et al.*, 1999). According to results in Table 14, the optimally developed gluten was able to essentially sorb all the added acid. In this thesis, the buffering capacity of the gluten was manipulated with both acetic and hydrochloric acid in order to obtain 4 pH values (5.8, 5.3, 4.8 and 4.3) as described in 3.2.2.2. The increase in the concentration of added acids not only led to lower dough pH values, but also to weaker doughs with shorter optimal mixing times (Table 8). An explanation for the occurrence of these chemical and physical changes in gluten is likely attributable to changes in gluten conformation.

Overmixing of doughs has been studied more exhaustively than under mixing and thus there is more information about it. As a result of prolonged mixing times, breakage of mainly disulphide bonds within the protein occurs. Smaller polymers result, with an expected exposure of hydrophilic amino acid side-chains (Danno & Hoseney, 1982; Hoseney & Brown 1983) such as

positively charged ones. The exposure of positively charged amino acids may contribute to lowering the pH of the overmixed hydrochloric acid doughs (Table 7). However, this simplistic model does not explain the increase in pH value of the overmixed acetic acid dough (Table 6), showing the relevance of the type of conjugated base of the acid and its effect on the conformation and electrostatics of the gluten polymers.

To account for the viscoelastic properties of gluten, two theories have been proposed: MacRitchie and Lafiandra (1997) suggested that polymers within gluten form entanglement points (small areas of the polymers interacting with each other) that are separated by long regions that do not interact. Later, Belton (1999) suggested a network structure of gluten where the proteins interact with each other through a train mechanism (high hydrogen bond density) with regions of no interaction (loops). Both models state the importance of the existence of non-covalent bonds and interactions in between aligned proteins in conferring both elasticity and viscosity to the gluten doughs.

Hoseney & Brown (1983) suggested that at low pH, as the result of an excess of positive charges, gluten polymers repel each other, and this prevents the protein from interacting with itself (via secondary bonds). Moreover, Bennett & Ewart (1962) stated that the presence of hydrogen ions in the dough led to a destruction of secondary bonds through repulsion of positively charged side-groups in the proteins. As a result of the increased repulsion between proteins, the gluten structure changed and so too did its mechanical properties (Table 10). Galal *et al.* (1978) proposed a model to represent this repulsion within the gluten proteins due to the presence of acid (Fig. 12).

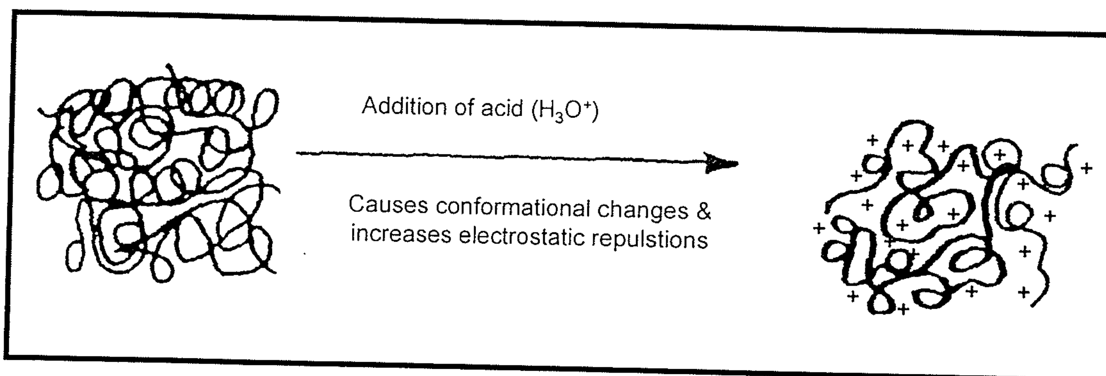


Figure 12. Possible effects of pH on wheat gluten proteins (Adaptation from Galal *et al.*, 1978)

Wu and Dimler (1963[a]) found that the isoelectric point of gluten was 7.5, so that at the range of pH covered in this study (pH values from 5.8 to 4.3) an overall positively charged gluten matrix is expected. When acids were incorporated into the dough, hydronium ions were expected to affect alignment and bonding in the gluten matrix due to interactions of these positive ions with hydrophilic amide and basic amino acid side-groups (i.e., asparagine, glutamine, lysine and arginine) (Bennett & Ewart, 1962). Moreover, two amino acids present in the gluten, histidine and glutamic acid, were expected to change their charge as pH was altered due to traversing their isoelectric point (Table 15).

Table 15. Side-chain charging of amino acids

pH	Net charge of His	Net charge of Glu
6.3	$0 \Rightarrow +1$	-1
5.8 – 4.8	+1	-1
4.3	+1	$-1 \Rightarrow 0$

His pK_R= 6.43 (Wu & Dimler, 1963[b])
Glu pK_R= 4.25 (Cheftel & Cuq, 1985)

Based on Belton's loop and train gluten model (1999) the change in positive charges resulting from the changes in these amino acid charges is expected to enhance repulsion within polymers and increase the amount of polymer in the loop configuration (Fig. 13).

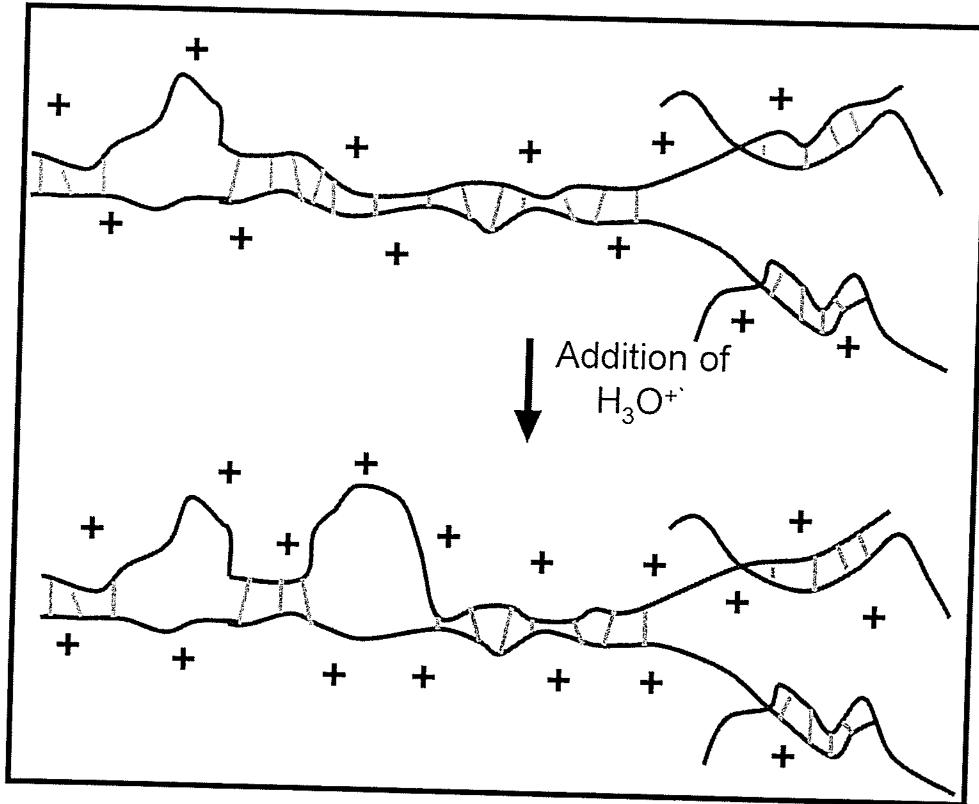


Figure 13. Hydrogen ions (H^+) included in the gluten matrix during mixing (Adapted from Belton, 1999)

As a result of the increase in the amount of polymer in the loop configuration, the gluten structure was expected to be weaker and less elastic. Results showing a weaker dough (Fig. 9 & Table 8) corresponded with the work of Danno & Hoseney (1982), who explained that shorter mixing times suffice to develop a dough when the proteins are charged, since repulsion of positive charges in the protein led to faster hydration. Viscoelastic properties of doughs

can be explained with Belton's model (1999), where it was proposed that stretching of the polymer results in deformation of the loops first (since these are the more compliant part of the structure) followed by the trains (where hydrogen bonds between the trains are broken). Upon relaxation of the applied stress the restoring force of the loops is related to their conformational entropy, while restoration of the trains is due to the re-formation of hydrogen bonds (Belton, 1999).

Therefore, hydrogen bonding within gluten polymers plays an important role in gluten viscoelasticity (i.e., energy recovery), when the applied stress is released. The stress used in this work was too small for disrupting molecular bonds and thus creating permanent deformation of the loops (since the gluten was stressed within the linear viscoelastic region). However, the induced electrostatic repulsions between the protein polymers, as a result of the change in pH could break the hydrogen bonds within them. Less hydrogen bonds would reduce the contact points between gluten polymers, generating bigger loops. Moreover, the decrease in hydrogen bonds would not allow the same recovery of energy applied in an oscillation test and thus, a less elastic dough was obtained (lower G' values) (Table 10). However, the breakage of hydrogen bonds did not significantly affect ($P \leq 0.01$) tan δ (Table 10) and thus the decrease in hydrogen bonding did not enhance the dissipation of strain energy in the gluten structure. A breakage of hydrogen bonds and disruption of hydrophogic interactions would happen more easily when compared with disulphide bonds since the bond energies of the formers are lower than for the

latter. A free energy of 3-7 kcal/mol is needed to break a hydroben bond, 3-5 kcal/mol are needed to unfold a nonpolar side-chain from the protein interior, and 50 kcal/mol are needed to break a disulphide bond (Wood *et al.*, 1981).

When doughs were undermixed gluten polymers were not totally unfolded and aligned, therefore different viscoelastic properties of these gluten doughs were expected (Létang *et al.*, 1999). The lower G' values for undermixed control doughs (no acids addition) can be explained based on their lower pH values; the exposure of acidic side-chains led to repulsion between proteins, and thus to a less elastic dough. The non-alignment of glutenin polymers due to electrostatic repulsions affected the tan δ of gluten doughs in a complicated way with no obvious trend and thus no easy explanation (Table 9). When acid was added to the mixing formula, both rheological properties, G' and tan δ, did not follow any constant trend and thus interpretation and prediction of their structural significance is difficult. These results highlight the complexity of underdeveloped doughs and thus the relevance of optimal development of the dough (as the result of an optimal mixing time) when explaining its rheological properties.

In earlier studies (Kerr *et al.*, 1993; Létang *et al.*, 1999), the rheology of overmixed doughs has been studied. But the effects of prolonged mixing times on dough structure have not been completely elucidated and thus dough viscoelasticity is not predictable. In this study, the breakage of bonds as the result of over mixing led in general to less elastic dough with lower tan δ values compared to those optimally mixed. When considering over mixing and addition of acids to the gluten, the effects on rheological properties were more complex

without following a trend. Therefore, aside from the weakening effect of over mixing and addition of acids on gluten, no other properties of gluten can be predicted.

When testing gluten doughs under vacuum some differences in dough rheology were found. Due to the differences in water absorption of the doughs mixed by different mixers, a direct comparison of the vacuum results with those obtained from the mixograph is not feasible (Autio *et al.*, 2001), although data agreed with studies where higher water contents gave lower G' and G" values (Khatkar *et al.*, 1995; Létang *et al.*, 1999). Following Pauling's theory (1945) where charged proteins hydrate more, and Létang & co-workers' model (1999) where alignment of polymers is required in order for hydration and formation of links in the gluten matrix, it is possible that the presence of acids in a system with less available water, affected the hydration of gluten and thus its rheological properties (Autio *et al.*, 2001).

When mixing under vacuum, fewer air bubbles are entrapped within the gluten network compared with mixing at atmospheric pressure (Hoseney, 1984; Elmehdi, 2001). The overall loss of internal air surface would lead to a gluten structure where less of proteins encountering the hydrophobic residues in the gluten become exposed as a result of the hydrophobic surfaces associated with the large number of small gas cells (Torres *et al.*, 2000). The higher elasticity (G' values) of acetic acid doughs compared to the hydrochloric acid ones was not affected by the presence/absence of air when mixing (Tables 11 & 12). Therefore the mechanism of action -at a pH of 4.3- through which different anions are incorporated (or not) into the gluten matrix, does not depend on the

amount of air incorporated into the dough, even though the amount of bubbles in the dough did have a significant effect on tan δ. In hydrochloric acid treated gluten doughs, the rearrangement of molecular structure associated with loss of gas cell surface ares allows more hydrophobic interactions between polymers, and the stiffness increases. It does not for the acetic acid doughs since the hydrophobic nature of the acetate ions interfere with the development of hydrophobic bonds that can potentially form following the loss of internal air surface.

The presence of more air bubbles as the result of atmospheric-mixing gave a higher tan δ value for the acetic acid dough than that for the hydrochloric acid one. This result showed that acetic acid is able to affect the viscoelasticity of the dough at a dough pH of 4.3 when atmospherically mixed, giving a more elastic and viscous dough (i.e., with both a higher G' and G" value). A possible explanation for this outcome is a change in conformation of the gluten, where hydrophobic residues get exposed and stabilised in the presence of acetate ions but not by chloride ions, since the former are more hydrophobic than the latter ones (Marcus, 1985). Another explanation is based on the fact that hydrophobic interactions are weaker than hydrogen bonds (Voet & Voet, 1990). The resultant hydrophobic interactions are then unable to store the applied strain energy (giving lower G' values). Because of the structural changes, the applied energy is now dispersed (giving higher tan δ values).

The weakening effect of acids on gluten doughs has been extensively studied (Galal *et al.,* 1978; Sekhon *et al.*, 1995; Larsson, 2002; Shiau & Yeh,

2001), but the effect of different types of acids has been hardly reported (Bennett & Ewart, 1962). In this work it was noticed that the type of acid was an important factor in affecting gluten's chemical (i.e., pH) and mechanical properties (i.e., optimal mixing time, G' and tan δ). In order to get the same pH value of gluten doughs when mixed with the two acids, a much higher concentration of hydrochloric acid was needed compared with acetic acid. Bennett & Ewart (1962) suggested that the effect of different acids may be related to the acid's ability to donate hydronium ions (acid dissociation). According to Table 14, Bennett & Ewart's suggestion (1962) did not apply, since both acetic and hydrochloric acids at all concentrations were practically completely dissociated and sorbed by the gluten dough. Another important difference was that the dough was significantly ($P \leq 0.01$) less elastic when a pH value of 4.3 was reached in the presence of acetic acid, whereas G' reached a plateau from a pH of 5.8 downwards when hydrochloric acid was added.

An explanation for the effects of type of acids on gluten properties could focus then on the type of anion (conjugated base of the acid). Preston (1989) suggested the importance of anion type on dough structure; anions are thought to mask positive charges present in a protein. Although the mechanism for this shielding is unknown, it seems plausible for the chloride to be included during mixing in the gluten structure in an easier and faster way than for the acetate, considering their molar volume differences and ionic mobilites in water (molar hydrated volume: 46.9 cm^3/mol for acetate *vs.* 24.2 cm^3/mol for chloride; ionic mobilities in water at 25°C: 76.3 cm^2/Vs for acetate *vs.* 40.9 cm^2/Vs for chloride)

(Marcus, 1985; Brey, 1978 as cited in Voet & Voet, 1990). A proposed mechanism in this thesis starts with the increase of positive charges in the gluten, i.e., the already positive character of gluten, and the increase in net positive side-chain charging due to first histidine and eventually glutamic acid as pH is decreased (Wu & Dimler, 1963[ab]; Cheftel & Cuq, 1985). As the result of this net charge of the gluten proteins, electrostatic repulsions are expected and thus increased breakage of hydrogen bonds with decrease of pH (Bennett & Ewart, 1962: Hoseney & Brown, 1983). The breakage of these bonds will lead to a more open gluten structure, which following Belton's model (1999), gives as a result bigger loops (Fig. 5). Considering the increase in size of the loops, the smaller the available anions, the easier their sorption. Once the anions have interacted with the protein side- chains a shielding of the positive charge of the gluten proteins will happen, and either a neutralisation or a partial negative charge might predominate (Chang, 1996). As the result of the partial negative charge of the gluten proteins, more small cations (i.e. hydronium ions with a net molar hydrated volume of 6.4 cm^3/mol) (Marcus, 1985) will be attracted to the structure and the same process could repeat over and over. The end-point of this process could be the filling of the loops with ions.

According to the previous suggested mechanism, acetate ions would have a size restriction for getting into the gluten loops, until the loops are big enough to allow them to be sorbed. This size restriction seemed to be surpassed at a pH of 4.3, where the concentration of both acids were of the same order of magnitude (Tables 6 & 7). The pH value of 4.3 indicates that by then more amino acid residues (i.e., glutamic acid) got positively charged

(Cheftel & Cuq, 1985) and thus higher electrostatic repulsions were expected. Following the modification of Belton's model (1999) of the creation of bigger loops due to a higher incidence of breakage of hydrogen bonds, differences in mechanical properties of the dough are expected even at the same pH of the doughs. This theory, based on the shielding and selective admission of ions in the gluten loops and thus breakage of hydrogen bonds, then corresponds to the rheological results. These results are encountered in differences in mixing times (Table 8), G' and tan δ values (Table 10), i.e., a weaker and less elastic acetic dough was found at a pH of 4.3 when compared with the hydrochloric acid dough.

3.5 CONCLUSIONS

Changes in pH affect the conformation of gluten doughs and thus their rheological properties. The elasticity of gluten is affected as the result of electrostatic interactions generated by the presence of a net positive charge on the proteins. The main amino acid residues responsible for changes in the electrostatic charge over the pH of interest (6.3 to 4.3) are histidine and glutamic acid. As a result of electrostatic repulsions hydrogen bonds between proteins are broken and thus the gluten doughs at pH values from 5.8 to 4.3 are less elastic (with lower G' values) than at a pH of 6.3.

When acids are present in the gluten and the electrostatic repulsions between amino acid polymers occur, shielding of the positive net charge of the gluten takes place by the corresponding anions of the acids.

The effect of acetic acid on the gluten structure and its mechanical properties (mixing time and G') was significantly different than that of hydrochloric acid at a dough pH of 4.3. Therefore, at this pH value, differences in the type of the conjugated base are important for determining the rheological properties of gluten doughs. More studies regarding effect of different acids on gluten structure are recommended.

The results from this study could have broad applications either in the scientific community (obtaining insights into structure and mechanical behaviour) or in the baking and packaging industries (understanding and thus predicting the functionality of gluten under acidic and vacuum conditions).

4. MANUSCRIPT II

Ultrasonic determinations of gluten doughs as affected by acids, salts and their combination

ABSTRACT

Functional properties of gluten doughs and films are sensitive to the presence of acids, salts, and their combination. The effect of different pH values and types of salt (with and without addition of acid) on gluten structure, and thus its functionality, has been extensively analysed by many workers. The techniques used for the analysis of gluten frequently destroy the sample and are difficult to apply in an on-line process. Ultrasonic techniques have the advantage of being non-destructive and being amenable to on-line implementation. This part of my thesis studied the effect of acids and salts on the ultrasonic characteristics of gluten doughs. Gluten doughs were mixed to optimal consistency time and to the same hydration level using either acids or salts or a combination of them. For the acidic doughs, acetic and hydrochloric acids were added to change the pH of the gluten in five decrements from 6.3 to 4.3. For the salty doughs, three chloride salts (at equivalent ionic strength) were used (NaCl, $MgCl_2$, and $AlCl_3$), either with or without acetic acid. Differences between gluten doughs were assessed by differences in mechanical, physicochemical and ultrasonic properties. The mechanical and

physicochemical properties included mixing time, pH and density. Ultrasonic determinations included ultrasonic velocity, attenuation coefficient, and longitudinal loss modulus (β''). The type of acid (acetic *vs.* hydrochloric) had an effect on all the assessed properties of the gluten dough. The effects of acids on gluten structure were more pronounced at the lowest pH value (4.3). The addition of acetic acid at the highest concentration led to a weaker and less dense gluten dough, where the ultrasonic velocity increased. It was surmised that the effect of cation type influenced the conformation of the gluten dough by the formation of salt bridges between the proteins. The relevance of cation type was more pronounced in the presence of acetic acid. In general, the attenuation coefficient was high in the gluten doughs, a finding that was attributed to strong dissipation of the sound waves. Addition of acids led to an opening of the protein structure due to electrostatic repulsions between the polymers as the result of an increase in positive charges on amino acid side-chains. In the case of hydrochloric acid, the chloride ion appeared to show some shielding of the positive charges, suggesting that in this case the gluten polymers adopted a more compact structure with less bound water. With respect to the chloride salts, the effect of cations on the gluten structure was attributed to the formation of salt links between gluten polymers.

4.1 INTRODUCTION

Gluten proteins are responsible for conferring the unique viscoelastic properties that are associated with wheat flour doughs (Eliasson & Larsson, 1993; Lindsay & Skerritt, 1999). Furthermore, gluten has properties that make it a promising film material for the packaging industry (Gennadios *et al.*, 1993). Acids, salts and their combination are common ingredients involved in the processing steps of gluten products (during fermentation of flour doughs and gluten film formation) or used as additives (Galal *et al.*, 1978; Gennadios *et al.*, 1993; Gontard & Ring, 1996; Cuq *et al.*, 1998; Takeda *et al.*, 2001). The effects of changes in the pH of gluten and the presence of salts and their combination have been observed by many researchers over the past five decades. However, the mechanisms by which they affect the functional properties of the gluten products has not been completely elucidated (Bennett & Ewart, 1962; Kim & Bushuk, 1995). Moreover, the mechanism by which the combination of salts and acids affect the structure and the properties of gluten proteins is even less clear (Galal *et al.*, 1978; Bakhoum & Ponte, 1982).

The development of the technologically important structure of gluten depends upon hydration of the proteins. Therefore the structural stability of gluten is determined not only by interactions within and between the polymers, but also between the polymers and the water. Gluten polymers are directly stabilised by covalent bonds, ionic forces, hydrophobic interactions and hydrogen bonds (Belitz *et al.*, 1986). Indirectly, gluten proteins are also

stabilised by the structure of the water present in the gluten system (Preston, 1981; Balla *et al.*, 1998). The stabilising forces of the structure of gluten proteins have been reported to be modified by the pH of the gluten, as well as by the addition of salts (Galal *et al.*, 1978; Hutchens & Yip, 1990).

When acids are present in flour doughs or gluten films, some of the amino acid side-groups in the gluten proteins get positively charged (Wu & Dimler, 1963[ab]). The increase in positive charges induces mutual repulsion of these charged amino acids and thus salt-bridges, hydrogen bonds and hydrophobic interactions between the gluten polymers are ruptured. The rupture of stabilising forces in the gluten leads to protein conformational changes that affect the functionality of gluten doughs and films (Bennett & Ewart, 1962; Gennadios *et al.*, 1993; Shiau & Yeh, 2001).

Anions affect gluten conformation by shielding the positive charges extant on the amino acid side-residues. The masking of charges allows the gluten polymers to interact and aggregate, giving as a result a stronger gluten dough (Galal *et al.*, 1978; Hoseney, 1994). The effect of the cations and anions in gluten systems (either doughs or films) is highly dependent on the salt concentration. In general, it has been reported that low concentrations of salt (<0.05 M) alter the gluten structure independently of the ion type. At higher salt concentrations, the effect of ions on gluten is related to an enhancement of hydrophobic interactions between the proteins (Preston, 1981; Kinsella & Hale, 1984; Preston, 1989). When chloride salts are used, a compact gluten structure is observed as the result of the drawing of water molecules from the protein polymers to the ions. The strengthening of gluten proteins in the presence of

divalent cations has been related to the formation of additional cross-links between the gluten polymers (Nayal & Di Cera, 1994; Balla *et al.*, 1998).

The combination of acids with salts leads to conformational changes of the gluten doughs as well. The amino acid residues are exposed as the result of addition of acids and then allowed to interact due to the shielding effect of positive charges by the salts. Some of the groups that are exposed and now able to interact may be hydrophobic residues or metal binding sites that in the presence of salts give as a result a compact and a strong gluten structure (Galal *et al.*, 1978; Hutchens & Yip, 1990)

Ultrasound is an emerging technique that is based on the principle of propagating sound at high frequencies (from 20 kHz to over 100 MHz) into a body and using the sound propagation properties to understand the material and structural properties of the body. Nowadays it is used for the analysis of food systems (Samari, 1994; Verdier & Piau, 1997; Juodeikiene & Basinskiene, 2004). Its has a number of technological and economical advantages including: measurements are rapid and precise, optically opaque systems can be assessed, on-line determinations during processing are possible, it is non-destructive and non-invasive, it is relatively cheap, and is available as an easy-to-use commercial instrument (McClements, 1997; Létang *et al.*, 2001; Elmehdi & Kovacs, 2003; Coupland, 2004; Scanlon, 2004). However, ultrasound also has some disadvantages when used in food systems due to the high attenuation of many samples (especially when gas bubbles are present). And thus the interpretation of the data may be difficult (Samari, 1994; McClements, 1997; Verdier & Piau, 1997; Coupland, 2004; Resa *et al.*, 2004).

Ultrasonic determinations can be related to structural, compositional and physical properties of food products (McClements, 1997; Elmehdi *et al.*, 2003; Scanlon, 2004). The velocity of ultrasonic propagation and the attenuation coefficient are two parameters that have been previously used for evaluating the structural and physical properties of food systems. Some examples of foods that have been analysed in this manner include: fruit juices, brine, oils, alcoholic drinks, fats, salad creams, meat, fish, fruits, vegetables, dairy products, eggs and flour doughs (McClements, 1997; Létang *et al.*, 2001; Elmehdi *et al.*, 2003). In the case of homogeneous materials, the ultrasonic velocity is related to their density and elastic moduli, whereas for heterogeneous foods, the ultrasonic properties depend on reflections, scattering, refraction and absorption mechanisms (McClements, 1997).

Due to its advantages in probing the composition and structure of food systems, low-intensity ultrasound appears to be a useful technique for studying the effects of acids, salts and their combination on the properties of gluten proteins. The hypothesis of this work is that addition of different types of acids, and addition of different types of salts, with and without addition of acid, will lead to conformational changes in the gluten dough that can be monitored and explained by ultrasonic determinations.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Commercial wheat gluten (spring wheat premium quality) purchased from Premier Cereals, Inc. (Winnipeg, MB) was used. The water and protein compositions (N x 5.7), as analysed by AACC methods (44-15A & 46-13, AACC 1983) were: 6.0 ± 0.09% and 70 ± 1.7% respectively. Acetic acid, hydrochloric acid, sodium chloride, magnesium chloride and aluminium chloride were purchased as reagent grade from Fisher Scientific (Nepean, ON). Distilled water was used for dilutions.

4.2.2 Methods

4.2.2.1 **Gluten Dough Preparation.** Commercial wheat gluten flour was rehydrated to 210% (10 g / 21 mL) moisture content on a flour weight basis. For creation of acidic and salty doughs, a 10-g Mixograph with a controlled temperature water circulating system at 25 °C and a speed of 88±2 rpm (Micro mixer, TMCO, Lincoln, NE, USA) was used. The mixograph plots were analysed using the Mixsmart® Computerized Data Analysis software (V 3.4, TMCO, Lincoln, NE, USA). All doughs were mixed to full development of the gluten, for which the term optimal mixing time (OMT) was used. OMT refers to mixing for 10% past the time required to reach the peak in the mixograph. The mixing of all doughs was done at least in triplicate.

Since pH measurements were important for the experiments, the pH of the doughs was determined immediately after mixing, again at least in triplicate. For pH determinations of doughs, a Fisher Scientific pH Meter, model 915 (Pittsburgh, PE), with a silver/silver chloride pH electrode, was used. To determine the pH of the gluten dough, the electrode was inserted directly into the dough (Salovaara, 1982; Larsson, 2002). This method was later validated by comparing the pH readings with the results using a SENTRON Hot-Line probe (LanceFET; Roden, The Netherlands), which is designed for using on similar type of food products.

Another set of dough samples was mixed for ultrasound measurements. Each dough was removed from the mixing bowl and cut into five pieces. Each dough piece was placed between a pair of glass plates (dimensions of 2.82 x 100 x 100 mm) and clamped with metallic clips (clamps). The glass plates were purchased from BOYD Autobody & Glass (Dominion Centre, Winnipeg, MB). Separation between the glasses was achieved with plastic spacers inserted between the glass plates. Dough pieces of different thicknesses (0.5, 0.74, 1.02, 1.49 and 2.06 mm) were achieved with the spacers. The assembly is shown in Fig. 14.

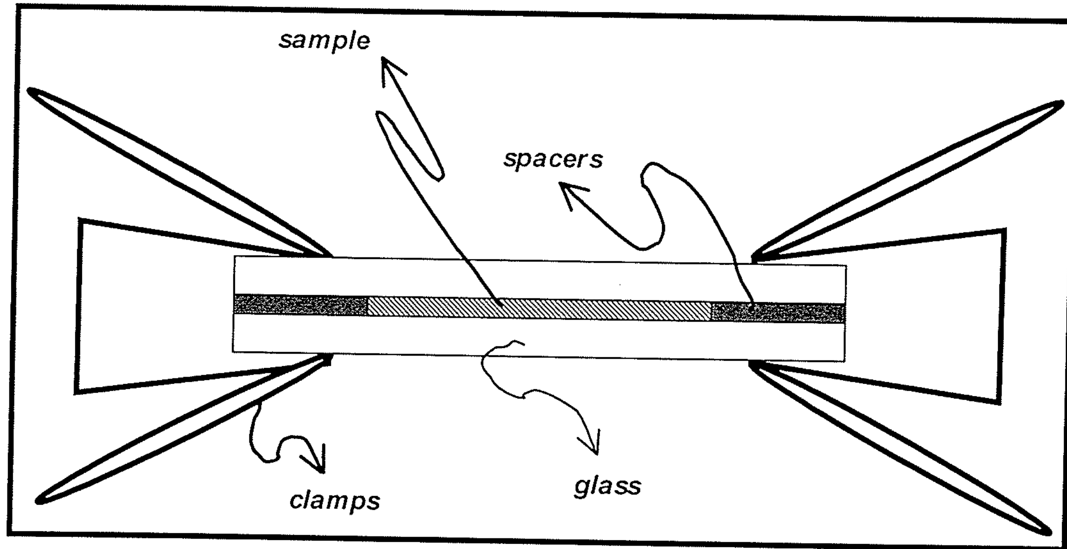


Figure 14. Assembly of gluten sample in between glass plates for ultrasound measurements, side view

The five assemblies of glass plates with gluten dough assemblies were kept in a covered container (Tupperware) for 90 min at room temperature. The placement in the container prevented dough moisture loss and allowed the dough to rest and relax the stresses from the mixing (Georgopoulos *et al.*, 2004). During this resting time, shaping of dough samples for ultrasonic measurements occurred as well.

Gluten doughs formulated according to different treatments were analysed by ultrasound. The three main treatments of this part of my thesis were based on acids and salts added to the gluten flour with the mixing water and are described as follows.

4.2.2.2 **Acidic Doughs.** To lower the pH of the dough without yeast addition, two types of acid were used: acetic and hydrochloric. In this way, the effect on gluten properties of two simple acids (one organic and one inorganic) was

tested. The pH of the doughs was brought down from 6.3 to 4.3 in five steps. A pH of 6.3 was the measured pH when gluten flour was mixed with distilled water and 4.3 is within the range of lowest pH values (4-4.3) that wheat dough reaches in the sourdough process (Clarke *et al.*, 2002). The concentration of acids in the mixing water needed to decrease the pH of the dough is based on previous experiments (Manuscript I) (Table 16).

Table 16. Acid solutions used for bringing down the pH of gluten dough

Desired pH of dough	HAc Concentration (mM)	HCl Concentration (mM)
6.3 (control)	0	0
5.8	3.5	32.9
5.3	8.8	115.6
4.8	17.5	165.3
4.3	177.1	265.4

HAc Acetic acid
HCl Hydrochloric acid

4.2.2.3 **Salty Doughs with and without Addition of Acid.** Previous results (Manuscript I) showed that addition of acids affected the rheological properties of gluten dough. The decrease in the elastic modulus of acidic gluten was first noticed at a dough pH value of 5.8. In order to understand the effects of acids and chloride ions on gluten structure, it was decided to add chloride salts to the dough in combination with acetic acid. Addition of chloride salts without acetic acid was used as the control treatment. The three chloride salts were: sodium, magnesium and aluminium (the last two were hexahydrated). A concentration of 3.51 mM of acetic acid was used for bringing down the pH of the doughs to 5.8. In order to keep the ionic strength (S) of the gluten doughs constant, the

concentration of salts added was based on the ionic strength of the hydrochloric solution necessary for obtaining a gluten dough of 5.8 in previous experiments (Manuscript I) as given by Eq. 16,

$$S_{ss} = S_{HCl} - S_{HAc}, \tag{16}$$

where S_{ss} is the ionic strength of the salt solution [mol], S_{HCl} is the ionic strength of the hydrochloric acid solution at a pH value of 5.8 = 33.05 x 10 $^{-3}$ mol, and S_{HAc} is the ionic strength of the acetic acid solution at a pH value of 5.8 (3.5 x 10 $^{-3}$ mol). This way the effect of chloride anions on the gluten structure at the same ionic strength as with hydrochloric acid but in the presence of three different types of cations was tested.

From the ionic strength of the salt solution (S_{ss}), the necessary concentration of the chloride salt was obtained (Table 17) as follows:

$$S_{ss} = 1/2[\Sigma^{n}_{i}(m_i z_i^2)_n], \tag{17}$$

where S_{ss} is the ionic strength of the salt solution [mol], m_i is the moles of ionic species i to n[mol], and z_i is the electrostatic charge of species i to n.

Table 17. Concentration of chloride salts used for gluten dough preparation

Salt	Concentration (mM)
NaCl	29.55
$MgCl_2.6\ H_2O$	9.85
$AlCl_3.6\ H_2O$	4.93

4.2.3 Density measurements

For density measurements, separate samples of the gluten doughs were prepared. A Mettler AE160 scale (Fisher Scientific, Pittsburgh, PE) was used for all weight determinations.

Gluten was mixed as described above (see 4.2.2.1); all treatments, controls, acid, salty and acidic salty doughs were prepared. After mixing, each gluten dough was removed from the mixing bowl and allowed to rest and flatten in between the plates of a Glutork2020 (Glutomatic System, Perten Instruments AB, Huddinge, Sweden) for 90 min at room temperature. In order to prevent dough moisture loss, the Glutork2020 was placed in a covered container (Tupperware) for the full 90 min alongside trays of water. Gluten disks were cut from the flattened dough sheet with a metal cork borer of 12 mm diameter and they were then weighed directly.

A clean and dried empty gravimetric bottle was weighed, filled with deionised water (at 20 °C) and weighed again. The gluten disk, previously weighed, was placed into the gravimetric bottle. The water displaced by the gluten disk was dried off the bottle, and the bottle was weighed again. The density of the gluten samples was calculated as follows:

$$\rho_{dough} = m_{dough} / V_{dough}, \qquad (18)$$

where ρ_{dough} is the density of gluten dough [g/cm^3], m_{dough} is the mass of gluten dough disk [g], and V_{dough} is the volume of gluten dough disk (equivalent to volume of water displaced from the bottle when the gluten disk is inserted) [cm^3].

4.2.4 Low-intensity ultrasound measurements

The ultrasound equipment was comprised of (Fig. 15):

- A Portable Ultrasonic Non-destructive Digital Indicating Tester (PUNDIT 6, CNS Farnell Ltd., Borehamwood, Hertfordshire, UK) that generated the voltage pulse.
- 50 Ω BNC cables that sent the pulse to the transducers.
- Two transducers (Panametrics) nominal frequency of 50 kHz: one that passed the resultant ultrasonic pulse to the glass plate assembly (Fig. 14), and the other one that received the signal after it had been transmitted through the assembly and transformed it back into an electromagnetic signal.
- A receiver amplifier (PUNDIT 6) that amplified the signal coming from the receiving transducer.
- A digital oscilloscope (Tektronix TDS 420 A, Chicago, IL) that displayed the signal.
- A computer that registers the resulting information.

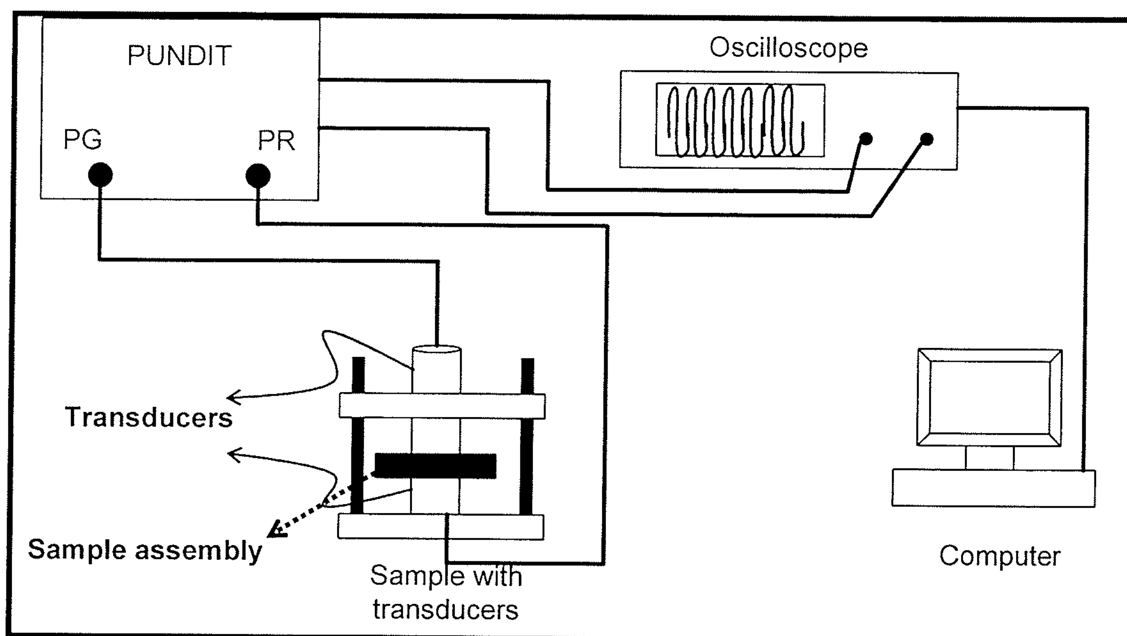


Figure 15. Block diagram of experimental set-up of the ultrasound equipment. PG is the voltage pulse to be sent to the generating transducer, PR is the received pulse coming from the detecting transducer

4.2.5 Ultrasound parameters for gluten doughs

Reference signals were taken each day prior to the testing of each set of samples. To acquire the reference signal a thin layer of coupling agent (Ultragel II, ultrasonic couplant, Sonotech Inc., Bellingham, WA) was put between the transducers. Gluten samples were then analysed at the five different thicknesses. Later it was decided to discard the results for the thinnest samples (0.5 mm) for all the treatments, due to the appearance of holes once the samples had been flattened to this thickness. The ultrasonic velocity and attenuation coefficient (α) of gluten doughs were determined, as these characterise the way in which the ultrasonic signal propagates through the gluten (Elmehdi, 2001). For analysing the results, the ultrasound wave data were first plotted on a time domain diagram

(signal amplitude *vs* time) in Origin (Microcal Origin, version 6.0, Microcal Software Inc., Northampton, MA); a typical pulse is shown in Fig. 16. From the waveforms, it was apparent that multiple reflections occurring within the glass plates were complicating the signal of the gluten doughs. As the result of this observation, it was decided to follow three different procedures in order to asses the best way of determining the ultrasonic velocity.

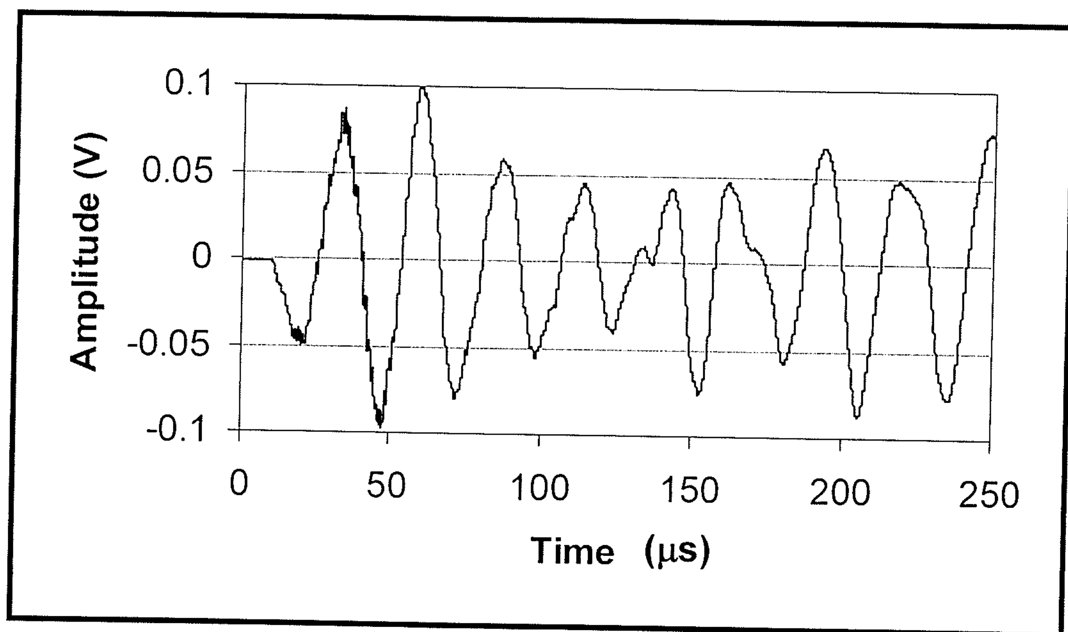


Figure 16. Typical ultrasonic waveform after passing through the gluten dough

The first procedure, called time domain (TD) analysis, consisted of the direct reading of the transit time at the point where the wave passed through the time axis (*x*). Each thickness of the sample had a different transit time, and so times were plotted against distance (sample thickness). The inverse of the slope of this graph permitted the velocity for a given sample to be determined since,

$$t = bd, \quad (19)$$

where t is the transit time [s], b is the slope (1/velocity) [s/m], and d is the thickness of gluten sample [m]. The second method used for the determination of the ultrasonic velocity was called the truncated-fast-Fourier-transform (T-FFT) procedure. The data were truncated (from 8 to 40.2 μs) and transformed by fast Fourier transform; the data were analysed in the frequency domain (Williams, 1999). Phase angle data (derived from the FFT) were taken at 30 kHz, since the signal showed its maximum at that frequency. From the phase data, transit time was calculated as described by Eq. 20 and plotted against thickness (Fig. 17),

$$t = \phi / (2\pi f), \qquad (20)$$

where t is the transit time [s], ϕ is the phase angle [rad], and f is the selected frequency (30 kHz). From the slope, the inverse of the velocity through the gluten dough was obtained (Eq. 19). The third procedure (MM) consisted of the application of a mathematical model that considered the reflections in the glass plates (Sukhovich, 2002). The MM consisted of a program that calculated the transmission of the ultrasonic wave through a layered medium (gluten samples assembled between the glass plates, Fig. 14). In order to correct for internal reflections, the settings for the program included known parameters of the glass plates (layer one and three), such as: density (2.48 g/cm^3), phase velocity (5.6 mm/μsec), thickness (2.82 mm) and attenuation coefficient (0.005 mm^{-1}). The two parameters known for layer two (gluten dough), density (Tables 20 & 21) and thickness (from 0.5 to 2.06 mm), were also set in the program. Starting with trial values of the ultrasonic velocity and the attenuation in the gluten dough, the predictions of the MM were compared with the experimental data for the

transmited phase and amplitude. The values of velocity and attenuation in the gluten dough that gave the best fit to the data were then determined iteratively. As a result, the program determined the phase and the attenuation coefficient of the gluten dough after correcting for the phase shifts due to multiple reflections in the glass. After comparing the results using the three methods of analysis (TD, T-FFT, and MM), the T-FFT (frequency domain) procedure was chosen as the best for ultrasonic velocity results. These results showed no systematic differences when analysed by the T-FFT method as compared from the results obtained from the MM method, with the advantage of being a simplier procedure to follow (Table 18).

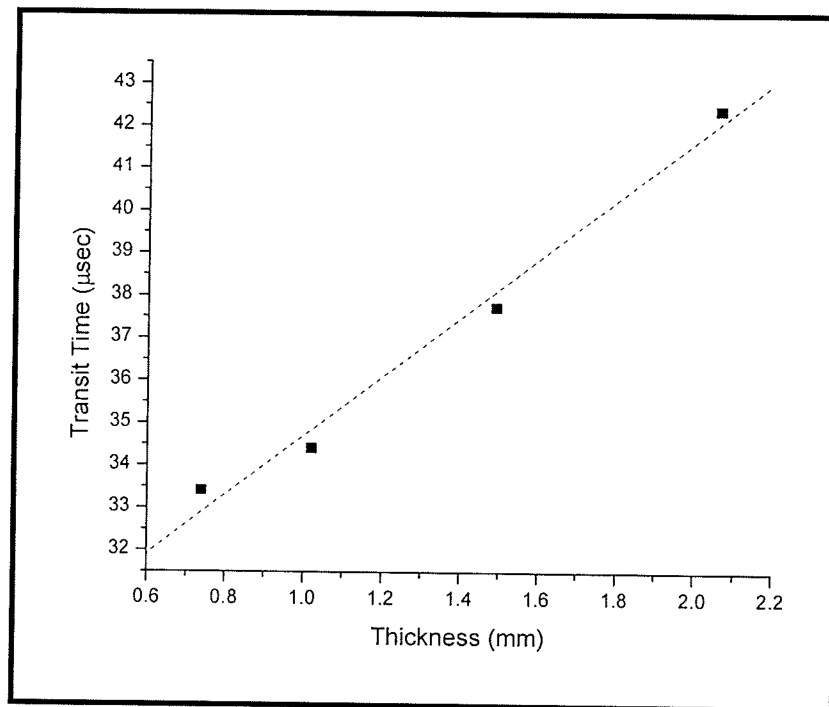


Figure 17. Typical results for the thickness dependence of the transit time, from which the ultrasonic velocity was determined

To obtain the attenuation coefficient, three analysis methods were used as well. The first one consisted of the direct reading of the signal amplitude from the first peak that occurred in the time domain data (TD method). For the second method the data were truncated and fast Fourier analysed (T-FFT method) as described in the previous paragraph, and the amplitude was read directly from the magnitude of the FFT at 30 kHz. The third method consisted of using the mathematical model (MM) above described for predicting the amplitude of the ultrasonic waves through the gluten doughs. The amplitude readings from each method were then plotted *vs* distance (thickness) using a log scale on the y axis (Fig. 18), from which the attenuation coefficient was obtained using Eq. 21,

$$\log A = \log A_0 + (-\alpha x/2)(\log(e)), \qquad (21)$$

where the slope is $((-\alpha/2) \log(e))$ and α is -2(slope) / 0.434. Eq. 21 is obtained from

$$A = A_0 \, e^{-\alpha(x/2)}, \qquad (22)$$

where A is the signal amplitude at a thickness x [V], A_0 is the signal amplitude at the leading edge of the dough sample [V], α is the attenuation coefficient [mm^{-1}], and x is the thickness of the dough sample [mm] (Fig. 18).

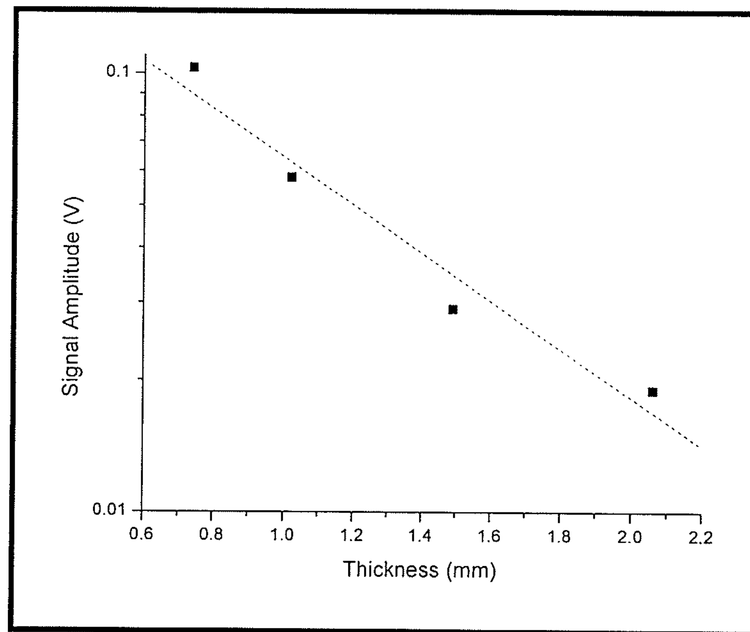


Figure 18. Typical results for the thickness dependence of the transmitted amplitude, from which the attenuation coefficient of gluten dough was obtained

Based on the attenuation coefficient results obtained with the three methods, it was decided that the TD method was more appropiate since it simplified the analysis work and no systematic differences were found. An example of the ultrasonic velocity and attenuation coefficient results obtained for the same sample by the three procedures is shown in Table 18.

Table 18. A typical example for comparing the three methods when obtaining the ultrasonic velocity and attenuation coefficient of the gluten dough

Prodecure	Ultrasonic velocity determinations (m/s)	Attenuation coeffiecient determinations (mm^{-1})
TD	92.5	3.0
T-FFT	96.3	3.1
MM	96.1	3.7

TD Time domain method
T-FFT Truncation-fast-Fourier-transform method
MM Mathematical model method

4.2.6 Statistical analysis

Statistical analysis was conducted with Statistical Analysis System software (the SAS system for Windows V8, SAS Institute Inc., Cary, NC, USA). Data were evaluated by analysis of variance (ANOVA) with a criterion of $P \leq 0.01$ to detect significant differences among treatments; Scheffe's multiple-comparison procedure was used.

The treatments analysed were:

- Effect of acid concentration on ultrasonic velocity and attenuation coefficient (α) of gluten doughs, comparing types of acid at the same pH of the dough and the same acid at different pH values.
- Effect of salts on ultrasonic velocity, β and α, comparing different salts with and without acetic acid.
- Effect of acetic acid on ultrasonic velocity, β and α of salty doughs.

For the analysis of ultrasound properties, two criteria were taken into account in order to determine the validity of replicates for each treatment. The first criterion was related to the goodness of the fit of the slope ($r^2 \geq 0.90$) obtained from the dependence of time against distance (velocity) and amplitude against distance (α). The second criterion was that the coefficient of variance (Covar) within replicates should not exceed a value of 40%, since higher Covar values denote low reproducibility of the treatment. Following the previous critera the raw data analysed for velocity and α consisted of at least a duplicate of the values *per* dough, and in many cases in triplicate.

4.3 RESULTS

4.3.1 Effects of acids and salts on gluten dough development

Gluten doughs were mixed to optimal consistency, regardless of the type of treatment (i.e., addition of acids, salts or their combination). Differences in mixing times were noticed for each type of dough. In general, the presence of acids weakened gluten doughs since shorter mixing times were necessary for optimal consistency of the gluten. The addition of salts (with and without the combination of acetic acid) made gluten doughs "stronger". An illustration of this was the need to shift the mixograph spring (attached to the arm that holds the mixing bowl) so that the movement of the mixing bowl would be restricted. The addition of acetic acid to the salty gluten doughs caused no significant differences ($P \leq 0.01$) in mixing times when compared to those for the corresponding salty doughs. Magnesium and aluminium salts made gluten doughs stronger (with longer mixing time) when compared to doughs mixed with sodium chloride. Mixing time for salty doughs with and without the addition of acetic acid are shown in Table 19.

Table 19. Effect of salts on mixing time of gluten doughs

Treatment	Salt + HAc concentration (mM)	Mixing time (min)
Control dough	0 + 0	10.5*
HAc	0 + 3.5	9.1*
NaCl	29.5 + 0	7.5 ± 0.29 b
NaCl + HAc	29.5 + 3.5	7.7 ± 0.24 b
$MgCl_2$	9.8 + 0	9.0 ± 0.03 a
$Mg\ Cl_2$ + HAc	9.8 + 3.5	9.5 ± 0.33 a
$AlCl_3$	4.9 + 0	9.5 ± 0.33 a
$Al\ Cl_3$ + HAc	4.9 + 3.5	9.7 ± 0.29 a

* Values obtained from Manuscript I. These mixing times are not directly comparable to the following ones (for salty doughs), since a shift on the mixograph spring (from 10 to 15) occurred for the mixing of the salty doughs
HAc Acetic acid
Mixing time values are the mean ± SD , n= 3
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

The results of the mixing experients for the salty doughs are consistent with previous studies by different researchers (Hlynka, 1962; Galal, *et al.*, 1978; Danno & Hoseney, 1982) who noticed the strengthening effect of salts on the mechanical properties of flour doughs. Furthermore, the effect of different cations on gluten doughs from this work corresponded with those obtained by Butow *et al.* (2002), who noticed the importance of different cations on altering the mixing properties of flour doughs. The general belief is that the presence of salts leads to conformational changes of gluten polymers. Salts suppress electrostatic repulsions within and between the polymer chains (Galal *et al.*, 1978), as opposed to promotion of repulsions which is the case with acidification of the gluten dough.

4.3.2 Effects of acids and salts on gluten dough physicochemistry

The pH of the gluten dough was decreased in five steps from 6.3 (control dough, no acids addition) to 4.3. The decrement in pH was attained with the addition of both acetic and hydrochloric acid. Despite the change in pH of the gluten dough, its density remained practically the same. In the case of the addition of acetic acid, there was no clear trend in density variation with acid concentration; density of the gluten was lowest at a pH of 5.8 (Table 20). The density of the dough with the lowest pH value (4.3), after the addition of hydrochloric acid, was significantly higher ($P \leq 0.01$) compared to the other doughs (Table 21).

Table 20. Density of acetic acid gluten doughs

Dough pH	Dough density (g/cm^3)
6.3	1.07 ± 0.007 a
5.8	1.02 ± 0.013 b
5.3	1.05 ± 0.018 ab
4.8	1.03 ± 0.014 ab
4.3	1.03 ± 0.020 ab

Density values are the mean ± SD, n=4
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

Table 21. Density of hydrochloric acid gluten doughs

Dough pH	Dough density (g/cm^3)
6.3	1.07 ± 0.007 b
5.8	1.04 ± 0.015 b
5.3	1.05 ± 0.008 b
4.8	1.03 ± 0.015 b
4.3	1.11 ± 0.016 a

Density values are the mean ± SD, n=4
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

Addition of salts lowered the pH of the dough in comparison to the control dough (with a pH value of 6.3) (Table 22). However, when acetic acid was added, pH was not further reduced as a result of the presence of the salt (since 3.5 mM would have attained a value of pH 5.8 on its own). Density of the gluten dough was affected by the type of cation (i.e., Mg^{2+}, Al^{3+}), the higher the valence number, the lower the density of the gluten; the only salt that did not affect gluten dough density was sodium chloride ($P \leq 0.01$) (Table 22).

Table 22. pH and density of salty gluten doughs, with and without addition of acetic acid

Salty dough	Dough pH	Dough density (g/cm^3)
NaCl	6.0 ± 0.05 ab	1.07 ± 0.003 a
NaCl + HAc	5.8 ± 0.04 d	1.07 ± 0.007 a
$MgCl_2$	6.1 ± 0.04 a	1.06 ± 0.007 ab
$MgCl_2$ + HAc	5.9 ± 0.11 c	1.05 ± 0.006 ab
$AlCl_3$	6.0 ± 0.08 bd	1.04 ± 0.010 b
$AlCl_3$ + HAc	5.7 ± 0.04 d	1.04 ± 0.007 b

HAc Acetic acid
pH and density values are the mean ± SD, n=15 for pH & n=4 for density
Means in a column with the same letter are not significantly different ($P \leq 0.01$)

4.3.3 Effects of acids and salts on the ultrasonic properties of gluten doughs

In general, no significant differences ($P \leq 0.01$) in the ultrasound results (velocity or α) of the gluten doughs were found as the result of changes in the pH of the doughs. Therefore it was decided to interpret the data based on the trends followed by each ultrasonic parameter within the range of pH studied.

In the presence of acetic acid, the velocity of the ultrasound propagating through the gluten dough increased, until the gluten dough pH was 4.3, at which

point the ultrasonic velocity decreased (Fig. 19). For hydrochloric acid doughs, ultrasonic velocity did not show such a change in its trend when the concentration of the acid was increased to attain a pH of 4.3 (Figs. 19). The attenuation coefficient (α) of acetic doughs showed an increase as the pH dropped in the range of pH from 6.3 to 4.8 (Fig. 20).

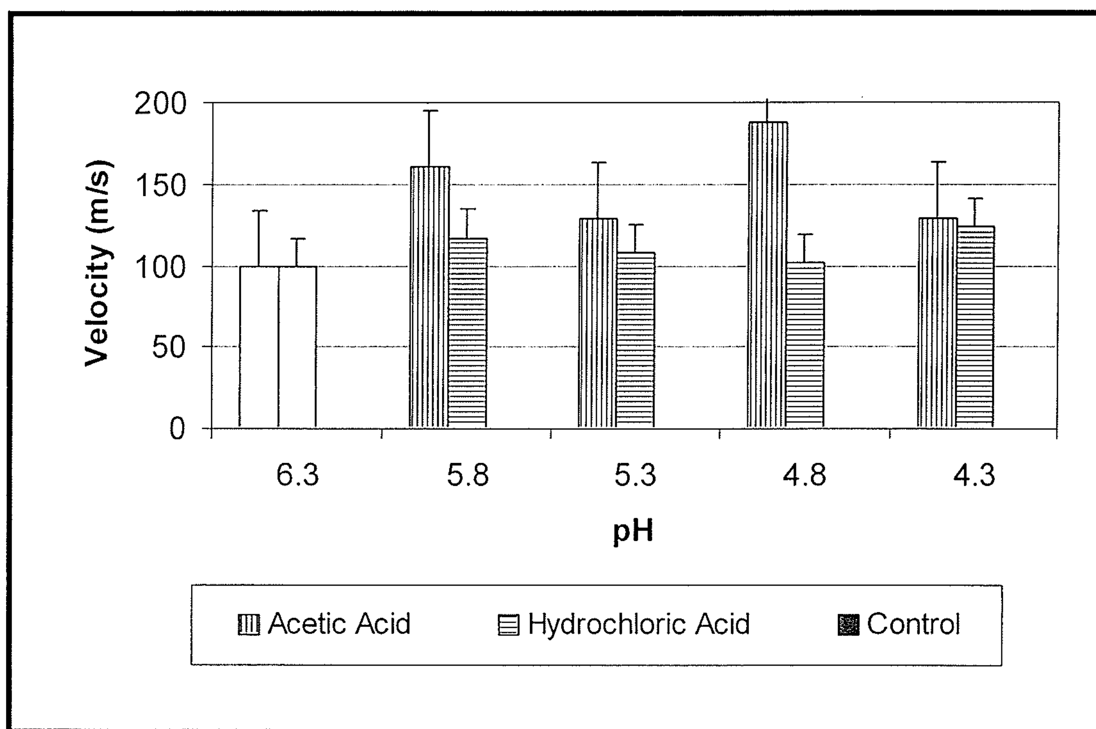


Figure 19. Effect of type and amount of acid on ultrasonic velocity through gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all dough pH values obtained with the same acid)

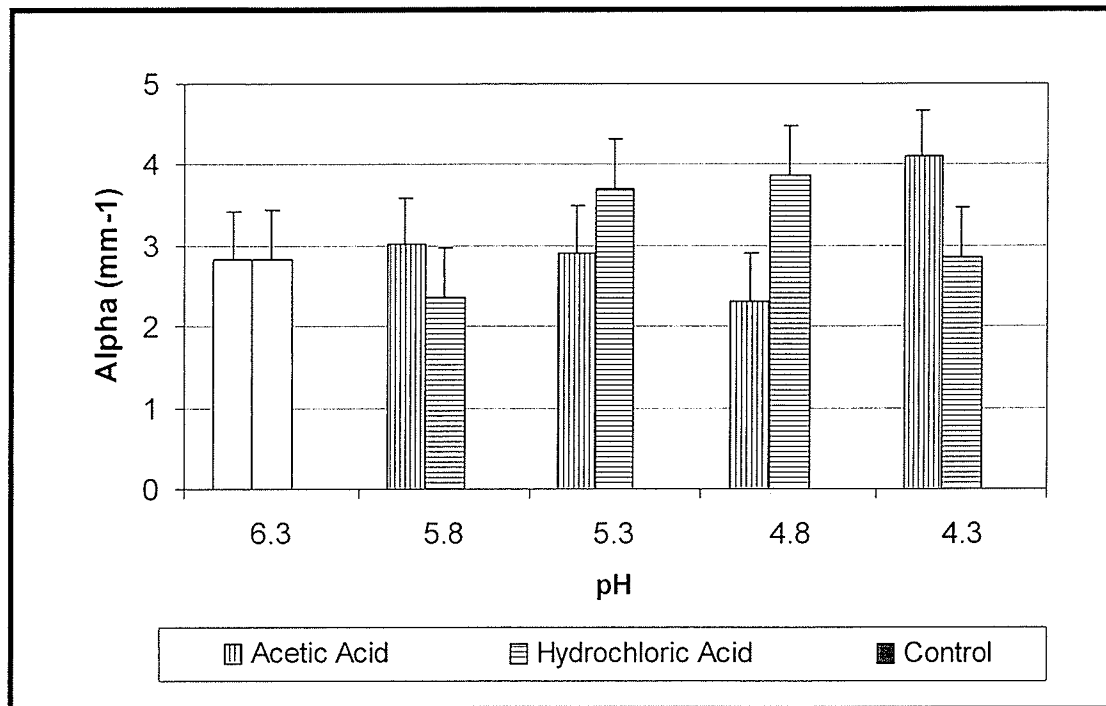


Figure 20. Effect of type of and amount of acid on the attenuation coefficient (alpha) of gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all dough pH values obtained with the same acid)

Values obtained here for ultrasonic velocity are similar to those previously reported by Elmehdi and Kovacs (2003) for gluten doughs from soft wheat (velocity value around 125 m/s). The pH value of 4.3 had an interesting effect on gluten doughs, this value representing a key point in the effect of acids on the ultrasonic and mechanical properties of the doughs.

When the statistical analysis was performed on the salty gluten doughs, again no significant differences were found, and thus the results are interpreted in terms of trends in the ultrasonic parameters. The presence of salts did not affect the ultrasonic parameters of the gluten dough (velocity and α). However, when acetic acid was added, the velocity of the salty doughs exhibited an increasing trend with cation valence (Fig. 21).

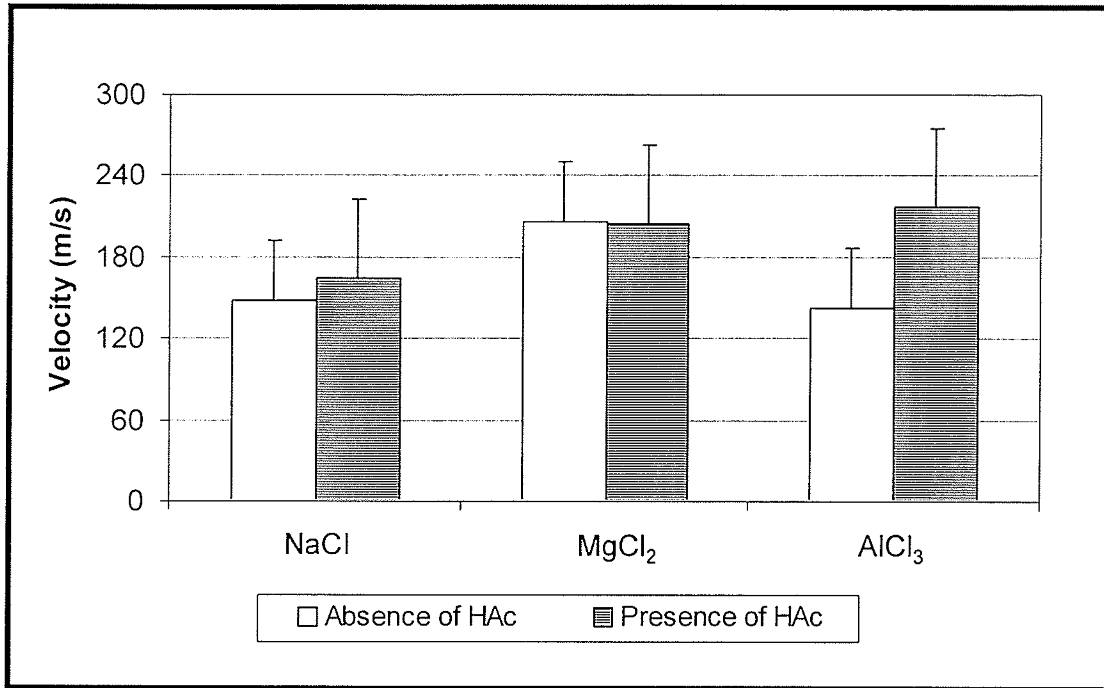


Figure 21. Effect of type of salt on ultrasonic velocity through gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all types of salt with or without acetic acid)

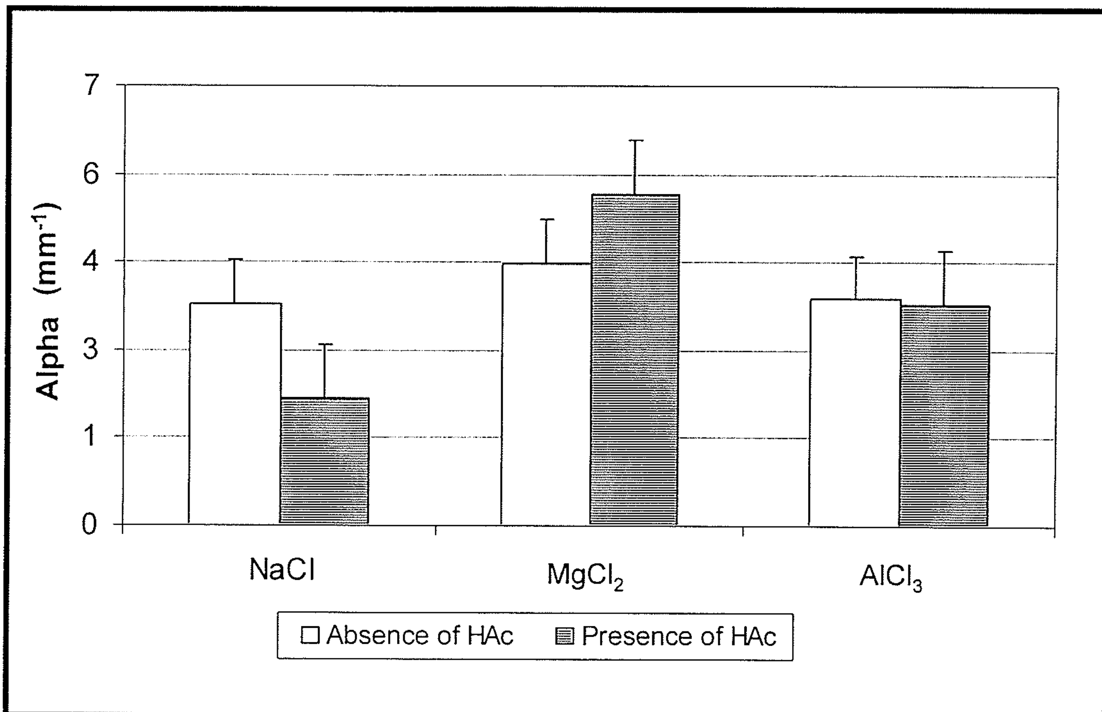


Figure 22. Effect of salt type on the attenuation coefficient (alpha) of gluten dough ($n \geq 2$, error bars represent the average of the standard deviations for all types of salt with or without acetic acid)

On average, the attenuation coefficient of sound propagating through the gluten doughs was 3 and 3.2 mm^{-1}, respectively, when hydrated with acetic and with hydrochloric acid (Fig. 20). Therefore, the large attenuation means that treating the gluten as an elastic material is not entirely valid. As a result, the elastic or storage longitudinal modulus (β') and the viscous or loss longitudinal modulus (β") can be calculated from the density, velocity and attenuation coefficient results, as given by,

$$\beta' = [\rho v^2 (1-\alpha^2 v^2/4\omega^2)] / (1+\alpha^2 v^2/4\omega^2)^2 \quad \Leftrightarrow \quad (\alpha v/\omega < 1), \qquad (23)$$

$$\beta'' = [2\rho v^3 (\alpha/2\omega)] / (1+\alpha^2 v^2/4\omega^2)^2, \qquad (24)$$

(Elmehdi, 2001). However, the attenuation coefficient was so large that the longitudinal storage modulus became negative for some pH values. Because the physical meaning of a negative storage modulus has being questioned (Létang *et al.*, 2001), just the β" and not the β' values are reported (Fig. 23).

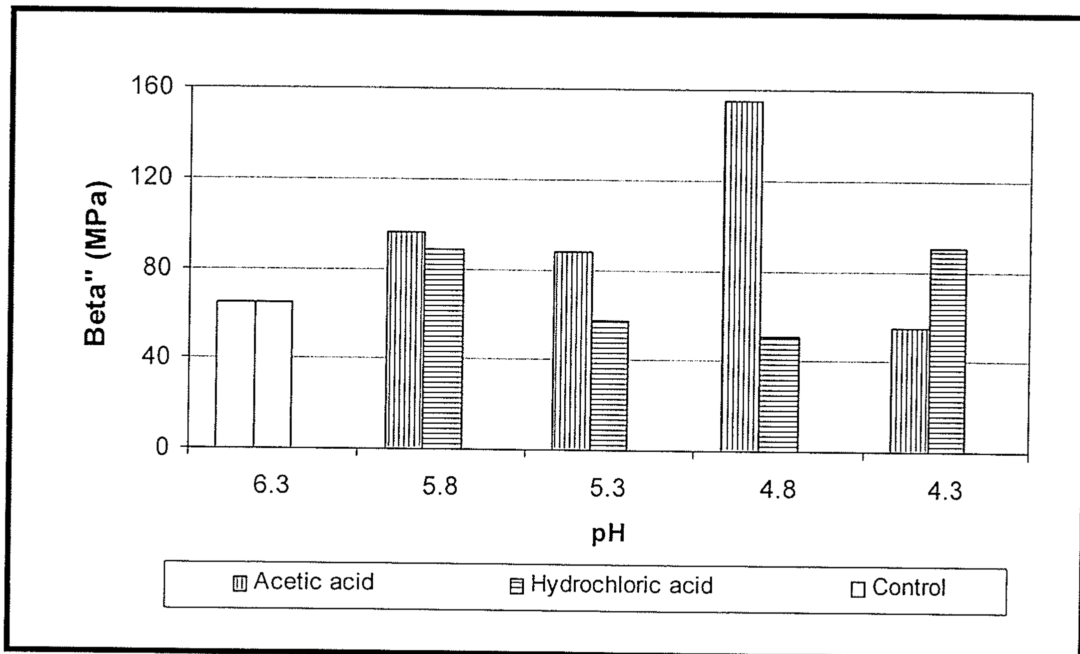


Figure 23. Effect of type of acid and pH on the loss longitudinal modulus (beta") of gluten dough

In a similar manner, the attenuation coefficients of the salty gluten doughs with and without addition of acetic acid (average values of 3.8 and 3.6 mm^{-1}, respectively) were very high. Therefore, only β" results are presented (Fig. 24).

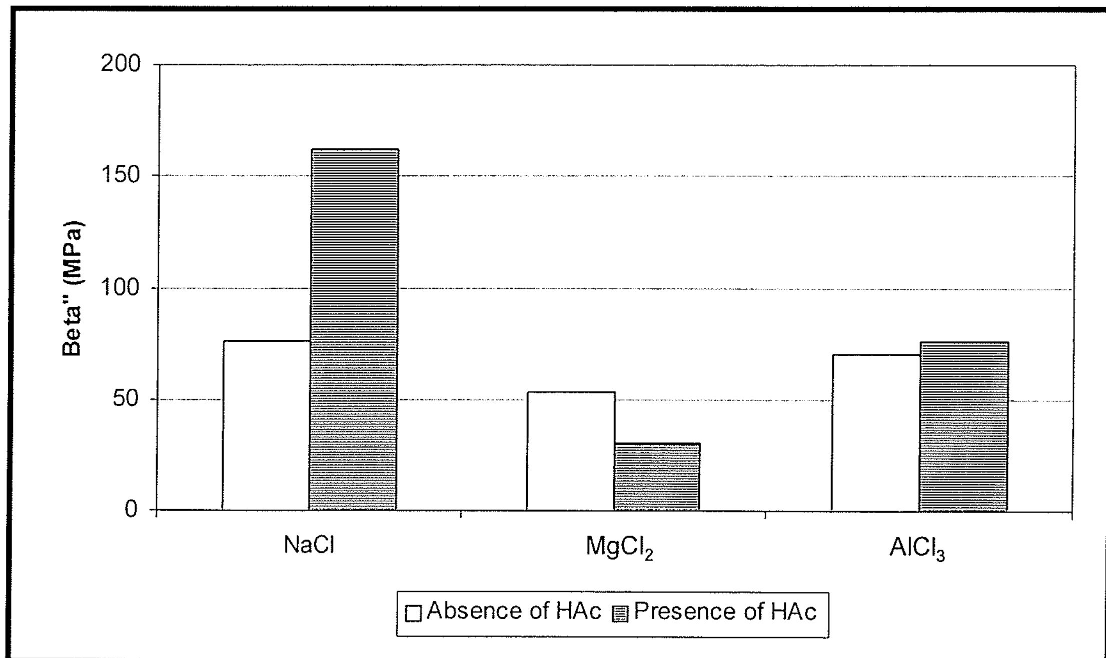


Figure 24. Effect of salt type with and without addition of acetic acid on the loss longitudinal modulus (beta") of gluten dough

4.4 DISCUSSION OF RESULTS

The pH of the control gluten dough is below the isoelectric point of the proteins (7.5), and thus a net positive charge on the gluten polymers is expected (Wu & Dimler, 1963[a]). The number of positive charges in the gluten dough is then enhanced when either acid or hydrochloric acid solutions are added (Cheftel & Cuq, 1985). As the result of an excess of positive charges, electrostatic repulsions take place between the gluten polymers (Bennett & Ewart, 1962). These repulsions cause a breakage of non-covalent bonds (i.e., hydrogen bonds), which led to a weaker dough with shorter mixing times (Smith and Mullen 1970; Galal *et al.*, 1978).

Following the "train and loop" model proposed by Belton (1999), hydrogen bonds play a very important role in stabilising the gluten structure. As a result of the addition of acids and the breakage of hydrogen bonds, an opening of the protein structure is likely (more and bigger loops). The opening of the loops may allow the entrance of the anions (Ac^- and Cl^-) in a selective manner. Chloride ions are smaller and faster than acetate ions, as denoted by their hydration volume (93.6 cm^3/mol for Cl^- *vs.* >113 cm^3/mol for Ac^-) (Marcus, 1985) and their ionic mobilities (76×10^{-5} cm^2/Vs for Cl^- *vs.* 41×10^{-5} cm^2/Vs for Ac^-) (Brey, 1978 as cited in Voet & Voet, 1990). The size and mobility of chloride ions therefore enables them to be sorbed more easily by the gluten structure compared to acetate ions. A higher suppression of positive charges by the conjugated base in the gluten dough when acidified with hydrochloric acid would lead to the

further incorporation of more hydronium ions in the gluten. Therefore, higher concentrations of hydrochloric acid were needed to obtain the same pH of gluten doughs compared to when acetic acid was added (Tables 20 & 21). According to this theory, chloride ions show a positive effect in terms of buffering capacity.

Differences in density of the most acidic gluten dough (with a pH of 4.3) showed as well that the type of anion had an important effect on gluten structure (Tables 20 & 21). Although it is proposed in this thesis that chloride ions are sorbed by the gluten proteins, it is not expected that all of these ions are entrapped between the polymers. Therefore it is still possible that there is suppression of positive charges on the surface of the protein as the result of electrostatic interactions between chloride ions and positively charged amino acids (Voet & Voet, 1990). As the result of a higher capacity for electrostatic shielding, chloride ions would likely induce more hydrophilic and hydrophobic interactions between protein polymers compared to what is achievable by acetate ions (Preston 1981, 1984, 1989). The increase in protein interactions led to both a stronger (longer mixing time) and a denser gluten dough at a pH value of 4.3 when hydrochloric acid was added in comparison to acetic acid dough at the same pH (Table 21).

The addition of acids led to a more charged and thus hydrophilic gluten structure that hydrates faster (Danno & Hoseney, 1982). Acetic acid has high affinity for glutamine residues, preventing them from interacting with each other so that they do not form intermolecular hydrogen bonds (Bennett & Ewart, 1962; Wellner *et al.*, 2003). In this way, hydrogen bonds between water molecules and glutamine residues are enhanced and thus more water is bound to the

protein polymers when acetic acid is added. Bound water to the protein has been shown to cause the ultrasonic velocity to be higher than when present as free or bulk water due to less frictional forces between the protein polymers (Ross *et al.*, 2004). Therefore the trend of an increase in the ultrasonic velocity with a reduction in pH for the acetic acid gluten dough is more evident than in the case of hydrochloric acid (Fig. 19).

Another possible explanation for the increase in ultrasonic velocity is based on the extent of electrostatic repulsions between positively charged side-chains on the protein polymers and their effect on gas cell sizes (Elmehdi *et al.*, 2003). As the result of addition of acids, the electrostatic repulsions between gluten polymers increases and thus hydrogen bonds between gluten polymers are broken. Following Belton's loop and train model (1999), the disruption of hydrogen bonds between the gluten proteins leads to an increase in the size of the loops. Since chloride ions are smaller (Marcus, 1985), it seems possible for them to be included more easily in the "induced loops" compared to the acetate ions.

The sorbed chloride ions will reduce the electrostatic repulsions between the gluten polymers, whereas the lack of counter ions inside the loops (in the case of addition of acetic acid) will not allow regulation of the electrostatic repulsions. The size of the induced loops in the presence of acetic acid will then be bigger than when hydrochloric acid is added, although the size of the loops will still not be enough for allowing the entrance of acetate ions. As the result of bigger loops in the dough with acetic acid, smaller gas cells are expected. Differences in size of the gas cells entrapped in doughs leads to differences in

ultrasonic velocity, whereby the presence of smaller gas cells is reflected by higher ultrasonic velocity (Elmehdi *et al.*, 2003). Lower electrostatic repulsions in the gluten dough with hydrochloric acid, due to greater suppression of positive charges, would lead as well to a lower pressure (and less shrinkage) of the gas cells (Létang *et al.*, 2001).

The generally high attenuation of gluten doughs may be attributed to scattering of the ultrasonic wave (due to a non homogeneous system), and to high viscous dissipation (related to the absorption of the ultrasonic energy) (Samari, 1994). In general, a high attenuation coefficient in protein systems is due to the protonation of the acid and base groups in the protein (Dunn, 1969). Therefore, the higher the concentration of the acid in the dough, the higher the protonation of the amino acid side-residues and thus the higher the attenuation coefficient of the gluten dough (Fig. 20). Moreover, the increase in the attenuation coefficient of the gluten dough with acetic acid may be due to the increase in bound water in the gluten dough (Létang *et al.*, 2001). Changes in the bulk viscosity of flour doughs have been attributed to changes in the continuity between their phases; the more discontinuities in the dough, the higher the dissipation of the ultrasound (Létang *et al.*, 2001). Therefore, the acetic acid (with and without the addition of salts) must have the effect of increasing the discontinuity of the gluten dough as shown by changes in the viscous dissipation (Figs. 23 & 24). The creation of a more compact structure due to the addition of hydrochloric acid when compared to acetic acid, may create a more continuous gluten dough with less noticeable dissipation of the ultrasound.

When salts are used as additives for dough preparation, electrostatic repulsions within the gluten polymers are suppressed and the interactions between hydrophobic amino acid side-residues enhanced (Preston, 1981, 1984, 1989; Butow *et al.*, 2002). These interactions lead to stronger doughs (Table 19) (Danno & Hoseney, 1982). Although the effect of cations on dough functionality has been generally considered as negligible, in this work their importance was observed as it was by He *et al.* (1992) and Butow *et al.* (2002). Chloride salts of different cations (i.e., Na^{+}, Mg^{2+}, and Al^{3+}) showed differences in mixing times (Table 19). Although $MgCl_2$ and $AlCl_3$ had a lower concentration of chloride ions for suppressing the positive charges in the dough, their addition increased the mixing time. Therefore, it appears likely that salt bridges of the cations with internal protein functional groups (i.e., their oxygen atoms) took place (Nayal & Di Cera, 1994; Balla *et al.*, 1998; Rao & Seib, 1999; Butow *et al.*, 2002).

Of the three cations (Na^{+}, Mg^{2+}, and Al^{3+}) that were used as chloride salts, the least chaotropic and the one with the smallest hydration volume is the sodium ion (Damodaran & Kinsella, 1982; Marcus, 1985). Therefore, sodium is the most sterically favoured of the cations for sorption by the gluten proteins. Due to its less chaotropic nature, sodium will promote non-polar aggregation of the protein side-chain residues and thus enhance hydrophobic interactions. As the result of greater hydrophobic interactions, a more compact gluten structure is obtained when compared to doughs with magnesium and aluminium salts (He *et al.*, 1992; Butow *et al.*, 2002). This difference in volume of the structure led to a decrease in density of the salty doughs as the valency number of the cations

increased (Table 22). The concentration of chloride ions was higher when added as the sodium salt than when added as $MgCl_2$ and $AlCl_3$ (Table 19). Therefore more chloride ions were available for drawing away water molecules from the gluten proteins (Balla *et al.,* 1998), which led to a more compact gluten structure and thus a denser dough (Table 22). The presence of salts in the gluten also appeared to affect gluten conformation, exposing acidic amino acid side chains, since the pH of the dough went down even in the absence of acetic acid.

Conformational changes of the gluten proteins take place when salts are added to dough (Galal *et al.*, 1978; Danno & Hoseney, 1982; Belton, 1999; Butow *et al.*, 2002). The changes probed here using ultrasound may be related to the effect of chloride ions on the availability of water in the dough system and on the effect of the type of cations on the protein polymers. When aluminium chloride was added, the concentration of chloride ions was less and thus less shielding of the positive charges of the gluten proteins occurred (Table 19). Therefore a higher pressure is expected to be exerted on the gas cells by the matrix, and thus smaller cells are expected in the dough with aluminium chloride than in the gluten dough made with sodium and magnesium chloride. This difference in size of the gas cells, leads again to the differences in ultrasonic velocity (Fig. 21). Another factor to be considered is the ability of magnesium and aluminium ions to form salt bridges (He *et al.*, 1992; Butow *et al.*, 2002). It is important to notice that without the addition of acetic acid, the increase in the ultrasonic velocity with cation valency is not observed and thus an enhancement of electrostatic repulsions has to occur for the effect of salts to be noticed.

4.5 CONCLUSIONS

The addition of either acetic or hydrochloric acid led to conformational changes in gluten doughs, surmised to be due to broken hydrogen bonds. The type of conjugated base (anion) was relevant when accounting for anion effects on gluten structure when the acids were added. Two mechanisms are proposed to explain anion effects on gluten structure. The first mechanism follows the loop and train model for gluten proteins (Belton, 1999). The size of the loops acts as a selective barrier for anions to be sorbed by the gluten polymers. The second mechanism is the affinity of acetic acid for glutamine residues, which disrupts glutamine-glutamine hydrogen bonds and enhances water-glutamine bonds. The reduction in mixing time as the result of addition of acetic acid to gluten doughs (and thus decreased pH) was consistent with the increase in ultrasonic velocity, except at a pH of 4.3. In general, a weaker acetic acid dough results in a faster ultrasonic velocity with higher dissipation of ultrasound energy.

Cation type (at the same ionic strength and low concentrations: below 0.05 M) proved to be an important factor for determining the effect of chloride salts on the properties of gluten dough. Magnesium and aluminium ions promote the formation of salt bridges of the gluten polymers, while the effect of sodium ions is due to their higher non-chaotropic nature. The effect of chloride ions on gluten structure is related to their ability to draw away water molecules from the protein polymers. The addition of salts enhances the interaction of hydrophobic residues within the gluten polymers. The effect of salts is

enhanced with the addition of acetic acid as assessed by density and ultrasonic velocity determinations.

5. MANUSCRIPT III

^{1}H NMR spectroscopy of gluten proteins under acidic conditions

ABSTRACT

Proton nuclear magnetic resonance (^{1}H NMR) is nowadays recognised as a very useful tool in the analysis of biological molecules. Moreover, the use of gradient pulses has made NMR a spectroscopic technique with the ability to measure molecular diffusion coefficients. Pulsed gradient NMR has been quite extensively used in porous media, but less so in food science applications. Nevertheless, pulsed gradient NMR has proved to be useful for analysing the properties of some food systems, including those of wheat gluten. Wheat gluten constitutes a biological polymer with broad applications that possesses unique viscoelastic properties. The addition of acids to wheat flour and gluten doughs has been shown to affect the functional properties of the gluten proteins. Although these effects have been studied for over 50 years, a complete understanding of the mechanisms by which the functional properties are altered is still far from complete. This part of my thesis studied the effect of acids when added to gluten doughs, as assessed by pulsed gradient ^{1}H NMR. Gluten doughs were prepared from Canadian and Australian gluten, with the addition of either water, acetic or hydrochloric acid. The Canadian gluten dough was also prepared with deuterium. The parameters analysed from the ^{1}H NMR spectra

were nuclear resonance (chemical shifts), spin-spin (T_2) relaxation times and diffusion coefficients (D) of the molecules. From the ^1H NMR spectra, water, lipids and protein components were identified. With the use of two-dimensional (2D) Laplace inversion, D/T_2 correlation maps were obtained. From these, two main components were observed for both Canadian and Australian gluten doughs (with and without the addition of acids). A component with a D value of 2.7×10^{-10} m^2/s and a T_2 value of 0.03 s was attributed to water linked to the protein polymers. The other component was attributed to free lipids present in the gluten doughs (D value of 2×10^{-12} m^2/s and T_2 value of 0.1 s). Individual studies of T_2 relaxation times were performed by using 1D Laplace inversion. Four components with different T_2 values were found for both gluten doughs at very short (< 10 ms), short (20-50 ms), long (100-200 ms), and very long relaxation times (>500 ms). As the result of addition of acids, differences were observed between the control and acidic gluten doughs from the ^1H NMR spectra and the T_2 determinations. The resonance signal of 1.9 ppm attributed to lipids was enhanced on the ^1H NMR spectra. Very short T_2 relaxation time (<10 ms) for the water present in the acidic gluten doughs was encountered, while this T_2 relaxation time component attributed to aliphatic regions disappeared. The results from the ^1H NMR spectra and the T_2 results suggest conformational changes of the gluten proteins due to the presence of acids. These conformational changes promote hydration of the gluten polymers as the result of breakage of bonds between amino acid side-residues, as well as the exposure of aliphatic amino acids and lipids likely due to the loss of hydrophobic interactions.

5.1 INTRODUCTION

Proton nuclear magnetic resonance (^1H NMR) spectroscopy is a powerful analytical technique. NMR has a number of capabilities, e.g., distinguishing between chemical species in a sample, as well as being able to determine molecular structure, concentration and diffusion (Gladden & Alexander, 1996; Callaghan & Gil, 1999; Seymour *et al.*, 2000; Calucci *et al.*, 2003).

One commonly used technique for NMR measurements is the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. From CPMG pulsed NMR, direct measurement of the relaxation times of the nuclear spins is obtained (Calucci *et al.*, 2003; Zhang & Hirasaki, 2003). Spin-spin (T_2) relaxation time is an NMR parameter that relates to the rotational motion and diffusion of the molecules within the sample. Therefore, T_2 values are indicative of aspects of molecular structure and the nature of interactions of some components in a sample (Gil *et al.*, 1997; Roudaut *et al.*, 1998).

Another NMR technique that is widely used is the Pulsed Gradient Spin Echo (PGSE) technique. The PGSE technique allows the measurement of the Brownian or random translational motion of molecules. This motion is determined using the PGSE method without the need for concentration gradients within the sample, so that it determines the true molecular self-diffusion coefficient (D) of the nuclei of interest. D is obtained from the attenuation of the echo amplitude of the NMR signal as a function of diffusion of the molecules after the application of a magnetic gradient (Stejskal & Tanner,

1965; Price, 1997; Antalek, 2002; Callaghan *et al.*, 2003[ab]). A correlation between T_2 and D gives information about the distinction between molecular interaction and molecular motion (Kleinberg & Horsfield, 1990; Godefroy & Callaghan, 2003).

The widespread utilisation of NMR can be attributed to its specificity, non-destructive nature and usefulness in the determination of water movement, a measurement which can also indicate water-association (Umbach *et al.*, 1992; Cherian & Chinachoti, 1996). NMR has found a large number of applications in food systems, although mainly in liquid foods such as: wine, fruit juices, beer and olive oil (Gladden & Alexander, 1996; Ogrinic *et al.*, 2003; Duarte *et al.*, 2004). Furthermore, some NMR studies have been made on semi-solid foods (mangoes, apples, cheese, cereals) and some food components (proteins and lipids) (Belton *et al.*, 1997; Gil *et al.*, 2000; Gil *et al.*, 2002; Godefroy & Callaghan, 2003).

A biopolymer with wide applications within the food and packaging industry is the storage proteins (prolamins) of wheat, known as gluten proteins (Magnuson, 1985; Cuq *et al.*, 1998; Redl *et al.*, 1999). The importance of these proteins relies basically on their viscoelasticity when hydrated (Popineau *et al.*, 1994; Wadhawan, 1988; Eliasson & Larsson, 1993; Gras *et al.*, 2001). Although gluten proteins are of great importance, a complete understanding of the mechanisms by which their functional properties are altered as a result of processing conditions is still far from complete. This lack of knowledge has been generally attributed to the complex composition of gluten as well as to the many intra- and inter-molecular interactions that take place during the

development of the dough (Grant *et al.*, 1999; Calucci *et al.*, 2003). Therefore, the ability of PGSE NMR to determine structural properties and molecular dynamics in a non-invasive way, indicates that it is a very promising spectroscopic technique for studying the complex gluten proteins. A number of NMR studies have been carried out in order to probe the molecular structure of gluten proteins and to examine their response to different factors (such as hydration and temperature). These studies are generally based on measuring the nature of hydration and motion of water molecules associated with the proteins (Umbach *et al.*, 1992; Belton *et al.*, 1995; Roudaut *et al.*, 1998; Grant *et al.*, 1999; Wang *et al.*, 2001; Esselink *et al.*, 2003[b]; Feeney *et al.*, 2003). However, not many NMR studies have been done examining the effect of processing products or additives on the properties of gluten doughs (Cherian & Chinachoti, 1997; Wellner *et al.*, 2003). During fermentation of dough, the generation of carbon dioxide by yeast causes the pH to drop (Miller *et al.*, 1994). This leads to changes in the charge on the gluten polymers (Galal *et al.*, 1978). It has been shown by rheological techniques that when acids are used to alter dough pH, they have a significant effect on gluten dough viscoelasticity. In general, these effects have been attributed to conformational changes of the gluten dough (Bennett & Ewart, 1962; Galal *et al.*, 1978; Sekhon *et al.*, 1995; Shiau & Yeh, 2001; Larsson, 2002).

This part of my thesis focussed on the study of the effects of acids on gluten doughs as assessed by NMR spectroscopy. The general hypothesis is that the presence of acids will promote conformational changes in the gluten

proteins that can be detected by parameters measured by NMR (chemical shift, T_2 and D).

5.2 MATERIALS AND METHODS

5.2.1 Materials

Two commercial wheat glutens were used, a Canadian and an Australian one. The Canadian flour gluten came from spring wheat premium quality and was purchased from Premier Cereals, Inc. (Winnipeg, MB). The Australian flour gluten was purchased from Healtheries of NZ Limited (Auckland, NZ). Both Canadian and Australian glutens were analysed for their water and protein composition (N x 5.7), by AACC methods (44-15 A & 46-13, respectively, AACC 1983). The results of the moisture analysis were 6 ± 0.1% for the Canadian gluten and 7 ± 0.2% for the Australian one. The protein content was 70%±1.7 for the Canadian gluten and 72%±0.9 for the Australian. Acetic acid and hydrochloric acid (reagent grade) and D_2O (99%) were purchased from Merck (Auckland, NZ). Distilled water was used for dilutions.

5.2.2 Methods

5.2.2.1 **Gluten Dough Preparation.** Both Canadian and Australian gluten flours were rehydrated with water. The necessary amount of water for rehydration of the flours was based on trial and error, as assessed by observation of the development of the gluten doughs. The former was rehydrated to 140% absorption on a flour weight basis (60 g/84 mL), while the latter was rehydrated to 125% absorption (60 g/75 mL). The Canadian gluten was also rehydrated with deuterium to the same absorption as with water.

Mixing was performed using a Breville Turbo Mixer with hooks for dough (EM, Breville PTY. Limited, China).

From previous results (manuscripts I and II), only the gluten doughs whose pH were at the extremes of the studied values (6.3 and 4.3) were chosen for this set of experiments. In order to bring down the dough pH to a value of 4.3 without yeast addition, two types of acid were used: acetic and hydrochloric. This way the effect of two monoprotic acids (one organic and one inorganic) on gluten structure was tested. The necessary concentration of acids in the mixing water needed to decrease the pH of the dough was calculated based on previous results (manuscript I and II) and adjusted for the % absorption required in this study. The resultant concentrations were:

- Canadian gluten: 265 mM acetic acid and 397 mM hydrochloric acid.
- Australian gluten: 297 mM acetic acid and 445 mM hydrochloric acid.

5.2.2.2 **pH Measurements and Mixing of the Doughs.** The control doughs (without acid addition) were mixed for 10.5 min, the acetic acid doughs for 3.2 min, and the hydrochloric acid doughs for 6 min. These values were based on previous experiments (Manuscript I). Immediately after mixing, the pH of the doughs was measured at least in triplicate by inserting a pH electrode directly into the doughs (Salovaara, 1982; Larsson, 2002). Determinations of pH were done with a Radiometer portable pH Meter, model 80 (Copenhagen, Denmark) in temperature compensation mode.

5.2.2.3 ^{1}**H NMR Measurements.** After mixing, a blade was used for taking approximately 2.5 g of the gluten dough, which was put into a 1 cm diameter NMR glass tube. After placing the piece of dough in the tube, it was closed from each end with teflon plugs. The sealed tube was placed in a covered container (Tupperware) for 90 min at room temperature in order to prevent dough moisture loss, while allowing the samples to relax from the stress of mixing (Godefroy & Callaghan, 2003; Georgopoulos *et al.*, 2004).

After resting, the tube was placed into the RF coil which was fixed to the NMR probe and the whole assembly was then inserted into a 7T superconducting magnet (AVANCE 300MHz Brüker NMR Spectrometer) equipped with a micro imaging attachment (Fig. 25).

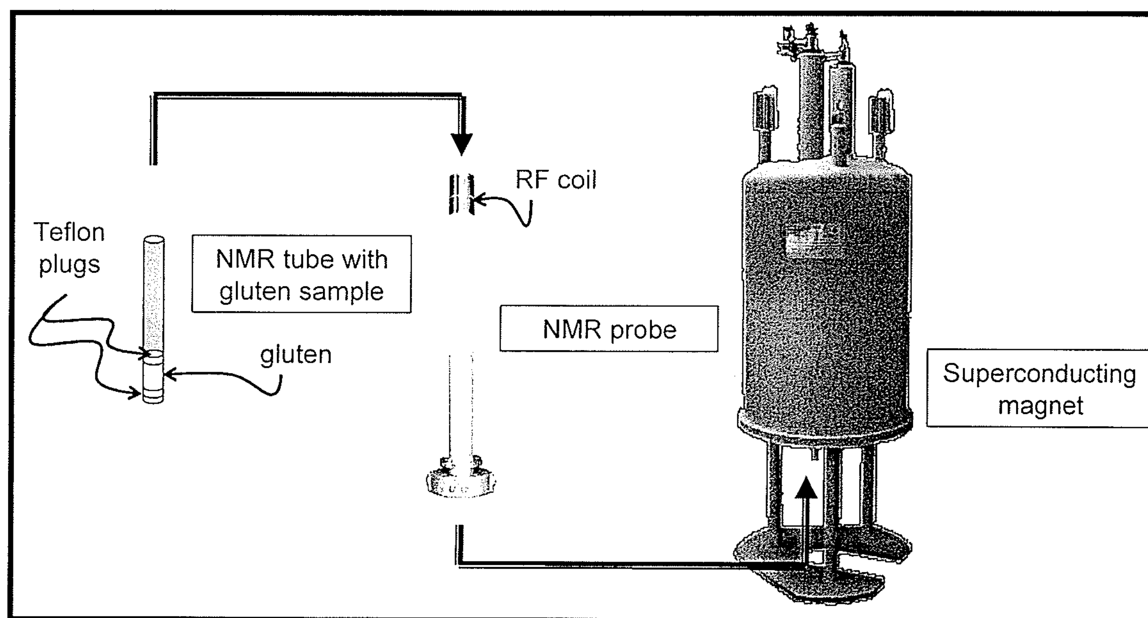


Figure 25. Positioning of the gluten sample within the superconducting magnet (Adaptation from Godefroy *et al.*, 2003)

5.2.3 ^{1}H NMR determinations

The relaxation time of the nuclear spins and the nuclear diffusion responses from the NMR signal were independently measured. The former response was determined from the Carr-Purcell-Meiboom-Gill (CPMG) pulsed train sequence, while the latter response was obtained using the Pulsed Gradient Spin Echo (PGSE) sequence. As shown in Figure 26, the CPMG pulse sequence was followed (and preceded) immediately by the PGSE sequence (Callaghan & Gil, 1999; Callaghan *et al.*, 2003[a]). The $90°_x$ RF pulse with duration of 12.5 ms and a $180°_y$ pulse of 25 ms were applied for the CPMG sequence, with a delay time (τ) between the pulses of 1 ms. The PGSE sequence consisted of 32 gradient pulses of a strength (Gmax) of 0.8 T/m, a duration (δ) of 7.5 ms and a delay between pulses (Δ) of 25 ms (Fig. 26). Eight scans of 40 kHz spectral width were obtained for the first replicates, while four scans of 20 kHz spectral width were obtained for the second replicate, in each case as 2048 data points. These scans were then averaged, automatically. Experiments were performed at 25°C. Each gluten dough was tested in duplicate, except for the Canadian gluten dough made with deuterium that was run just once.

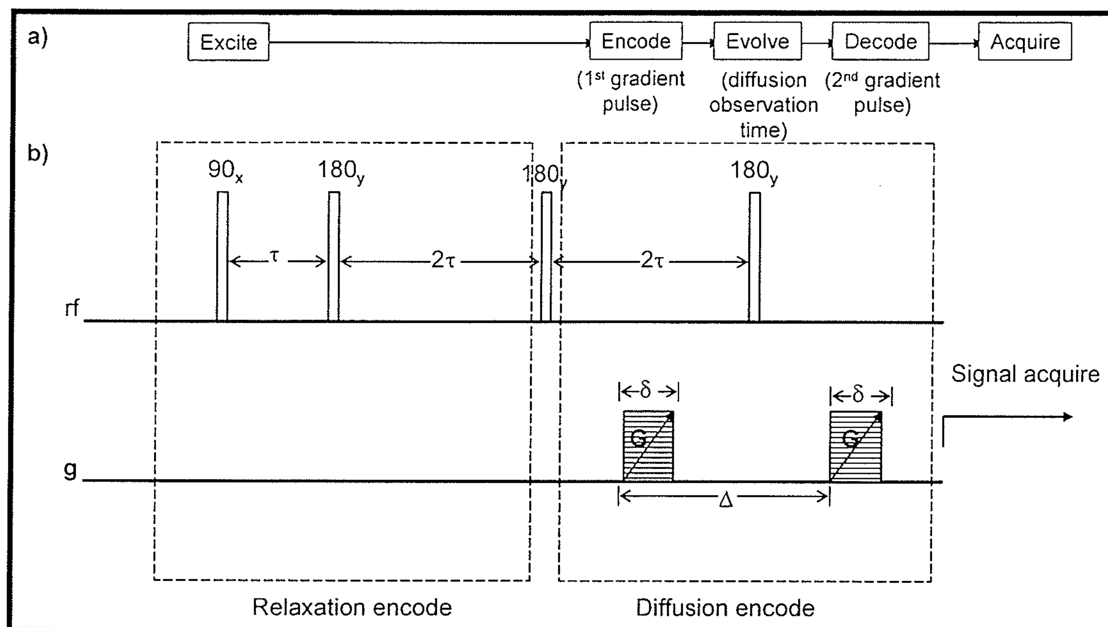


Figure 26. Essential components of PGSE NMR diffusion experiments (a). Schematic representation of a CPGM sequence followed (and preceded) by the PGSE sequence used for these experiments (b) (Adapted from Antalek, 2002 and Callaghan *et al.*, 2003[a]). See text for details of symbols

From the experiments, ^{1}H NMR spectra of intensity of the signal (a measure of the net magnetisation in the *xy* plane of the nuclear spins following the application of an RF pulse in either the *x* or the *y* direction) *vs.* frequency were obtained.

For analysing the data, the frequency data were transformed to ppm considering the spectral width, the frequency of the magnet (300 kHz) and the total number of data points in the spectra, as given by Eq. 25,

$$x = y * (z \text{ kHz}/300 \text{ kHz})/2048 \times 10^{6}, \qquad (25)$$

where *x* is the chemical shift [ppm], *y* is the data points (from 1 to 2048), *z* is the spectral width of either 40 or 20 kHz, and 2048 is the total number of data points. The chemical shift values obtained from Eq. 25 were adjusted to fit a

value of 4.7 ppm for the most intense peak (Callaghan, 2003, personal communication), since a chemical shift of 4.7 ppm has been attributed to water (Gil *et al.*, 1997), as shown in Eqs. 26 & 27,

$$a = b + 4.7. \tag{26}$$

where a is the fixed number for the adjustment and b is the chemical shift (from Eq. 26), whose value corresponds to the highest intensity for the ^1H NMR signal;

$$c = x - a, \tag{27}$$

where c is the adjusted chemical shift [ppm], x is the chemical shift from Eq. 25 [ppm], and a is the fixed number for the adjustment from Eq. 26.

^1H NMR spectra were obtained successively for both gluten doughs, from a region where no gradient was applied (bottom spectrum) to the maximum gradient (top spectrum) in thirty-two steps (e.g., Fig. 27). The data from the ^1H NMR spectra were analysed to obtain the diffusion coefficients (D) and the spin-spin (T_2) relaxation times for the chemical components in the gluten dough. D/T_2 correlation maps were obtained by the use of 2-dimensional (2D) Laplace inversion. For obtaining the individual T_2 values, 1D Laplace inversion was used (Callaghan *et al.*, 2003[a]; Godefroy & Callaghan, 2003). The programs used to extract T_2 and D values from the NMR signal after the application of the CPMG and PGSE sequences were developed by the MacDiarmid Institute, Victoria University of Wellington, New Zealand (Godefroy *et al.*, 2002).

5.3 RESULTS

5.3.1 ^{1}H NMR spectra for gluten doughs

Canadian and Australian gluten doughs prepared with water (referred to as control doughs) were studied by ^{1}H NMR spectroscopy. Only the bottom and top spectra, from the thirty-two ^{1}H NMR spectra obtained successively, were analysed for each gluten dough. The gluten doughs were prepared in duplicate, and thus a total of 4 spectra were analysed *per* gluten dough (2 bottom spectra and 2 top spectra). As an example, spectra for the Australian control doughs are shown in Fig. 27.

Several researchers have used deuterated water (D_2O) in order to diminish the signal from water protons and distinguish them from the protons of interest in the gluten proteins (Belton *et al.*, 1995; Cherian & Chinachoti, 1996; Gil *et al.*, 1997; Callaghan & Gil, 1999; Grant *et al.*, 1999; Gil *et al.*, 2001; Wang *et al.*, 2001; Calucci *et al.*, 2003; Wellner *et al.*, 2003). Therefore, Canadian gluten was hydrated with D_2O and its ^{1}H NMR signal analysed as well (Fig. 28).

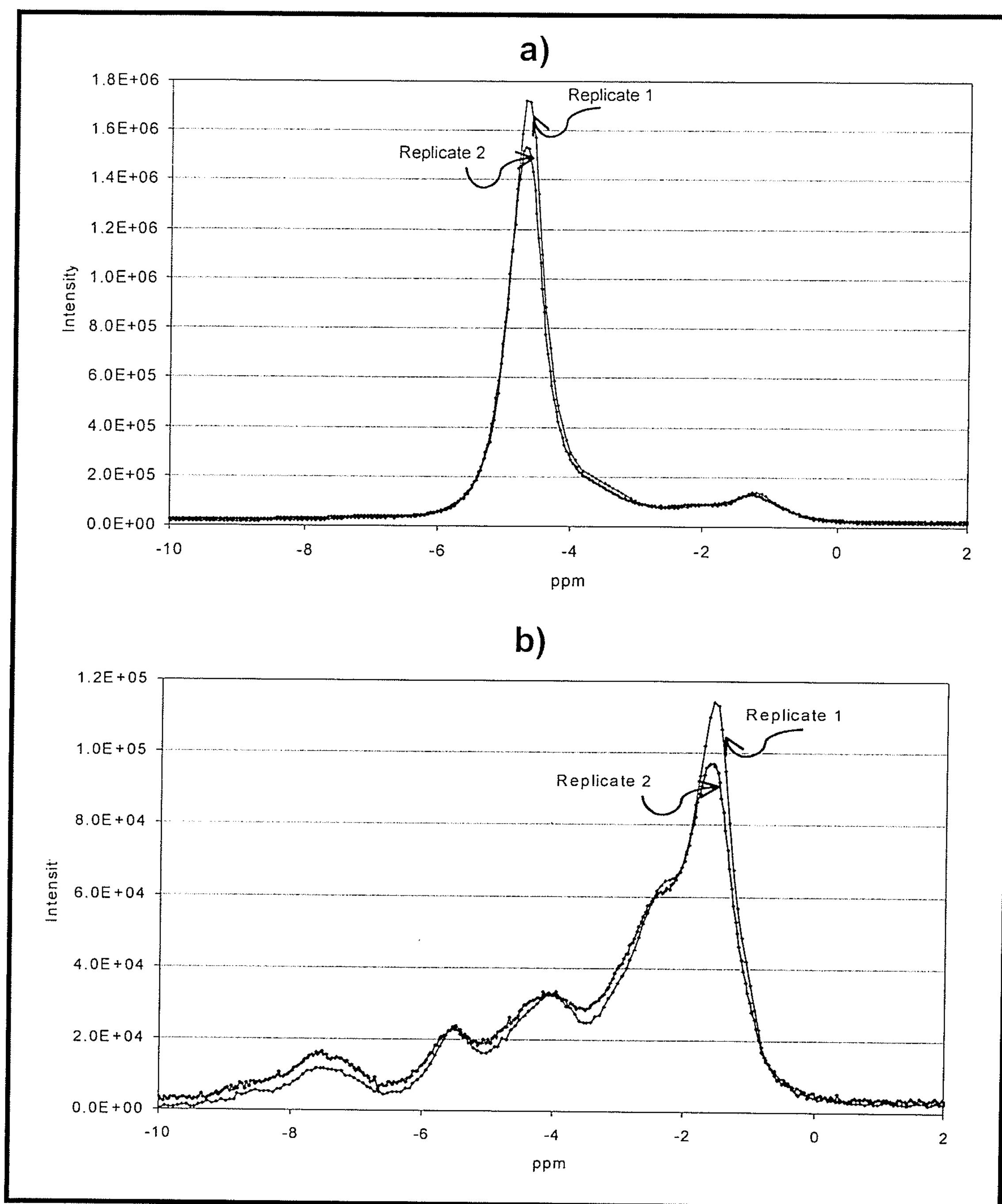


Figure 27. ^{1}H NMR spectra for Australian gluten hydrated to 125% with H_2O, where no gradient was applied (a) and where gradient was maximised (b). Note change in scale of intensity axis

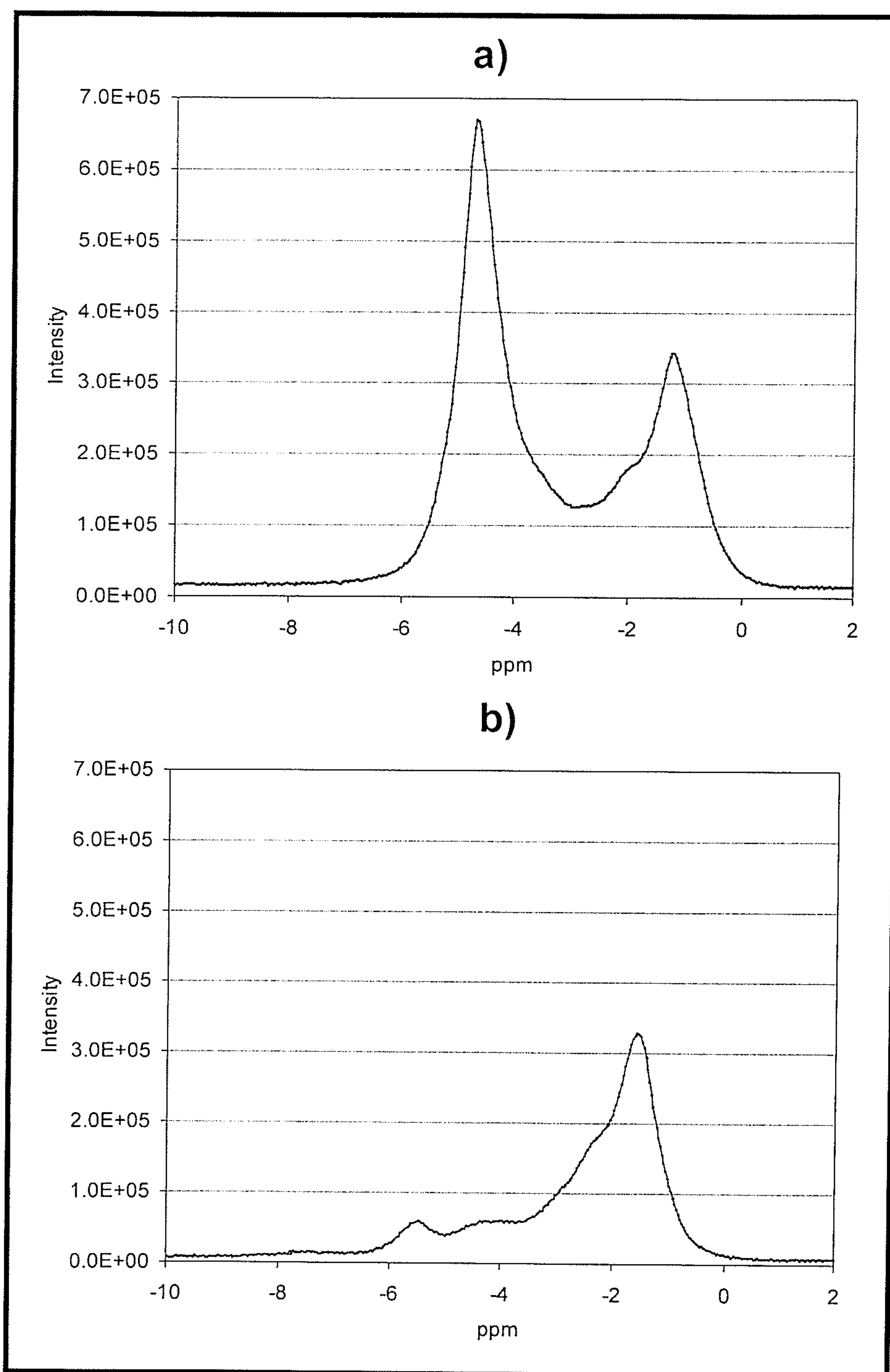


Figure 28. ^{1}H NMR spectra for Canadian gluten hydrated to 140% with D_2O, where no gradient was applied (a) and where gradient was maximised (b)

From a comparison of Fig. 27a and 27b, it is apparent that two well defined chemical shifts (peaks) were observed in the no-gradient spectra for both Canadian and Australian control gluten doughs. Six peaks were evident on the spectra where the maximum gradient was operating. The chemical shifts associated with the different glutens, and at the gradient extremes, are shown in Table 23.

Table 23. ^{1}H NMR chemical shift (ppm)[a] for wheat gluten doughs (average standard deviation in chemical shift = ±0.04)

	Canadian gluten with H_2O	Australian gluten with H_2O	Canadian gluten with D_2O
No Gradient	4.70	4.70	4.67
	3.46*	3.39*	#
	#	#	1.96*
	1.18	**1.18**	**1.18**
Maximum Gradient	**8.54**	**8.54**	Nd
	7.43	**7.43**	#
	5.48	5.51	5.51
	4.24	**4.11**	**4.18**
	2.22*	2.29*	2.29*
	1.51	1.51	1.54

[a] Numbers in bolds indicate broader peaks
* shoulder
\# shoulder present, but no inflexion point discernible
Nd Not detected

The chemical shifts detected in this study corresponded with those previously assigned to gluten proteins, lipids, water and residual deuterated water (HDO) in other studies (Knowles *et al.*, 1976; Gil *et al.*, 1997; Callaghan & Gil, 1999; Gil *et al.*, 2001; Calucci *et al.*, 2003). All the peaks observed for the Australian gluten dough were observed for the Canadian gluten dough when the gluten flours were hydrated with distilled water. A comparison for the dough

hydrated with D_2O was not possible, since the Australian gluten was not made up into a dough with D_2O.

Canadian and Australian gluten doughs were prepared with acetic and hydrochloric acid solutions in order to simulate the drop in pH of wheat doughs after fermentation (Clarke *et al.*, 2002). The acids adjusted the pH of both Canadian and Australian gluten doughs to a value of 4.3. Chemical shift values were compared between treatments (control dough and acid addition) for the same gluten dough. An example of this comparison for both the bottom and the top spectra is shown in Fig. 29 for the Canadian gluten dough.

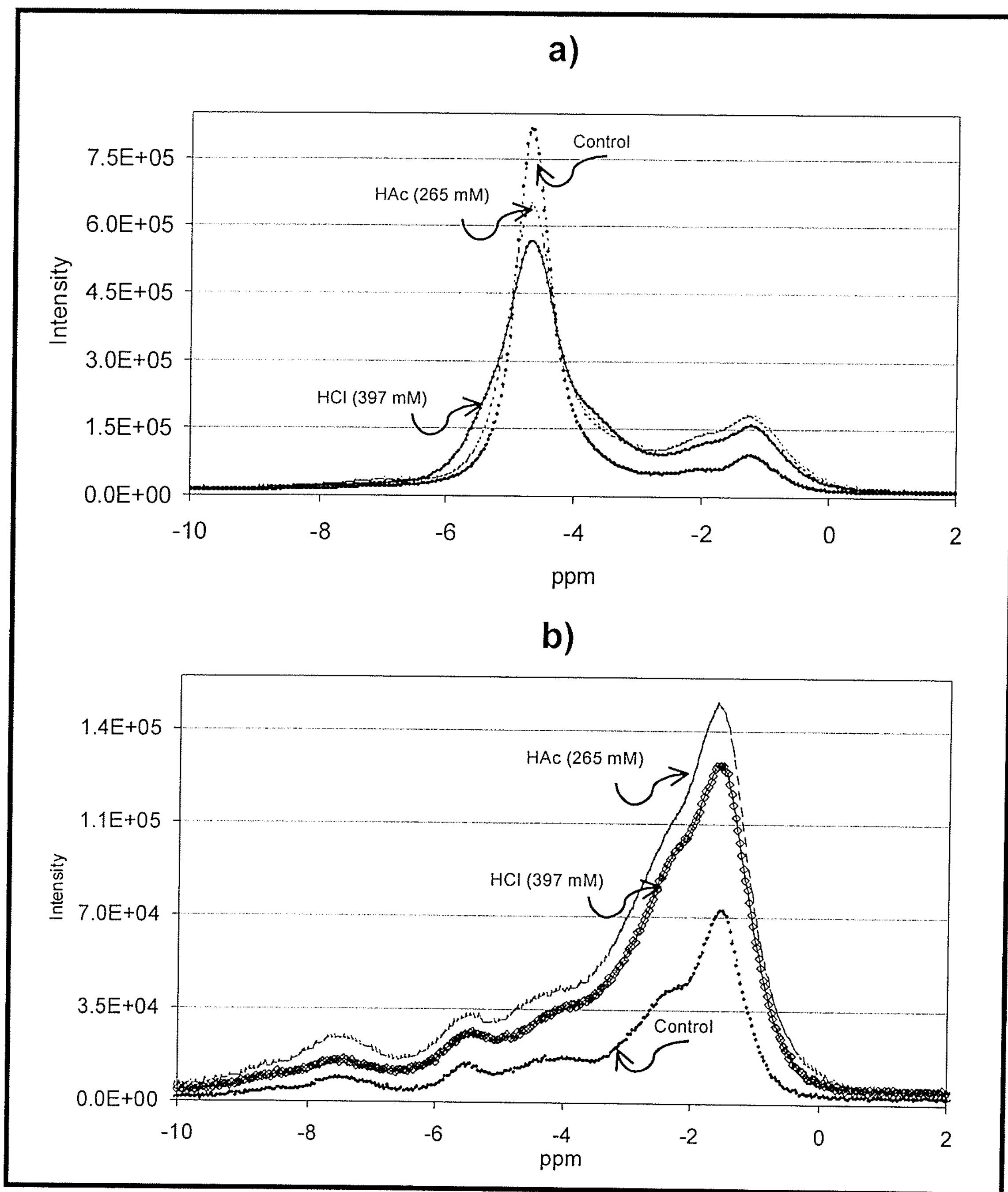


Figure 29. ^{1}H NMR spectra for Canadian gluten with no gradient (a) and maximum gradient (b), showing the effect of acetic and hydrochloric acid. Note change of scale of intensity axis

The presence of both acetic and hydrochloric acid in Canadian gluten dough made the resonance occurring at about 1.9 ppm more noticeable (Table 24). For the Australian gluten dough the chemical shift at 1.9 ppm became evident only after the addition of acetic acid (Table 25). No other differences were observed either between the gluten doughs of different national origin following acids' addition or between treatments (control doughs *vs.* acids addition).

Table 24. ^{1}H NMR chemical shift (ppm)[a] for Canadian wheat gluten dough (average standard deviation in chemical shift = ±0.04)

	Control dough	Acetic acid dough	Hydrochloric acid dough
No Gradient	4.70	4.70	4.70
	3.46*	#	3.53*
	#	1.90*	1.90*
	1.18	**1.18**	**1.18**
Maximum Gradient	**8.54**	**8.54**	**8.54**
	7.43	**7.43**	**7.43**
	5.48	5.54	5.48
	4.24	**4.18**	**4.11**
	2.22*	2.33*	2.16*
	1.51	1.54	1.50

[a] Numbers in bolds indicate broader peaks
* Shoulder
\# Shoulder present, but no inflexion point discernible

Table 25. ^{1}H NMR chemical shift (ppm)[a] for Australian wheat gluten dough (average standard deviation in chemical shift = ±0.04)

	Control dough	Acetic acid dough	Hydrochloric acid dough
No Gradient	4.70	4.70	4.70
	3.39*	#	3.46*
	#	1.90*	#
	1.18	*1.18*	*1.18*
Maximum Gradient	8.54	#	#
	7.43	**7.43**	**7.43**
	5.51	5.48	5.48
	4.11	**4.11**	**4.11**
	2.29*	2.29*	2.29*
	1.51	1.57	1.51

[a] Numbers in bolds indicate broader peaks
* Shoulder
# Shoulder present, but no inflexion point discernible

The chemical shift at approximately 1.9 ppm has been attributed to aliphatic amino acids and lipids present in the gluten (Callaghan & Gil, 1999; Gil *et al.*, 2001; Calucci *et al.*, 2003). The enhancement of the signal around 1.9 ppm for the Canadian gluten dough suggests conformational changes of the proteins as the result of acids addition. It has been proposed before that acid addition affects gluten structure in part by the exposure of hydrophobic residues. Therefore, the increase of the signal at 1.9 ppm is not surprising (Galal *et al.*, 1978).

5.3.2 2-D Diffusion (D) / spin-spin (T_2) relaxation correlation of gluten doughs

The effect of acids on both Canadian and Australian gluten doughs was assessed by the correlation of the diffusion coefficient (D) and the spin-spin relaxation time (T_2) of proton nuclear spins. The aim of this analysis was to distinguish rotational and translational motion of molecules in the gluten (Roudaut *et al*, 1998; Godefroy & Callaghan, 2003). The data that were analysed were selected based on the results obtained from the ^1H NMR spectra. Two main peaks were observed from the ^1H NMR spectra when no gradient was applied. One peak was well defined with a chemical shift of 4.7 ppm, the other one, at around 1.2 ppm, was a broader peak. In order to characterise these peaks, D/T_2 relaxation maps were obtained for Canadian and Australian control gluten doughs, as well as for the D_2O Canadian gluten. For the peak at 4.7 ppm the region from 3 to 6 ppm was integrated to provide the data in Table 26.

Table 26. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs* spin-spin (T_2) relaxation time for wheat gluten doughs in the region from 3 to 6 ppm of the ^1H NMR spectra

Sample	D (m^2/s)		T_2 (ms)	
	Rep 1	Rep 2	Rep 1	Rep 2
C-c	$2.9x10^{-10}$	$2.7x10^{-10}$	29	29
A-c	$2.5x10^{-10}$	$2.5x10^{-10}$	25	27
C-d	$2.9x10^{-10}$		40	

C-c Canadian control gluten dough (with water)
A-c Australian control gluten dough (with water)
C-d Canadian gluten dough with deuterium

The fast diffusion and short T_2 relaxation of the signal corresponded to that assigned to water linked to the protein polymers by Godefroy & Callaghan

(2003). Once the chemical shift at 4.7 ppm was analysed, the next region that was chosen for the 2D Laplace inversion analysis was the signal occurring in the chemical shift at about 1.2 ppm (analysed from the region from 0.6 to 1.6 ppm). This region was selected since the signal came from amidic protons and lipids (Callaghan & Gil, 1999; Gil *et al.*, 2001), which are thought to be affected by addition of acids (Galal *et al.*, 1978; Belton, 1999). Results of such a 2D Laplace inversion are presented in Table 27 and an example of the D/T_2 distribution maps is shown in Fig. 30.

Table 27. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs* spin-spin (T_2) relaxation time for wheat gluten doughs in the region from 0.6 to 1.6 ppm of the ^1H NMR spectra

Sample	D (m^2/s)		T_2 (ms)	
	Rep 1	Rep 2	Rep 1	Rep 2
C-c	3.3×10^{-12}	2.5×10^{-12}	117	103
A-c	2.9×10^{-12}	4.0×10^{-12}	94	90
C-d	1.9×10^{-12}		88	

C-c Canadian control gluten dough (with water)
A-c Australian control gluten dough (with water)
C-d Canadian gluten dough with deuterium

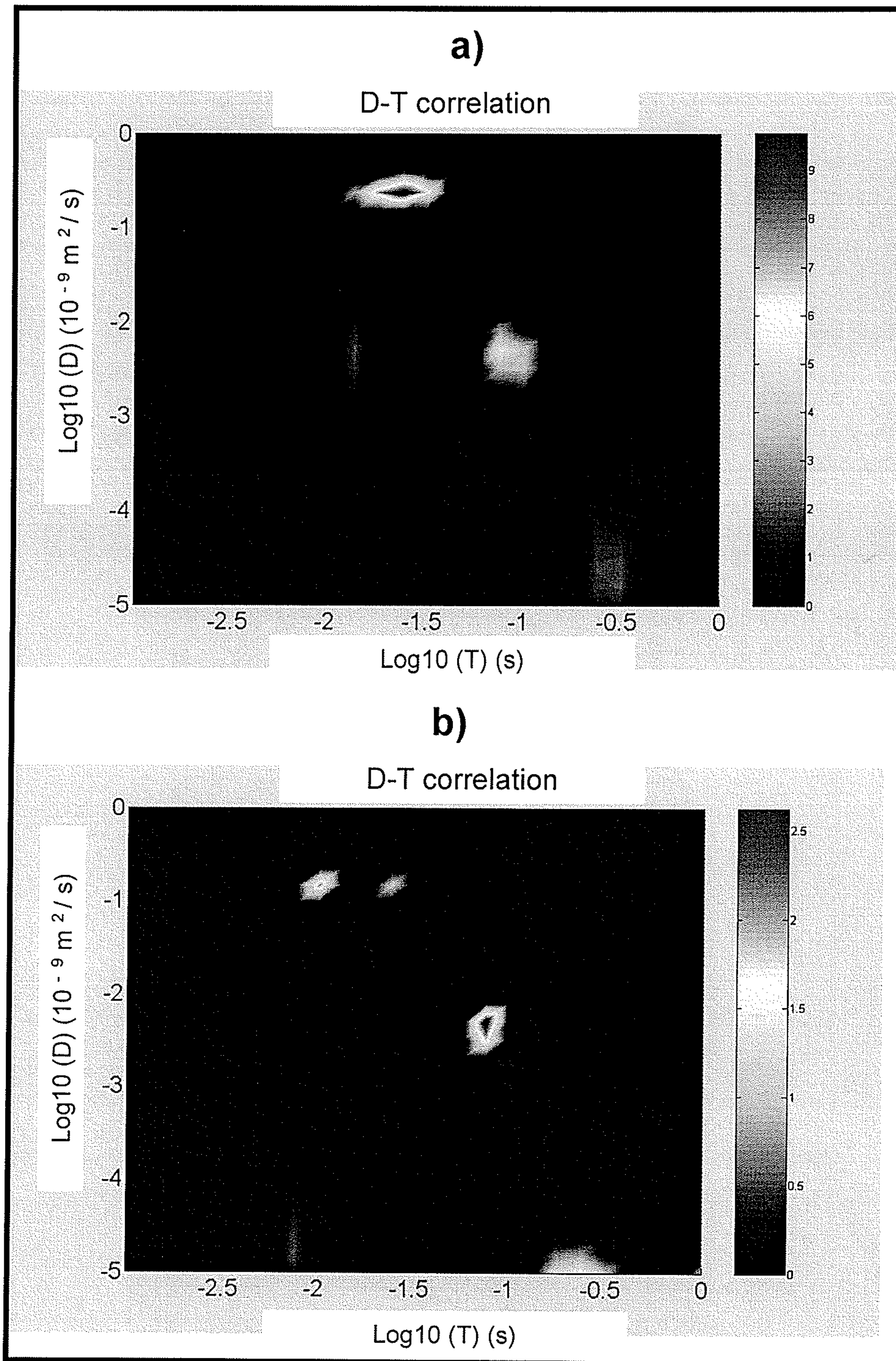


Figure 30. D *vs.* T_2 distribution maps for Australian gluten dough hydrated with water (a) and Canadian gluten dough hydrated with D_2O (b). Bar on right of graphs denotes intensity

As seen on the D *vs.* T_2 distribution maps (Fig. 30), the water peak was still present and prominent in the 0.6 to 1.6 ppm region. This is also true in the deuterated sample (Fig. 30b), and the latter result arises from exchange of protons between bound water and the D_2O and between protons in the environment and D_2O to crease deuterated water (HDO). However, another peak was detected which had the properties of a lower diffusion coefficient (about 2×10^{-12} *vs.* 2.7×10^{-10} m^2/s) and longer T_2 values (about 0.1 *vs.* 0.03 s) compared to bound water.

D/T_2 correlation maps were also obtained for both Canadian and Australian gluten doughs after acids addition. The ^1H NMR region from 3 to 6 ppm showed the same characteristics as for the control doughs and thus are not presented. The results corresponding to the spectral region of 0.6 to 1.6 ppm were also obtained and are compared with those obtained for the control doughs (Tables 28 & 29).

Table 28. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs* spin-spin (T_2) relaxation time for Canadian gluten doughs in the region from 0.6 to 1.6 ppm of the ^1H NMR spectra

Sample	D (m^2/s)		T_2 (ms)	
	Rep 1	Rep 2	Rep 1	Rep 2
C-c	3.3×10^{-12}	2.5×10^{-12}	117	103
C-HAc	1.8×10^{-12}	9.8×10^{-12}	302	207
C-HCl	1.8×10^{-12}	8.9×10^{-12}	271	236

C-c Canadian control gluten dough (with water)
C-HAc Canadian gluten dough with acetic acid
C-HCl Canadian gluten dough with hydrochloric acid

Table 29. 2D Laplace inversion correlation data of diffusion coefficient (D) *vs* spin-spin (T_2) relaxation time for Australian gluten doughs in the region from 0.6 to 1.6 ppm of the ^1H NMR spectra

Sample	D (m^2/s)		T_2 (ms)	
	Rep 1	Rep 2	Rep 1	Rep 2
A-c	$2.9x10^{-12}$	$4.0x10^{-12}$	94	90
A-HAc	$2.9x10^{-12}$	Nd	183	Nd
A-HCl	$5.4x10^{-12}$	Nd	200	Nd

A-c Australian control gluten dough (with water)
A-HAc Australian gluten dough with acetic acid
A-HCl Australian gluten dough with hydrochloric acid
Nd Not detected

The D and T_2 values obtained for both Canadian and Australian gluten doughs after acid addition did not seem to be affected by the low pH. In general, it was surmised that the high intensity of signals corresponding to the presence of water and lipids obscured other components in the correlation maps (Callaghan & Hubbard, 2003, personal communication).

5.3.3 1D spin-spin (T_2) relaxation analysis for gluten doughs

Analysis of T_2 relaxation times was performed on individual peaks of the ^1H NMR spectra since the water signal was very prominent on the D/T_2 correlation maps. The purpose of this extra analysis was because T_2 has been reported to be useful for indicating molecular conformation, molecular interactions and molecular mobility in food systems (Callaghan & Gil, 1999; Belton & Wang, 2001; Wellner *et al.*, 2003). T_2 relaxation values were determined for the four main resolved chemical shifts obtained from the maximum gradient spectra for both gluten doughs (e.g., Fig. 27-b). This would

permit identification of relaxation times associated with components which did not diffuse fast, i.e., components associated with the polymers in the gluten doughs. The four regions corresponded to chemical shifts of : 8.1-6.4 ppm, 6-4.9 ppm, 4.8-3.5 ppm and 3.4-0.2 ppm. In general, four T_2 components were evident from all regions of these 1D spin-spin relaxation analyses: components possessing very short relaxation times (<10 ms), short (20-50 ms), long (100-500 ms), and very long relaxation times (>500 ms). As discussed in 5.4, the components responsible for these relaxation times corresponded to those seen in previous analyses of gluten dough samples (Roudaut *et al*, 1998; Grant *et al.*, 1999; Wang *et al,* 2001; Gil *et al.*, 2001; Calucci *et al.*, 2003; Esselink *et al.*, 2003[ab]).

T_2 relaxation times attributable to the proteins themselves are very short and thus conformational changes in proteins were analysed by studying the relaxation times associated with water and the aliphatic regions as done in previous studies (Belton *et al.*, 1995; Gil *et al.*, 1997; Grant *et al.*, 1999; Wang *et al.*, 2001; Esselink *et al.*, 2003[b]; Godefroy & Callaghan, 2003). In order to understand the effect of acids on gluten doughs, it was decided to focus only on the chemical shift regions that have previously been attributed to water and aliphatic residues (4.7 ppm and 3.4-0.2 ppm, respectively) (Calucci *et al.* 2003; Gil *et al.* 2001). Relaxation times consistently found in both replicates for Canadian and Australian gluten doughs are presented in Tables 30 and 31, respectively.

Table 30. Presence/absence of spin-spin (T_2) relaxation time components for Canadian gluten doughs as the result of acid addition

T_2 values (ms)	Water region[a]			Aliphatic region[b]		
	Control	HAc	HCl	Control	HAc	HCl
< 10	✕	✓	✓	✓	✕	✕
20-50	✓	✓	✓	✓	✓	✓
100-500	✓	✓	✓	✓	✓	✓
>500	✓	✓	✕	✓	✓	✓

[a] Region integrated from 4.3 to 5.1 ppm of the ^{1}H NMR spectra
[b] Region integrated from 3.4-0.2 ppm of the ^{1}H NMR spectra
HAc Acetic acid
HCl Hydrochloric acid
✓ Present
✕ Not present

Table 31. Presence/absence of spin-spin (T_2) relaxation time components for Australian gluten doughs as the result of acid addition

T_2 values (ms)	Water region[a]			Aliphatic region[b]		
	Control	HAc	HCl	Control	HAc	HCl
< 10	✕	✓	✕	✓	✕	✕
20-50	✓	✓	✓	✓	✓	✓
100-500	✓	✓	✓	✓	✓	✓
>500	✓	✓	✓	✓	✓	✓

[a] Region integrated from 4.3 to 5.1 ppm of the ^{1}H NMR spectra
[b] Region integrated from 3.4-0.2 ppm of the ^{1}H NMR spectra
HAc Acetic acid
HCl Hydrochloric acid
✓ Present
✕ Not present

The four T_2 components from the water and the non-polar regions (aliphatic residues and lipids), generally showed consistency in both Canadian and Australian gluten doughs. Changes in the T_2 relaxation times as the result of acids addition may be attributed to conformational changes in gluten doughs when lowering the pH as assessed by other techniques (Galal *et al.*, 1978; Sekhon *et al.*, 1995; Shiau & Yeh, 2001).

5.4 DISCUSSION OF RESULTS

From the bottom H^1 NMR spectra (where no gradient was applied), a very prominent peak with a chemical shift at 4.7 ppm was observed, and this peak is attributed to water (Gil *et al.*, 1997). The intensity of this signal decreased when the gradient was applied, as a result of the rapid diffusion of the water molecules (Stejskal & Tanner, 1965; Price, 1997). As the water molecules diffused, the chemical shifts of other components became apparent in the top spectra (maximum gradient). The identities of these components were then assigned and were found to correspond to those previously reported; possible assignments for specific chemical shifts have been reported by Gil *et al.* (1997) and Calucci *et al.* (2003) (Table 32). In addition to these assignments, the chemical shift values occurring from 1.2-1.5 ppm are assigned to aliphatic residues, while those at 5.5 ppm are assigned to lipids and to residual starch (Callaghan & Gil, 1999; Gil *et al.*, 1997; Gil *et al.*, 2001).

Table 32. Possible assignments of ^{1}H NMR chemical shifts for gluten proteins

Chemical Shift (ppm)	Source: Gil *et al.*, 1997	Source: Calucci *et al.*, 2003
1.5-1.6	Leu γ; Gln γ; Pro β	Lipid –CH_2-CH_2-COO
1.9-2.0	Pro β γ; Gln β γ	Lipid –CH_2-CH=CH-
2.2-2.3	Pro β γ; Gln β γ; Leu γ	Lipid –CH_2-CH_2-COO
3.4-3.5	Pro δ	Pro δ; backbone protons
4.1-4.2	Leu α; Gln α	Pro δ; backbone protons
4.7-4.8	Water	Water
7.4	Gln δ	Gln δ; aromatic side-chains; backbone –NH
8.5	-	Gln δ; aromatic side-chains; backbone –NH

The masking effect of the presence of water on the signals due to amino acids and lipids was diminished, but not avoided, when D_2O was used to make up the gluten. This result has been previously reported by Callaghan & Gil (1999), who attributed the persistence of the peak at 4.7 ppm to the presence of residual deuterated water (HDO). When the Canadian gluten dough was prepared with D_2O, the signal with a chemical shift between 7.4 and 8.5 was barely or not detected (Table 23). The decrease of the signal in this region of the spectrum is the result of the exchange of amide protons from glutamine (Table 32) with deuterium (Callaghan & Gil, 1999).

From the ^1H NMR spectra for Canadian *vs.* Australian control gluten doughs just one difference was observed: the chemical shift at 8.5 ppm was not well defined for the Australian dough (Table 23). The difference is attributed to the different levels of hydration between the doughs (140% for Canadian, *vs.* 125% for Australian). Higher hydration levels inducing greater mobility in the protein structure, and the greater amount of hydrogen bonds associated with the water, both allow better detection of the protein structure (Gil, 1995).

The addition of both acetic and hydrochloric acid to the gluten dough is surmised to lead to the breakage of hydrogen interactions between protein polymers (Galal *et al.*, 1978). An apparent chemical shift occurring around 1.9 ppm was observed when no gradient was applied in the Canadian gluten (Table 24). This chemical shift has been previously attributed to the presence of glutamine and aliphatic residues (Gil *et al.*, 1997; Callaghan & Gil, 1999). Therefore, the addition of acids in this study corresponds to the hydration of glutamine residues as well as to the exposure of hydrophobic regions. The

results of this work therefore indicate more specifically the rupture of hydrogen bonds between glutamine residues following acid addition. As described by Belton (1999) in his train and loop model, hydrogen bonds between glutamine residues have a great influence on gluten structure. Moreover, Wellner and co-workers (2003) have reported the ability of acetic acid to link with the glutamine side-chain and thus block the ability of glutamine residues to form intermolecular hydrogen bonds. This latter effect was detected in the Australian gluten when acetic acid was added, but not observed with hydrochloric acid (Table 25).

Pulsed gradient spin-echo (PGSE) NMR has been previously used in order to examine water movement and the degree of water-polymer associations (Umbach *et al.*, 1992). D/T_2 correlation maps were analysed for the two main resolved resonance signals obtained in the ^1H NMR spectra for both Canadian and Australian gluten doughs. From here, the signal attributed to water corresponded to D and T_2 values of water linked to the protein polymers (Table 26) (Esselink *et al.*, 2003[a]; Godefroy & Callaghan, 2003). It is important to emphasise that the signal did not come from free water, since the D and T_2 values attributed to it are remarkably different: 2.5 to 3 x 10^{-9} m^2/s and 3 s, respectively (Umbach *et al.*, 1992; Grant *et al.*, 1999; Ono *et al.*, 1998). The values of D and T_2 for the gluten doughs and the D_2O dough for the region from 0.6 to 1.6 ppm of the ^1H NMR spectra (Table 27) were observed in all the gluten samples. These values ($D = 2 \times 10^{-12}$ m^2/s and $T_2 = 0.1$ s) corresponded to that previously identified as being due to lipids (Godefroy *et al.*, 2003) and water strongly linked to the gluten proteins (Umbach *et al.*, 1992).

The four sets of relaxation time obtained from the individual analysis of T_2 for both Canadian and Australian gluten doughs corresponded to previously assigned components in the results of other researchers:

- A very short T_2 component (<10 ms) corresponds to residual starch, aliphatic residues and water tightly linked to dough components (Gil *et al.*, 2001; Esselink *et al.*, 2003[ab]).
- A short T_2 component (between 20-50 ms) corresponds to water interacting with the gluten (Esselink *et al.*, 2003[a]).
- A long T_2 component (between 100-500 ms) corresponds to water interacting weakly with dough components (Grant *et al.*, 1999; Wang *et al*, 2001; Esselink *et al.*, 2003[a]).
- A very long T_2 component (>500 ms) corresponds to the lipid fraction (Roudaut *et al.*, 1998).

From Tables 30 and 31, it was observed that when acids were added to the gluten doughs, the aliphatic region corresponding to the very short T_2 component was not detected. A change in the region identified as coming from the water present in the gluten doughs was also detected as observed by the appearance of a very short T_2 component. These results seem to match a situation where addition of acids leads to conformational changes in gluten proteins by increasing the positive charge on the polymers (Wu & Dimler, 1967[ab]; Galal *et al.*, 1978; Eliasson & Larsson, 1993). The inducement of a net positive charge on the gluten proteins leads to a more evenly distributed water system tightly linked to the charges on the charged amino acids. This new

conformation, induced by the charging of some of the amino acids, also affects the hydrophobic residues as seen by the T_2 analysis of the aliphatic region (Tables 30 & 31). The hydrophobic amino acids are now exposed and are not close enough for interacting with each other by van der Waals forces (Galal *et al.*, 1978). As a result of the absence of aliphatic residues interacting with themselves, the very short T_2 component (<10 ms) corresponding to the aliphatic region is not detectable.

5.5 CONCLUSIONS

Pulsed gradient spin echo NMR was used to show that breakage of hydrogen bonds between amino acid residues and the exposure of hydrophobic side chains takes place at a pH value of 4.3 in gluten doughs. This was observed for commercial glutens from both Canadian and Australian sources. The findings correspond with Belton's train and loop model where glutamine residues are key amino acids in the formation of hydrogen bonds between gluten polymers, and where electrostatic repulsions fostered by acidification induce train to loop transitions in gluten. Acetic acid was found to have a higher affinity for glutamine residues compared to hydrochloric acid.

VI GENERAL DISCUSSION AND CONCLUSIONS

The first hypothesis which was to be tested in this thesis was that the addition of acetic and hydrochloric acids will lead to conformational changes in the gluten proteins, and as a result, a less elastic dough will be obtained. This hypothesis was probed with two fundamental rheological techniques (dynamic shear strain rheometry and low-intensity ultrasound).

According to Wu & Dimler (1963[ab]); the isoelectric point of the gluten proteins is 7.5, therefore a pH below this isoelectric point will lead to a net positive charge on the gluten polymers. Histidine and glutamic acid are the two amino acids whose net charge is altered within the range of pH covered in this study (Table 15) (Cheftel *et al.*, 1985). As the result of an excess in positive charges, electrostatic repulsions between gluten proteins take place, which leads to the breakage of hydrogen bonds between amino acid residues and a change in the conformation of the gluten proteins (Bennett & Ewart, 1962; Galal *et al.*, 1967; Hoseney & Brown, 1983). Following the loop and train model of Belton (1999), these results imply that there is the breakage of hydrogen bonds between proteins as a result of the addition of acids. This leads to the formation of more or larger loops where hydrogen bonds between amino acid residues and water molecules (or the conjugated base of the acid) can take place. The decrease in the number of hydrogen bonds acting between amino acid residues does not permit efficient storage of the applied strain energy and thus lower G' values are obtained as pH changes from 6.3 to 5.8 (first addition of acids) (Table

10). Since the strain energy is not stored, it is therefore dissipated (note that this is also consistent with the very large values of the longitudinal loss modulus that were observed for the acidic doughs). The addition of acids increases the hydrophilicity of the gluten proteins (Danno & Hoseney, 1982). This higher affinity for the water molecules is the result of an increase in the number of hydrogen bonds between amino acid side-residues and water molecules. The inducement of bonding between water and the protein polymers leads to more bound water in the dough as the result of acids' addition. Differences in bound and free water affect the propagation of ultrasound through the sample (Létang *et al.*, 2001; Ross *et al.*, 2004). It was observed that for gluten doughs whose pH was adjusted by acetic acid that the ultrasonic velocity through the gluten increased as dough pH decreased (Fig. 23).

^{1}H NMR was used to directly identify changes in molecular structure arising from the addition of acids. NMR readings from amino acid residues were detected by the application of PGSE NMR. Previously, the importance of glutamine residues in the stabilisation of the gluten proteins as assessed by NMR has been reported (Belton *et al.*, 1995, 2001; Callaghan & Gil, 1999; Wellner *et al.*, 2003). In this thesis, the breakage of glutamine residue hydrogen bonds due to acids' addition was corroborated as noticed by changes in the intensity of the NMR signal assigned to glutamine (Tables 24 & 25). 1D Laplace analysis of spin-spin relaxation times (T_2 values, Tables 30 & 31) showed an increase of water bound to the proteins and a decrease of hydrophobic components (aliphatic amino acids and lipids) with low T_2 relaxation times. These results are explained by the increase in the net positive charge of the

protein that leads to a more hydrophilic structure where more bound water is found. The charging of the protein polymers led as well to electrostatic repulsions that will not allow hydrophobic amino acids to interact with each other and thus the T_2 relaxation time coming from aliphatic components increases.

The breakage of hydrogen bonds between gluten polymers and the size of the induced loops were sensitive to the type of acid added to the dough. The importance of type of acids is based on observed differences in the necessary concentration of acids needed to lower the pH of the gluten dough (Table 4), as well as differences in the effects of acids on mixing time, G', and ultrasonic velocity (Tables 8 & 10, and Fig 23, respectively). The interactions between amino acid residues and the conjugated base may be sterically selective, whereby smaller anions can enter the loops and shield the positive charges that exist on the polymer chains. In the case of the anions that corresponded to the two acids (acetic and hydrochloric) used in this thesis, chloride ions were more likely to be incorporated into the loops due to their smaller size and faster ionic mobility (Marcus, 1985; Brey, 1978 as cited in Voet & Voet, 1990). Chemical shifts in the ^1H NMR spectra for Australian gluten doughs pointed to structural changes associated with glutamine residues that were prevalent for acetic acid, but not for hydrochloric acid.

As a result of the conjugate base effects that were observed, it was decided to study the effects of chloride salts in combination with acetic acid at the concentration where the gluten dough pH would be 5.8 when using acetic acid alone. The second main hypothesis then, was that the addition of acid will open up the gluten structure (creation of loops) so that chloride ions are

sterically allowed. The addition of more chloride ions, as chloride salts, without further positive charges (hydronium ions) would prove the hypothesis of a sterically selective profile of the loops. The effects of sodium, magnesium and aluminium salts of equal ionic strength (and thus different concentrations), in combination with acetic acid, on gluten structure were assessed by ultrasonic determinations. Conformational changes of the gluten proteins as the result of salts addition have been shown previously (Danno & Hoseney; Preston 1981; 1989; Butow *et al.*, 2002). The inclusion of chloride ions into the loops when acetic acid was added was manifest as differences in the rheological properties of gluten doughs as assessed by ultrasound (Figs. 21, 22 & 24). A lower number of anions (as when aluminium chloride was added in comparison to sodium chloride) leads to a lower degree of shielding of positive charges among the gluten polymers (Preston, 1981). The higher electrostatic repulsions that ensue, reduce the size of gas cells, and thus a larger ultrasonic velocity is measured (Fig. 21). The type of cation (related to differences in size and valency) was also relevant in the presence of acetic acid. It was surmised that sodium ions enhanced hydrophobic interactions whereas magnesium and aluminium ions promoted the formation of salt bridges between the protein polymers.

Two issues arise from this thesis associated with the main points that were not accomplished. Firstly, none of the two main hypotheses (i.e., the induced conformational changes in gluten due to the addition of different acids and salts, and the steric selectivity of gluten loops as assessed by salts) were proven from ultrasonic measurements. Secondly, the hypothesis related to the

steric selectivity of gluten loops as assessed by three chloride salts was not proven either.

Ultrasonic determinations are difficult to interpret when dealing with highly attenuating materials (McClements, 1997). The gluten dough is a very highly attenuating material (α of about 3 mm^{-1}) at low frequencies, so that the viscous or loss longitudinal modulus (β") was very high (values around 80 MPa). Therefore, ultrasonic determinations for gluten doughs may be difficult to interpret and use for the determination of structural changes arising from these selected processing conditions. Considering the many advantages of ultrasonic determinations, the suggestion for further work is therefore the assessment of the properties of gluten proteins at higher frequencies where the imaginary component (loss modulus) associated with the longitudinal modulus may not represent as large a problem when analysing the samples.

The second hypothesis, regarding the steric selectivity of gluten loops induced by the addition of acids, seems interesting although it could not be proved with the addition of chloride salts as assessed by ultrasound. Therefore it is suggested here to work with higher concentration of chloride salts, being careful to use salt concentrations below 0.5 M in order to see electrostatic and not lyotropic effects on the gluten proteins (Preston, 1981; 1989; Butow *et al.*, 2002).

A general conclusion related to the first hypothesis to prove in this thesis is that addition of acids to the gluten proteins affects their conformation by breaking hydrogen bonds between amino acid residues (mainly glutamine) and

disrupting hydrophobic interactions. The breakage of hydrogen bonds present in the gluten polymers affects the rheological properties of the gluten dough by decreasing its elasticity (reducing G' values), although the propagation of ultrasonic velocity through it increased. Regarding the second hypothesis, it can be concluded that the breakage of hydrogen bonds between protein residues led to the formation of loops among the gluten polymers that are sterically selective.

This work is relevant to both cereal science and the gluten industry. Insights into gluten structure were obtained corroborating the importance of glutamine residues, hydrogen bonds and hydrophobic interactions on the stabilisation of the structure of gluten. It was proved that processing conditions (addition of acids and salts) affect the rheological properties of the gluten proteins and thus can be used to manipulate their functionality. The addition of acids leads to a weaker and less elastic protein where hydrogen bonds and hydrophobic interactions are disrupted, and more so at lower pH values (4.3).

LITERATURE CITED

AACC. 1983. *Approved Methods of the American Association of Cereal Chemists*. 8th edition. Methods 2-52, 44-15, and 46-13. AACC, St. Paul, MN.

Antalek, B. 2002. Using pulsed gradient spin echo NMR for chemical mixture analysis: how to obtain optimum results. Concepts in Magnetic Resonance. 14(4):225-258.

Anderssen, R.S., Gras, P.W., and MacRitchie, F. 1998. The rate-independence of the mixing of wheat flour dough to peak dough development. Journal of Cereal Science. 27:167-177.

Attenburrow, G., Barnes, D.J., Davies, A.P., and Ingman, S.J. 1990. Rheological properties of wheat gluten. Journal of Cereal Science. 12:1-14.

Autio, K., Flander, L., Kinnuen, A.K., and Heinonen, R. 2001. Bread quality relationship with rheological measurements of wheat flour dough. Cereal Chemistry. 78:654-657.

Bagley, E.B., Dintzis, F.R., and Chakrabarti, S. 1998. Experimental and conceptual problems in the rheological characterization of wheat flour doughs. Rheologica Acta. 37:556-565.

Baker, J.C., and Mize, M.D. 1937. Mixing doughs in vacuum and in the presence of various gases. Cereal Chemistry. 14:721-734.

Bakhoum, M.T., and Ponte, J.G. 1982. Combined effects of sodium chloride and hydrochloric acid on wheat flour strength. Cereal Chemistry. 59:37-40.

Balla, A., Razafindralambo, H., Blecker, C., and Paquot, M. 1998. Interfacial properties of gluten monolayers spread on various chloride salt solutions. Effects of electrolytes, salt concentrations, and temperature. Journal of Agricultural and Food Chemistry. 46:3535-3539.

Belitz, H.-D., Kieffer, R., Seilmeier, W., and Weiser, H. 1986. Structure and function of gluten proteins. Cereal Chemistry. 63:336-341.

Belton, P.S. 1999. On the elasticity of wheat gluten. Journal of Cereal Science. 29:103-107.

Belton, P.S., Colquhoun, I.J., Grant, A., Wellner, N. 1995. FTIR and NMR studies on the hydration of a high-M_r subunit of glutenin. International Journal of Biological Macromolecules. 17(2):74-80.

Belton, P.S., Delgadillo, I., Gil, A.M., Roma, P., Casuscelli, F., Colquhoun, I.J., Dennis, M.J., and Spraul, M. 1997. High field proton NMR studies of apple slices. Magnetic Resonance in Chemistry. 35:52-60.

Belton , P.S., and Wang, Y. 2001. Fast field cycling NMR –applications to food. Pages: 145-154 in: *Magnetic Resonance in Food Science, A View to the Future.* Webb, G.A., Belton, P.S., Gil., A.M., Delgadillo, I. The Royal Society of Chemistry, Cambridge, Great Britain.

Bennett, R. and Ewart, J.A.D. 1962. The reaction of acids with dough proteins. Journal of the Science of Food and Agriculture. 13:15-23.

Blitz, J. 1963. *Fundamentals of Ultrasonics.* Butterworths. Londond, Great Britain. 214 pp.

Bloksma, A.H. 1975. Thiol and disulfide groups in dough rheology. Cereal Chemistry. 52:170r-180r.

Bohlin, L., and Carlson, T.L.-G. 1980. Dynamic viscoelastic properties of wheat flour dough: dependence on mixing time. Cereal Chemistry. 57:174-177.

Bovey, F.A, Jelinski, L., and Mirau, P.A. 1989. *Nuclear Magnetic Resonance Spectroscopy.* 2nd ed. Academic Press, Inc., San Diego, CA. 653 pp.

Brey, W.S. 1978. *Physical Chemistry and its Biological Applications.* Academic Press. P. 172. As cited in Voet & Voet, 1990.

Bull, H.B. 1951. *Physical Biochemistry*. 2nd ed. John Wiley & Sons, Inc., New York. NY. 355 pp.

Bushuk W. 1990. Foreword. Pages: *vii-viii* in: *Dough Rheology and Baked Product Texture.* Faridi, H., and Faubion, J.M. Van Nostrand Reinhold, New York, NY.

Butow, B.J., Gras, P.W., Haraszi, R., and Bekes. F. 2002. Effects of different salts on mxing and extension parameters on a diverse group of wheat cultivars using 2-g mixograph and extensigraph methods. Cereal Chemistry. 79:826-833.

Callaghan, P.T., and Gil, A.M. 1999. ^{1}H NMR spectroscopy of polymers under shear and extensional flow. Rheol Acta. 38:528-536.

Callaghan, P.T., Godefroy, S., and Ryland, B.N. 2003[a]. Diffusion-relaxation correlation in simple pore structures. Journal of Magnetic Resonance. 162:320-327.

Callaghan, P.T., Godefroy, S., and Ryland, B.N. 2003[b]. Use of the second dimension in PGSE NMR studies of porous media. Magnetic Resonance Imaging. 21:243-248.

Calucci, L., Forte, C., Galleschi, L., Geppi, M., Ghiringhelli, S. 2003. ^{13}C and ^{1}H solid state NMR investigation of hydration effects on gluten dynamics. International Journal of Biological Macromolecules. 32:179-189.

Campbell, I.D., and Dwek, R.A. 1984. *Biological Spectroscopy*. The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA. 404 pp.

Campos, D.T., Steffe, J.F., and Perry, K.W.N. 1997. Rheological behavior of undeveloped and developed wheat dough. Cereal Chemistry. 74:489-494.

Chang, R. 1996. *Química*. 3rd ed. McGraw-Hill Interamericana. D.F., Mexico. 1221 pp.

Castell-Perez, M.E., and Steffe, J.F. 1992. Viscoelastic properties of dough. Pages 77-102 in: *Viscoelastic Properties of Foods.* Rao, M.A., and Steffe, J.F. Elsevier Applied Sc., London, Great Britain.

Cheftel, J.C., and Cuq, J.-L. 1985. Amino acids, peptides, and proteins. Pages: 246-370 in: *Food Chemistry.* Fennema, O.R. 2nd ed. Marcel Dekker, Inc., New York, NY.

Cherian, G., and Chinachoti, P. 1996. ^{2}H and ^{17}O nuclear magnetic resonance study of water in gluten in the glassy and rubbery state. Cereal Chemistry. 73:618-624.

Cherian, G., and Chinachoti, P. 1997. Action of oxidants on water sorption, ^{2}H nuclear magnetic resonance mobility, and glass transition behaviour of gluten. Cereal Chemistry. 74:312-317.

Clarke, C.I., Schober, T.J., and Arendt, E.K. 2002. Effect of single strain and traditional mixed strain starter cultures on rheological properties of wheat dough and on bread quality. Cereal Chemistry. 79:640-647.

Clarke, C.I., Schober, T.J., Dockery, P., O'Sullivan, K., and Arendt, E.K. 2004. Wheat sourdough fermentation: effects of time and acidification on fundamental rheological properties. Cereal Chemistry. 81:409-417.

Coupland, J.N. 2004. Low intensity ultrasound. Food Research International. 37(6):537-544.

Cumming, D.B., and Tung, M.A. 1975. Dynamic shear behaviour of commercial wheat gluten. Canadian Institute of Food Science and Technology Journal. 8(4):206-210.

Cuq, B., Gontard, N., and Guilbert, S. 1998. Proteins as agricultural polymers for packaging production. Cereal Chemistry. 75:1-9.

Damodaran, S., and Kinsella, J.E. 1982. Effects of ions on protein conformation and functionality. Pages: 327-358 in: *Food Protein Deterioration, Mechanisms and Functionality.* Cherry, J.P. American Chemical Society, Washington, DC.

Danno, G., and Hoseney, R.C. 1982. Effect of sodium chloride and sodium dodecyl sulfate on mixograph properties. Cereal Chemistry. 59:202-204.

Dobraszczyk, B.J., and Morgenstern, M.P. 2003. Rheology and the breadmaking process. Journal of Cereal Science. 38:229-245.

Dreese, P.C, Faubion, J.M., and Hoseney, R.C. 1988[a]. Dynamic rheological properties of flour, gluten, and gluten-starch doughs. I. Temperature-dependent changes during heating. Cereal Chemistry. 65:348-353.

Dreese, P.C, Faubion, J.M., and Hoseney, R.C. 1988[b]. Dynamic rheological properties of flour, gluten, and gluten-starch doughs. I. Effect of various processing and ingredient changes. Cereal Chemistry. 65:354-359.

Dreese, P.C, Faubion, J.M., and Hoseney, R.C. 1988[c]. The effect of different heating and washing procedures on the dynamic rheological properties of wheat gluten. Cereal Foods World. 33:225-228.

Duarte, I.F., Barros, A., Almeida, C., Spraul, M., and Gil, A.M. 2004. Multivariate analysis of NMR and FTIR data as a potential tool for the quality control of beer. Journal of Agricultural and Food Chemistry. 52:1031-1038

Dunn, F. 1969. Absorption and dispersion of ultrasound in biological media. Pages: 205-332 in: *Biological Engineering*. Schwan, H.P. Inter-University Electronic Series, Vol. 9. McGraw-Hill Book Company, New York, NY.

Edwards, N.M., Dexter, J.E., Scanlon, M.G., and Cenkowski, S. 1999. Relationship of creep-recovery and dynamic oscillatory measurements to durum wheat physical dough properties. Cereal Chemistry. 76:638-645.

Eliasson, A.-C. 1990. Rheological properties of cereal proteins. Pages 67-110 in: *Dough Rheology and Baked Product Texture.* Faridi, H., and Faubion, J.M. Van Nostrand Reinhold, New York, NY.

Eliasson, A.-C., and Larsson, K. 1993. *Cereals in Breadmaking, a Molecular Colloidal Approach.* Marcel Dekker, Inc., New York, NY. 376 pp.

Elmehdi, H.M. 2001. An Ultrasonic Investigation of the Effect of Voids on the Mechanical Properties of Bread Dough and the Role of Gas Cells in Determining the Cellular Structure of Freeze-dried Breadcrumb. Ph.D. Thesis, University of Manitoba, Winnipeg, MB. 196 pp.

Elmehdi, H.M., and Kovacs, M.I.P. 2003. Using ultrasound to investigate the structural and functional properties of wheat gluten. The Preceeding of 3rd International Simposium on Food Rheology and Structure. Pp. 547, 548.

Elmehdi, H.M., Page, J.H., and Scanlon, M.G. 2003. Monitoring dough fermentation using acoustic waves. Transactions of the Institution of Chemical Engineers. 81(part C):217-223.

Elmehdi, H.M., Page, J.H., and Scanlon, M.G. 2004. Ultrasonic investigation of the effect of mixing time under reduced pressure on the mechanical properties of bread dough. Cereal Chemistry. 81:504-510.

Esselink, E.F.J., van Aalst, H., Maliepaard, M., and van Duynhoven, J.P.M. 2003[a]. Long-term storage effect in frozen dough by spectroscopy and microscopy. Cereal Chemistry. 80:396-403.

Esselink, E.F.J., van Aalst, H., Maliepaard, M., Henderson, T.M.H., Hoekstra, N.L.L., and van Duynhoven, J.P.M. 2003[b]. Impact of industrial dough processing on structure: a rheology, nuclear magnetic resonance, and electron microscopy study. Cereal Chemistry. 80:419-423.

FAO. 2004. *FAOSTAT Database.* [Online Database]. URL: www.fao.org (May 2004).

Faubion J.M. and Faridi, H.A. 1986. Dough rheology: its benefits to cereal chemistry. Pages 1-10 in: *Fundamentals of Dough Rhelology.* Faridi, H. and Faubion, J.M. ed. AACC, Saint Paul, MN.

Faubion, J.M., and Hoseney, R.C. 1990. The viscoelastic properties of wheat flour doughs. Pages: 29-66 in: *Dough Rheology and Baked Product Texture.* Faridi, H., and Faubion, J.M. Van Nostrand Reinhold, New York, NY.

Feeney, K.A., Wellner, N., Gilbert, S.M., Halford, N.G., Tatham, A.S., Shewry, P.R., and Belton, P.S. 2003. Molecular structures and interactions of repetitive peptides based on wheat glutenin subunits depend on chain length. Biopolymers. 72:123-131.

Funt Bar-David C.B. and Lerchenthal, C.H.H. 1975. Rheological and thermodynamic properties of gluten gel. Cereal Chemistry. 52:154r-160r.

Galal, A.M., Marston, E.V. and Johnson, J.A. 1978. Rheological dough properties as affected by organic acids and salt. Cereal Chemistry. 55:683-691.

Gan, Z., Ellis, P.R., and Schofield, J.D. 1995. Mini Review. Gas cell stabilisation and gas retention in wheat bread dough. Journal of Cereal Science. 21:215-230.

Gennadios, A., Brandenburg, A., Weller, C.L. and Testin, R.F. 1993. Effect of pH on properties of wheat gluten and soy protein isolate films. Journal of Agricultural and Food Chemistry. 41:1835-1839.

Georgopolous, T., Larsson, H., and Eliasson, A.-C. 2004. A comparison of the rheological properties of wheat flour dough and its gluten prepared by ultracentrifugation. Food Hydrocolloids. 18:143-151.

Gil, A.M. 1995. NMR studies of cereal proteins. Pages: 272-286 in: *Magnetic Resonance in Food Science.* Belton, P.S, Delgadillo, I., Gil, A.M., and Webb, G.A. The Royal Society of Chemistry, Cambridge, Great Britain.

Gil, A.M., Alberti, E., Tatham, A.S., Belton, P.S., Humpfer, E., and Spraul, M. 1997. Magic angle spinning NMR study of the hydration of the wheat seed storage protein omega-gliadins. Magnetic Resonance in Chemistry. 35:S101-S111.

Gil, A.M., Duarte, I.F., Delgadillo, I., Colquhoun, I.J., Casuscelli, F., Humpfer, E., and Spraul, M. 2000. Study of the compositional changes of mango during ripening by use of nuclear magnetic resonance spectroscopy. Journal of Agricultural and Food Chemistry. 48:1524-1536.

Gil, A.M., Alberti, E., and Santos, D. 2001. An insight into the structure of foods using ^{13}C and ^{1}H magic angle spinning (MAS) NMR: application to wheat dough.

Pages: 43-53 in: *Magnetic Resonance in Food Science, A View to the Future*. Webb, G.A., Belton, P.S., Gil., A.M., Delgadillo, I. The Royal Society of Chemistry, Cambridge, Great Britain.

Gil, A.M., Alberti, E., Tatham, A.S., Belton, P.S., Humpfer, E., and Spraul, M. 2002. A fast MAS ^{1}H NMR study of amino acids and proteins. Journal of Molecular Structure. 602-603:357-366.

Gladden, L.F., and Alexander, P. 1996. Applications of nuclear magnetic resonance imaging in process engineering. Measurement Science and Technollogy. 7:423-435.

Godefroy, S., and Callaghan, P.T. 2003. 2D relaxation/diffusion correlations in porous media. Magnetic Resonance Imaging. 21:381-383.

Godefroy, S., Ryland, B., and Callaghan, P.T. 2002. 2D Laplace Inversion Instruction Manual. McDiarmid Institute of Advanced Materials and Nanotechnology, Victoria University of Wellington, Wellington, New Zealand. 8 pp.

Godefroy, S., Creamer, L.K., Watkinson, P.J., and Callaghan, P.T. 2003. The use of 2D Laplace inversion in food material. Report to Fronterra. Fronterra Research Centre and McDiarmid Institute of Advanced Materials and Nanotechnology, Victoria University of Wellington, Wellington, New Zealand. 37 pp.

Gontard, N., Guilbert, S., and Cuq, J.L. 1992. Edible wheat gluten films: influence of the main process variables on film properties using response surface methodology. Journal of Food Science. 57(1):190-195.

Gontard, N., and Ring, S. 1996. Edible wheat gluten film: Influence of water content on glass transition temperature. Journal of Agricultural and Food Chemistry. 44:3474-3478.

Grant, A., Belton, P.S., Colquhoun, I.J., Parker, M.L., Plitjer, J.J., Shewry, P.R., Tatham, A.S., and Wellner, N. 1999. Effects of temperature on sorption of water by wheat gluten determined using deuterium nuclear magnetic resonance. Cereal Chemistry. 76:219-226.

Gras P.W., Anderssen, R.S., Keentok, M. Békés, F. and Appels. R. 2001. Gluten protein functionality in wheat flour processing: a review. Australian Journal of Agricultural Research. 52:1311-1323.

Hatefi, Y., and Hanstein, W.G. 1969. Solubilization of particulate proteins and nonelectrolytes by chaotropic agents. Proceedings of the National Academy of Sciences of the United States of America. 62:1129-1136.

He, H., Roach, R.R., and Hoseney, R.C. 1992. Effect of nonchaotropic salts on flour bread-making properties. Cereal Chemistry. 69:366-371.

Hlynka, J. 1962. Influence of temperature, speed of mixing, and salt on some rheological properties of dough in the farinograph. Cereal Chemistry. 39:286-303.

Hollander, M., and Wolfe, D. A. 1973. *Nonparametric Statistical Methods*. Wiley, New York, NY. 503 pp.

Holmes, J.T., and Hoseney, R.C. 1987. Chemical leavening: effect of pH and certain ions on breadmaking properties. Cereal Chemistry. 64:343-348.

Hornak, J.P. 2003. *The Basics of NMR*. [Online database]. URL: www.cis.rit.edu/htbooks/nmr/inside.htm (August 2003).

Hoseney, R.C. 1984. Gas retention in bread doughs. Cereal Foods World. 29:305-308.

Hoseney, R.C. 1992. Physical chemistry of bread dough. Pages: 443-457 in: *Physical Chemistry of Foods*. Schwartzberg, H.G., and Hartel, R.W. Marcel Dekker, New York, NY.

Hoseney, R.C. 1994. *Principles of Cereal Science and Technology*. 2nd ed. AACC, St. Paul, MN. 378 pp.

Hoseney, R.C. and Brown, R.A. 1983. Mixograph studies. V. Effect of pH. Cereal Chemistry. 60:124-126.

Huebner, F.R. 1977. Wheat flour proteins and their functionality in baking. The Bakers Digest. 51:25-31

Hussain, A., Lukow, O.M., and McKenzie, R.I.H. 1998. Rheological properties of gluten derived from wheat cultivars with identical HMW glutenin subunit composition. Journal of the Science of Food and Agriculture. 78:551-558.

Hutchens, T.W., and Yip, T.-T. 1990. Protein interactions with immobilized transition metal ions: quantitative evaluations of variations in affinity and binding capacity. Analytical Biochemistry. 191:160-168.

Janssen, A.M., van Vliet, T., and Vereijken J.M. 1996[a]. Rheological behaviour of wheat glutens at small and large deformations. Comparison of two glutens differing in bread making potential. Journal of Cereal Science. 23:19-31.

Janssen, A.M., van Vliet, T., and Vereijken J.M. 1996[b]. Rheological behaviour of wheat glutens at small and large deformations. Effect of gluten composition. Journal of Cereal Science. 23:33-42.

Juodeikiene, G., and Basinskiene, L. 2004. Non-destructive texture analysis of cereal products. Food Research International. 37(6):603-610.

Kasarda, D.D., Nimmo, C.C., and Kohler, G.O. 1971. Proteins and the amino acid composition of wheat fractions. Pages 227-618 in: *Wheat, Chemistry and Technology*. Pomeranz, Y. AACC, St. Paul, MN.

Kent, N.L. 1983. *Technology of Cereals, an Introduction for Students of Food Science and Agriculture.* 3rd ed. Pergamon Press, Oxford, Great Britain.

Kerr C., Hoseney R.C. and Faubion, J.M. 1993. Mixograph studies. VI. Combined effects of charge (pH), activated double-bond compounds, and oxidants on dough mixing properties. Cereal Chemistry. 70:633-636.

Khan, K., Tamminga, G., and Lukow, O. 1989. The effect of wheat flour proteins on mixing and baking - correlations with protein fractions and high molecular weight glutenin subunit composition by gel electrophoresis. Cereal Chemistry. 66:391-396.

Khatkar, B.S., Bell, A.E., and Schofield, J.D. 1995. The dynamic rheological properties of glutens and gluten sub-fractions from wheats of good and poor bread making quality. Journal of Cereal Science. 22:29-44.

Kim, H.R., and Bushuk, W. 1995. Salt sensitivity of acetic acid-extractable proteins of wheat flour. Journal of Cereal Science. 21:241-250.

Kinsella, J.E., and Hale, M.L. 1984. Hydrophobic associations and gluten consistency: effects of specific anions. Journal of Agricultural and Food Chemistry. 32:1054-1056.

Kleinberg, R.L., and Horsfield, M.A. 1990. Transverse relaxation processes in porous sedimentary rock. Journal of Magnetic Resonance. 88:9-19.

Knowles, P.F., Marsh, D., and Rattle, H.W.E. 1976. *Magnetic Resonance of Biomolecules, an Introduction to the Theory and Practice of NMR and ESR in Biological Systems.* John Wiley & Sons, London, Great Britain. 343 pp.

Larsson, H. 2002. Effect of pH and sodium chloride on wheat flour dough properties: ultracentrifugation and rheological measurements. Cereal Chemistry. 79:544-545.

Larsson, H., and Eliasson, A.-C. 1996. Phase separation of wheat flour doughs studied by ultracentrifugation and stress relaxaton. II. Influence of mixing time, ascorbic acid, and lipids. Cereal Chemistry. 73:25-31.

Larsson, H., Eliasson, A.-C., Johansson, E., and Svensson, G. 2000. Influence of added starch on mixing of dough made with three wheat flour differing in high molecular weight subunit composition: rheological behavior. Cereal Chemistry. 77:633-639.

Lásztity, R. 1984. *The Chemistry of Cereal Proteins.* CRC Press, Inc., Boca Raton, FL. 203 pp.

Létang, C., Piau, M., and Verdier, C. 1999. Characterization of wheat flour-water doughs. Part I: rheometry and microstructure. Journal of Food Engineering. 41:121-132.

Létang, C., Piau, M., Verdier, C., and Lefevre, L. 2001. Characterization of wheat flour-water doughs: a new method using ultrasound. 39:133-141.

Li, W., Dobraszczyk, B.J., and Schofield, J.D. 2003. Stress relaxation behavior of wheat dough, gluten, and gluten protein fractions. Cereal Chemistry. 80:333-338.

Lindsay, M.P., and Skerritt, J.H. 1999. The glutenin macropolymer of wheat flour doughs: structure-function perspectives. Trends in Food Science and Technology. 10:247-253.

MacRitchie, F. 1999. Wheat proteins: characterization and role in flour functionality. Cereal Foods World. 44(4):188-193.

MacRitchie, F. 1987. Evaluation of contributions from wheat protein fractions to dough mixing and breadmaking. Journal of Cereal Science. 6:259-268.

MacRitchie, F., and Lafiandra, D. 1997. Structure-function relationships of wheat proteins. Pages: 293-324 in: *Food and their applications.* Damodara, S., and Paraf A. Marcel Dekker Inc., New York, NY.

Magnuson, K. M. 1985. Uses and functionality of vital wheat gluten. Cereals Foods World. 30(2):179-181.

Mani, K., Eliasson, A.-C., Lindahl, L., and Trägardh, C. 1992. Rheological properties and breadmaking quality of wheat flour doughs made with different dough mixers. Cereal Chemistry. 69:222-225.

Marcus, Y. 1985. *Ion Solvation*. John Wiley & Sons Limited, Chichester, Great Britain. 306 pp.

McClements, D.J. 1997. Ultrasonic characterization of foods and drinks: principles, methods, and applications. Reviews in Food Science and Nutrition. 37(1):1-16.

Menjivar, J.A. Fundamental aspects of dough rheology. Pages: 1-28 in: *Dough Rheology and Baked Product Texture.* Faridi, H., and Faubion, J.M. Van Nostrand Reinhold, New York, NY.

Miller, R.A., Graf, E., and Hoseney, R.C. 1994. Leavened dough pH determination by an improved method. Journal of food Science. 59(5):1086-1090.

Minister of Industry. 2003. *Food Composition in Canada*. Cataloge no. 32-229-XIB. Part 1.2002. Statistics in Canada. [Online database]. Minister of Industry. Ottawa. URL: www.statcan.ca

Mohsenin, N.N. 1986. *Physical Properties of Plant and Animal Materials.* Gordon and Breach Science Publishers, New York, NY. 891 pp.

Nayal, M., and Di Cera, E. 1994. Predicting Ca^{2+}-binding sites in proteins. Proceedings of the National Academy of Sciences of the United States of America. 91:817-821.

Neter, J., Kutner, M., Nachtshem, and Wasserman, W. 1990. *Applied Linear Statistical Models: Regression Analysis of Variance, and Experimental Designs.* 3rd ed., Homewood, IL. 1181 pp.

Nierle, W., and Kersting, H.-J. 1998. Changes of physical properties of wheat gluten and starch as a function of removing some attending substances. Starch. 50(11-12):493-499.

Ogrinic, N., Kosir, I.J., Spangenberg, J.E., and Kidric, J. 2003. The application of NMR and MS methods for detection of adulteration of wine, fruit juices, and olive oil. A review. Analytical and Bioanalytical Chemistry. 376(4):424-430.

Ono, H., Yamada, H., Matsuda, S., Okajima, K., Kawamoto, T., and Ijima, H. 1998. ^{1}H-NMR relaxation of water molecules in the aqueous microcrystaline cellulose suspension systems and their viscosity. Cellulose. 5:231-247.

Pauling, L. 1945. The adsorption of water by proteins. Journal of the American Chemical Society. 67:555-557.

Popineau, Y., Bonenfant, S., Cornec, M., and Pezolet, M. 1994. A study by infrared spectroscopy of the conformations of gluten proteins differing in their gliadin and glutenin compositions. Journal of Cereal Science. 20:15-22.

Preston, K.R. 1981. Effects of neutral salt upon wheat gluten protein properties. I. Relationship between the hydrophobic properties of gluten proteins and their extractability and turbidity in neutral salts. Cereal Chemistry. 58:317-324.

Preston, K.R. 1984. Gel filtration and characterization of neutral salt extracted wheat gluten proteins varying in hydrophobic properties. Cereal Chemistry. 61:76-83.

Preston, K.R. 1989. Effects of neutral salts of the lyotropic series on the physical dough properties of a Canadian red spring wheat flour. Cereal Chemistry. 61:76-83.

Price, W.S. 1997. Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion: part 1. Basic theory. Concepts of Magnetic Resonance. 9:299-336.

Rao, V.K., and Seib, A. 1999. Effect of zinc and aluminum ions in breadmaking. Cereal Chemistry. 76:355-360.

Redl, A., Morel, M.H., Bonicel, J., Guilbert, S., and Vergnes, B. 1999. Rheological properties of gluten pasticized with glycerol: dependence on themperature, glycerol content and mixing conditions. Rheologica Acta. 38:311-320.

Resa, P., Elvira, L., Montero de Espinosa, F. 2004. Concentration control in alcoholic fermentation processes from ultrasonic velocity measurements. Food Research International. 37(6):587-594.

Rha, C.K., and Pradipasena, P. 1986. Viscosity of proteins. Pages: 79-120 in: *Functional Properties of Food Macromolecules*. Mitchell, J.R., and Ledward, D.A. Elsevier Applied Science Publishers, London, Great Britain.

Ross, K.A., Pyrak-Nolte, L.J., Campanella, O.H. 2004. The use of ultrasound and shear oscillatory tests to characterize the effect of mixing time on the rheological properties of dough. Food Research International. 37:567-577.

Roudaut, G., Van Dusschoeten, D., Van As, H., Hemminga, M.A., and Le Meste, M. 1998. Mobility of lipids in low moisture bread as studied by NMR. Journal of Cereal Science. 28:147-155.

Salovaara, H. 1982. Effect of partial sodium chloride replacement by other salts on wheat dough rheology and breadmaking. Cereal Chemistry. 59:422-426.

Samari, S. 1994. Ultrasonic inspection methods for food products. Lebensmittel Wissenschaft und Technologie. 27:210-213.

Sanders, J.K.M., and Hunter, B.K. 1987. *Modern NMR Spectroscopy, a Guide for Chemists.* Oxford University Press, Oxford, Great Britain. 308 pp.

Scanlon, M.G. 2004. Editorial: low-intensity ultrasound for food research and the food industry. Food Research International. 37(6):535-536.

Sekhon, K.S., Singh, N., Kaur, H., and Nagi, H.P.S. 1995. Improving the functional and bread making properties of sprouted Indian wheat. Journal of Food Processing and Preservation. 19:147-160.

Serna, S.S. 2001. *Química, Almacenamiento e Industrialización de los cereales.* AGT Editor, S.A. , D.F., México. 521 pp.

Seymour, J.D., Caprihan, A., Altobelli, S.A., Fukushima, E. 2000. Pulsed gradient spin echo nuclear magnetic resonance imaging of diffusion in granular flow. Physical Review Letters. 84(2):266-269.

Shellenberg, J.A. 1971. Production and utilization of wheat. Pages 1-10 in: *Wheat chemistry and thechnology*, 2nd ed. Pomeranz, Y. Ed. AACC, Saint Paul, MN.

Shewry, P.R., Popineau, Y., Lafiandra, D., and Belton, P. 2001. Wheat glutenin subunits and dough elasticity: findings of the EUROWHEAT project. Trends in Food Science and Technology. 11:433-441.

Shewry, P.R., and Halford, N.G. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. Journal of Experimental Botany. 53(370):947-958.

Shewry, P.R., Halford, N.G, Belton, P.S., and Tatham, A.S. 2002. The structure and properties of gluten: an elastic protein from wheat grain. Philosophical Transactions of the Royal Society of London. 357:133-142.

Shiau, S.-Y., and Yeh, A.-I. 2001. Effects of alkali and acid on dough rheological properties and characteristics of extruded noodles. Journal of Cereal Science. 33:27-37.

Singh, H., and MacRitchie, F. 2001. Use of sonication to probe wheat gluten structure. Cereal Chemistry. 78:526-529.

Smith D.E., and Mullen, J.D. 1970. Note on the effect of pH and oxygen on the mixing properties and protein solubility of flour doughs. Cereal Chemistry. 47:650-654.

Steffe, J.F. 1996. *Rheological Methods in Food Process Engineering.* 2nd ed. Freeman Press. East Lansing, MI. 418 pp.

Stejskal, E.O., and Tanner, J.E. 1965. Spin diffusion measurements: spin echoes in the presence of a time-dependent field gradient. The Journal of Chemical Physics. 42(1):288-292.

Sukhovich, A., 2002. Transmission through layered media program. Based on: *Waves in Layered Media*. 1960. Brekhovskikh, L.M. Academic Press, New York, NY. 561 pp.

Takeda, K., Matsumura, Y., and Shimizu, M. 2001. Emulsifying and surface properties of wheat gluten under acidic conditions. Journal of Food Science. 66(3):393-399.

Tanaka, K., Furukawa, K. and Matsumoto, H. 1967. The effect of acid and salt on the farinogram and extensigam of dough. Cereal Chemistry. 44:675-680.

Tanner, R.I., and Walters, K. 1998. *Rheology: an Historical Perspective.* Elsevier, Amsterdam, The Netherlands. 255 pp.

Torres, P.I., Vazquez-Moreno, L.., Ledesma-Osuna, A.I., and Medina-Rodriguez, C. 2000. Contribution of hydrophobic soluble gluten proteins, fractionated by hydrophobic interaction chromatography in highly acetylated agarose, to dough rheological properties. Cereal Chemistry. 77:702-707.

Tung, M.A. Unknown. Some rheological principles involved in food texture. Department of Food Science and Technology. Canadian Institute of Fisheries Technology. Technical University of Nova Scotia. Halifax, NS. 28 pp.

Umbach, S.L., Davis, E.A., Gordon, J., and Callaghan, P.T. 1992. Water self-diffusion coefficients and dielectric properties determined for starch-gluten-water mixtures heated by microwave and by conventional methods. Cereal Chemistry. 69:637-642.

Uthayakumaran, S., Newberry, M., Phan-Thien, N., and Tanner, R. 2002. Small and large strain rheology of wheat gluten. Rheologica Acta. 41:162-172.

Veraverbeke, W.S., and Delcour, J.A. 2002. Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. Critical Reviews in Food Science and Nutrition. 42(3):179-208.

Verdier, C., and Piau, M. 1997. Acoustic wave propagation in two-phase viscoelastic fluids: the case of polymer emulsions. Journal of the Acoustical Society of America. 101(4):1868-1876.

Voet, D., and Voet, J.G. 1990. *Biochemistry*. John Wiley & Sons, New York, NY. 1223 pp.

Von Hippel, P.H., and Schleich, T. 1969. The effects of neutral salts on the structure and conformational stability of macromolecules in solution. Pages: 417-574 in: *Structure and Stability of Biological Macromolecules.* Timasheff, S.N., and Fasman, G.D. Marcel Dekker, Inc., New York, NY.

Wadhawan, C.K. 1988. Fundamental Studies on Vitality of Gluten for Breadmaking. Ph.D. Thesis, University of Manitoba, Winnipeg, MB. 275 pp.

Wang, Y., Belton, P.S., Bridon, H., Garanger, E., Wellner, N., Parker, M.L., Grant, A., Feillet, P., and Noel, T.R. 2001. Physicochemical studies of caroubin: a gluten-like protein. Journal of Agricultural and Food Chemistry. 49:3414-3419.

Weak E.D., Hoseney, R.C. Seib, P.A., and Biag, M. 1977. Mixograph studies. I. Effect of certain compounds on mixing properties. Cereal Chemistry. 54:794-802.

Wehrle, K., Grau, H. and Arendt, E.K. 1997. Effects of lactic acid, acetic acid, and table salt on fundamental rheological properties of wheat dough. Cereal Chemistry. 74:739-744.

Weipert, D. 1990. The benefits of basic rheometry in studying dough rheology. Cereal Chemistry. 67:311-317.

Weipert, D. 1992. Descriptive and fundamental rheometry in a new light. Cereal Foods World. 37(1):15-24.

Wellner, N., Bianchini, D., Clare Mills, E.N., Belton, P.S. 2003. Effect of selected Hofmeister anions on the secondary structure and dynamics of wheat prolamins in gluten. Cereal Chemistry. 80:596-600.

Williams, E.G. 1999. *Fourier Acoustics, Sound Radiation and Nearfield Acoustical Holography.* Academic Press. San Diego, CA. 306 pp.

Wood, W.B., Wilson, J.H., Benbow, R.M., and Hood, L.E. 1981. *Biochemistry, A Problems Approach*. 2nd Ed. The Benjamin/Cummings Publishing Co. Menlo Park, CA. 498 pp.

Wu Y.V., and Dimler, R.J. 1963[a]. Hydrogen-ion equilibria of wheat gluten. Archives of Biochemistry and Biophysics. 102:230-237.

Wu Y.V., and Dimler, R.J. 1963[b]. Hydrogen-ion equlibria of wheat glutenin and gliadin. Archives of biochemistry and biophysics. 103:310-318.

Zhao, F.-J., Salmon, S.E., Withers, P.J.A., Evans, E.J., Monaghan, J.M., Shewry, P.R., and McGrath, S.P. 1999. Responses of breadmaking quality to

sulphur in three wheat varieties. Journal of the Science of Food and Agriculture. 79:1865-1874.

Zhang, G.Q., and Hirasaki, G.J. 2003. CPMG relaxation by diffusion with constant magnetic field gradient in a restricted geometry: numerical simulation and application. Journal of Magnetic Resonance 163:81-99.

APPENDIX 1

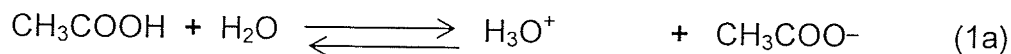
Assessment of the amount of hydronium ions (H^+) incorporated in the gluten matrix during mixing

When acid is added to form the gluten doughs, the pH is lowered. If no hydrogen ions were sorbed by the gluten matrix, the pH of the resultant gluten doughs would be approximately the same value as the acid solution. Because dough pH is higher in comparison to the pH of the acid solution, it means that some of the hydronium ions (H_3O^+) of the solution have been sorbed by the gluten matrix. In order to determine this amount of sorbed ions, some considerations have to be taken into account: the concentration of H_3O^+ coming from the acids used, the initial concentration of H_3O^+ in the gluten dough (before acid addition), and the final concentration of H_3O^+ in the gluten dough (after acid addition).

Concentration of H_3O^+ coming from acetic and hydrochloric acid

In order to calculate the concentration of H_3O^+ ($[H_3O^+]$) donated by acetic and hydrochloric acids, their acid dissociation constants (K_a) have to be considered. Hydrochloric acid is an acid which dissociates approximately 100% when diluted in water and thus it can be said that all the H_3O^+ coming from this acid are available for sorption by the gluten. On the other hand, acetic acid has a K_a of 1.75×10^{-5}, and therefore its dissociation at the concentrations in which it was added to the gluten have to be calculated as follows.

At equilibrium, the same amount of H_3O^+ and acetate ions (CH_3COO^-) are expected (Eq. 1a).



$$CH_3COOH + H_2O \rightleftharpoons H_3O^+ + CH_3COO^- \quad (1a)$$

Knowing the K_a of acetic acid and the concentration at which it was used as the mixing water ([CH_3COOH]), the concentration of both ions (H_3O^+ and CH_3COO^-) can be obtained considering the dissociation constant of acetic acid, as given by,

$$K_a = ([H_3O^+] [CH_3COO^-]) / [CH_3COOH], \quad (2a)$$

where K_a is the dissociation constant of acid, $[H_3O^+]$ is the concentration of hydronium ions, $[CH_3COO^-]$ is the concentration of acetate ions, and $[CH_3COOH]$ is the concentration of the acetic acid. If the condition: $\{(x / [CH_3COOH]) (100)\} < 5$ is true, then simplification of Eq. 2a can be performed for equilibrium conditions, so that the following Eq. can be used,

$$K_a = x^2 / [CH_3COOH], \quad (3a)$$

Rearranging and substituting values in Eq. 3a, the dissociation (x) of the acid is obtained as follows,

$$x = \{(1.8 \times 10^{-5}) ([CH_3COOH])\}^{1/2}, \quad (4a)$$

When the above condition is not met, then other calculations have to be done (for more information see Chang, 1996). For the concentrations at which acetic acid was used in this thesis, this simplifying condition was accomplished except for the acetic acid concentration of 3.5 mM. From Eq. 4a (including the pertinent corrections using higher-order terms), the concentration of H_3O^+ ions coming from acetic acid that would be available for sorption by the gluten dough were

then obtained. Concentration of available H_3O^+ ions coming from both acetic and hydrochloric acid solutions are shown in Table 1a.

Table 1a. Concentration of hydronium ions (H_3O^+) coming from acetic and hydrochloric acid

$[CH_3COOH]$ (mM)	$[H_3O^+]$ from CH_3COOH (mM)	[HCl] (mM)	$[H_3O^+]$ from HCl (mM)
3.5	0.24	32.9	32.9
8.8	0.40	115.6	115.6
17.5	0.56	165.3	165.3
177.1	1.79	265.4	265.4

$[CH_3COOH]$ Concentration of acetic acid used as mixing water
[HCl] Concentration of hydrochloric acid used as mixing water

Initial and final concentration of H_3O^+ in the gluten doughs

The concentration of H_3O^+ that was originally in the gluten dough (5.13×10^{-7} M), was calculated with Eq. 5a, considering the experimental result for the pH of the control dough. This concentration of H_3O^+ includes the contribution of H_3O^+ coming from the water (approximately 1×10^{-7} M, since $pH_{water} = 7$) as well as the protons liberated by the gluten proteins (Eq. 5a).

From the experimentally measured pH values of the control dough (pH of 6.3) and the acid gluten doughs (close to the desired targets of 5.8, 5.3, 4.8 and 4.3), the concentration of free H_3O^+ ions was calculated, as given by Eq. 6a,

$$[H_3O^+]_{AA} = [H_3O^+]_{GF} - ([H_3O^+]_{GC} + [H_3O^+]_{w}), \qquad (5a)$$

where $[H_3O^+]_{AA}$ is the concentration of H_3O^+ in the gluten due to acids addition, $[H_3O^+]_{GF}$ is the concentration of H_3O^+ in the gluten after acids addition, $[H_3O^+]_{GC}$

is the concentration of H_3O^+ in the control gluten dough, and $[H_3O^+]_W$ is the oncentration of H_3O^+ in the water. In order to obtain the mentioned concentrations, the definition of pH has to be considered, as given by,

$$pH = - \log [H^+]. \tag{6a}$$

Calculations of sorbed H_3O^+ by the gluten dough

The concentration of H_3O^+ ions sorbed by the gluten doughs can be calculated once the concentration of H_3O^+ ions coming from the acids (acetic and hydrochloric) is known, as well as the concentration of H_3O^+ ions originally in the gluten dough, as given by Eq. 7a,

$$[H_3O^+]_{SG} = [H_3O^+]_{HB} - [H_3O^+]_{AA}, \tag{7a}$$

where $[H_3O^+]_{SG}$ is the concentration of H_3O^+ sorbed by the gluten, $[H_3O^+]_{HB}$ is the concentration of H_3O^+ of the acid solution, and $[H_3O^+]_{AA}$ is the concentration of H_3O^+ in the gluten due to acids addition.

The % of H_3O^+ sorbed was obtained considering the concentration of H_3O^+ sorbed by the gluten ($H_3O^+{}_{SG}$) in respect to the original concentration of H_3O^+ correspondent to the acid solution ($H_3O^+{}_{HB}$), as given by Eq. 8a,

$$\% \text{ sorbed } H_3O^+ = (100)([H_3O^+]_{SG}) / ([H_3O^+]_{HB}). \tag{8a}$$

From the data shown in Table 2a, it can be seen that around 100% of the H_3O^+ coming from the acids were sorbed by the gluten proteins. Based on the principle of Le Chatelier (Chang, 1996), the chemical equation (Eq. 1a) would be driven to the products' side as the result of sorption of H_3O^+ by the gluten matrix.

This would enhance further dissociation of the acetic acid until no more undisociated acetic acid would be present. Therefore, complete dissociation of the acetic acid was assumed in this thesis (Table 2a).

Table 2a. Hydronium ions (H_3O^+) sorbed into the gluten matrix during mixing

	Acid concentration (mM)	Concentration of sorbed H^+ (M)	% of H^+ that are sorbed
HAc	3.5	2.42×10^{-4}	99.8
	8.8	3.94×10^{-4}	99.2
	17.5	5.52×10^{-4}	98.2
	177.1	1.76×10^{-3}	98.3
HCl	32.9	3.30×10^{-2}	≈100
	115.6	1.16×10^{-1}	≈100
	165.3	1.65×10^{-1}	≈100
	265.4	2.65×10^{-1}	≈100

HAc Acetic acid
HCl Hydrochloric acid

Acid concentration and H^+ concentration is equivalent since both acids are monoprotic and are essentially fully dissociated