Universitat Autònoma de Barcelona

Departament de Ciència Animal i dels Aliments
Facultat de Vetèrinaria
Universitat Autònoma de Barcelona

DEVELOPMENT OF GLUTEN-FREE BREAD FORMULATIONS

Begoña Miñarro Vivas

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Marta Capellas Puig i Elena Albanell Trullás, professores titulars del Departament de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona,

FAN CONSTAR que la llicenciada en Ciència i Tecnologia dels Aliments Begoña Miñarro Vivas ha realitzat, sota la seva direcció, el treball titulat "Development of gluten-free bread formulations" que presenta per optar al grau de Doctor en Ciència dels Aliments.

I perquè així consti, signen el present document a Bellaterra (Cerdanyola del Vallès), el 2 d'abril del 2013

A mis padres, Felipe Miñarro y Begoña Vivas

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Publications and presentations related to the thesis

- ## Publications

Title: Influence of unicellular protein on gluten-free bread characteristics

Authors: B. Miñarro, I. Normahomed, B. Guamis, M. Capellas

Reference: European Food Research and Technology 2010, 231(2):171-179

Title: Effect of legume flours on baking characteristics of gluten-free bread

Authors: B. Miñarro, E. Albanell, N. Aguilar, B. Guamis, M. Capellas

Reference: Journal of Cereal Science 2012, 56(2):476-481

Title: Detection of low-level gluten content in flour and batter by near infrared reflectance spectroscopy (NIRS)

Authors: E. Albanell, B. Miñarro, N. Carrasco

Reference: Journal of Cereal Science 2012, 56(2):490-4959

Title: Liquid whey as an ingredient to formulate gluten-free bread

Authors: B. Miñarro, E. Albanell , N. Aguilar, B. Guamis, M. Capellas

Reference: Journal of Bioprocess Technology (sent)

- **Scientific communications**

Title: Development of gluten-free bread
Authors: B. Miñarro, I. Normahomed, M. Capellas, B. Guamis
Meeting: BIET'07 European Meeting on Baking Ingredients, Enzymes, and Technology
Place and Date: 16-17 May, 2007, Barcelona. Spain
Type of communication: Poster communication

Title: Novel technologies for gluten-free bread development
Authors: B. Miñarro, M. Capellas, E. Albanell
Meeting: 9th European Young Cereal Scientist and Technologist Workshop
Place and Date: 25-27 May, 2010, Budapest. Hungary
Type of communication: Oral communication

Title: New ingredients in gluten-free formulations
Authors: N. Aguilar, B. Miñarro, E. Albanell, B. Guamis, M. Capellas
Meeting: 10th European Young Cereal Scientist and Technologist Workshop
Place and Date: 23-25 May, 2011, Helsinki. Finland
Type of communication: Oral communication

Title: Effect of legume flours on baking characteristics of gluten-free breads
Authors: B. Miñarro, E. Albanell, M. Capellas,
Meeting: Second international Symposium on Gluten-Free Cereal Products and Beverages
Place and Date: 8-11 June, 2010, Tampere. Finland
Type of communication: Poster communication

Title: Functionality of whey as a gluten-free ingredient
Authors: B. Miñarro, N. Aguilar, B. Guamis, E. Albanell, M. Capellas
Meeting: AACC International Annual Meeting
Place and Date: 16-19 Palm Springs, 2011, California. USA
Type of communication: Oral and Poster communication

Title: Whey as a gluten-free bread ingredient

Authors: B. Miñarro, E. Albanell, N. Aguilar, B. Guamis, M. Capellas,

Meeting: 11th European Young Cereal Scientists and Technologists Workshop

Place and Date: 9-11 May, 2012, Barcelona. Spain

Type of communication: Oral communication / Member of the organizing committee

Abstract

Celiac patients cannot tolerate gluten, the protein from wheat, rye and barley that gives to dough the viscoelastic properties required to develop bakery products of good quality. Its lack results in liquid batter rather than dough, yielding breads with a crumbling texture, pale colour and poor mouth-feel. Many studies have been carried out in the last years, testing potential ingredients and complex formulations with the aim of reproducing gluten functionality and developing gluten-free breads as similar as possible to wheat ones.

In this thesis, unicellular protein, legume flours and cheese whey have been studied as potential protein sources to improve gluten-free bread characteristics. Preliminary trials were performed to develop basic gluten-free formulations and optimize the bread making process. In the first study, starch, starch-vegetable and flour based formulations were prepared, and unicellular protein was added to increase bread protein content. Inclusion of unicellular protein caused a decrease in bake loss, an increase in hardness and a darkening of crumb and crust color. In a second study, four formulations prepared with legume protein sources (chickpea flour, pea protein isolate, carob germ flour and soya flour) were compared, with the aim of substituting soya flour. Carob germ flour bread presented the lowest specific volume and the highest hardness. Chickpea bread exhibited the best baking characteristics and, in general, good sensory behaviour, although its taste decreased consumer preference. Both chickpea flour and pea protein isolate could be promising alternatives to soya flour, due to its high allergenicity. A third study was performed to evaluate the effect of water and/or soya substitution by fresh and ripened liquid cheese whey in gluten-free bread. Combination of ripened or fresh whey and soya flour increased batter visco-elasticity and decreased bread specific volume. Breads with water or ripened whey and without soya were the most preferred by consumers, probably due to the softest texture and colour. In summary, specific improvements in gluten-free bread baking and sensory characteristics have been achieved during this research on gluten-free protein sources.

Finally, the last study included in this thesis demonstrates that NIRS methodology can predict accurately the concentration of gluten content in flours and batters. However, it should not be considered as a reliable method for determining gluten content contamination in gluten-free products.

Resumen

Los celíacos no toleran el gluten, la principal proteína presente en el trigo, el centeno y la cebada, que da a la masa las propiedades viscoelásticas necesarias para desarrollar productos panificables de buena calidad. Su ausencia en pan sin gluten, resulta en pastas líquidas en lugar de masas, originando panes con una textura quebradiza, color pálido y, en general, una calidad pobre. Han sido numerosos los estudios realizados en los últimos años, probando potenciales nuevos ingredientes y complejas formulaciones que permitieran imitar la funcionalidad del gluten y desarrollar panes sin gluten lo más similares posibles a los de trigo.

En esta tesis se han estudiado la proteína unicelular, harinas de leguminosas y el suero de quesería, como potenciales fuentes proteicas para mejorar las características del pan sin gluten. Se realizaron pruebas preliminares para conseguir una formulación de pan sin gluten básica y optimizar su proceso de fabricación. En el primer estudio, se añadió proteína unicelular a las formulaciones basadas en almidón, almidón-vegetal y harina, con el objetivo de aumentar el contenido proteico del pan. La inclusión de proteína unicelular causó una disminución de las pérdidas por cocción, un aumento de la dureza y el oscurecimiento del color de la corteza y la miga. En un segundo estudio, se compararon cuatro harinas de leguminosas (harina de garbanzo, aislado de proteína de guisante, harina de germen de garrofín y harina de soja), con el objetivo de sustituir la harina de soja, debido a su alta alergenicidad. El pan elaborado con germen de garrofín presentó el menor volumen específico y la dureza más alta. Los panes de garbanzo mostraron las mejores características panarias y, en general, un buen perfil sensorial, aunque su sabor disminuyó la preferencia del consumidor. Tanto la harina de garbanzo como el aislado de proteína de guisante podrían ser una alternativa prometedora a la harina de soja. En un tercer estudio se evaluó el efecto de la sustitución del agua y/o la harina de soja por suero líquido de quesería en pan sin gluten. La combinación de suero fresco o madurado y soja aumentó la viscoelasticidad de la masa y disminuyó el volumen específico del pan. Los panes preferidos por los consumidores fueron los elaborados con agua o suero madurado y sin soja, probablemente debido a su esponjosidad y color. En resumen, se han conseguido mejoras específicas en las características panarias y sensoriales del pan sin gluten mediante la investigación de potenciales fuentes proteicas sin gluten.

Finalmente, el último estudio de esta tesis demuestra que la metodología NIRS puede predecir con exactitud el contenido de gluten en harinas y masas. Sin embargo, no debe ser considerada como un método fiable para determinar la contaminación de gluten en productos sin gluten.

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Abbreviation Key

CD	Celiac disease
CLSM	Confocal laser scanning microscopy
CMC	Sodium carboxymethyl cellulose
DNA	Deoxyribonucleic acid
EMA	IgG and IgA anti-endomysial
G´	Storage modulus
G''	Elastic modulus
GF	Gluten-free
HLA	Human leukocyte antigens
HPMC	Hydroxypropyl methyl cellulose
LC	Liquid chromatography
LC–MS	Liquid chromatography–mass spectrometry
MS	Mass spectrometry
NIRS	Near infrared reflectance spectroscopy
PCR	Polymerase chain reaction
PPM	Parts per million
QC-PCR	Quantitative competitive polymerase chain reaction
RH	Relative humidity
TG2	Transglutaminase 2
TGA	Anti-tissue transglutaminase

Chapter 1

Interest of the study, objectives and working plan

1.1 Interest of the study

Currently, 1% of European population is suffering from celiac disease, which demands a strict gluten-free diet for the entire life. Gluten is part of some common cereals and, therefore, of basic foods included in the Mediterranean diet, like bakery products. The market for gluten-free products has shown considerable growth in the last years. However, consumer acceptance of these products is limited by their poor taste, texture and mouthfeel.

Gluten in wheat dough is a plastic-elastic substance consisting of gliadin and glutenin. Both proteins together give to wheat dough the elastic and extensible properties able to develop good quality bakery products. Its lack in gluten-free breads has led technologists to search for ingredients and technologies able to reproduce its unique properties. Although substantial work has been carried out (mainly involving the incorporation of starches, different sources of proteins and hydrocolloids into a gluten-free flour base) with the aim of improving the structure and overall acceptability of gluten-free breads, they still largely differ from wheat breads.

Alternative ingredients, such as animal and vegetable proteins sources, should be studied to test their ability to replace gluten and form similar network structures in gluten-free bread. The interaction between components in bread formulations and their influence on texture and flavour formation must be analysed in the search for particular improvements. Sensory and nutritional enhancement should be ensured by using proper raw materials and combinations. And finally, evaluation of consumer preferences and needs should be part of the research and allow the development of inexpensive and widely accepted gluten-free products.

1.2 Objectives

General Objective

The general objective of this dissertation was to study the effect of different ingredients on gluten-free bread physico-chemical and organoleptic characteristics. Additionally, the use of near infrared reflectance spectroscopy to detect low gluten levels was evaluated.

Specific Objectives

In order to achieve the general objective, the specific objectives were:

- To study the effect of unicellular protein addition in gluten-free bread physico-chemical characteristics, consumer acceptance and shelf-life.
- To study the effect of different legume flours addition in gluten-free batter rheology and fermentation; and in bread physico-chemical characteristics, consumer acceptance and shelf-life.
- To study the effect of liquid whey addition in gluten-free batter rheology and fermentation; and in bread physico-chemical characteristics, consumer acceptance and shelf-life.
- To develop a NIRS calibration for gluten determination in flour and batter, suitable to detect low gluten levels.

1.3 Working Plan

About 40 preliminary trials were carried out to develop a gluten-free bread basic formulation comprised by corn starch, sugar, salt, hydrocolloids, soya flour, baking powder, emulsifier, shortening, yeast and water. Once this formulation was obtained, additional or alternative ingredients such as dairy proteins, vegetable proteins, unicellular protein, buckwheat, whey or legume flours were added to improve the basic formulation or in substitution of one of the ingredients. Table 1.1 summarizes the specific ingredients tested during our study.

A specific gluten-free bread making process was optimized for the formulations evaluated in this study, as detailed in Fig. 1.1.

The experimental design of the assays performed in the different works is schematically represented as follows:

- Fig. 1.2. Influence of unicellular protein on gluten-free bread characteristics.
- Fig. 1.3. Effect of legume flours on baking characteristics of gluten-free bread.
- Fig. 1.4. Liquid whey as an ingredient to formulate gluten-free bread.
- Fig. 1. 5. Detection of low-level gluten content in flour and batter by near infrared reflectance spectroscopy (NIRS).

Table 1.1 Specific ingredients tested in bread development studies

	Experiment 1						Experiment 2				Experiment 3								
	S	**V**	**F**	**S_{up}**	**SV_{up}**	**F_{up}**	**Chickpea**	**Pea**	**Soya**	**Carob**	**5W**	**2.5W**	**0W**	**5R**	**2.5R**	**0R**	**5F**	**2.5F**	**0F**
Buckwheat flour	•	•	•	•	•	•													
Rice flour			•			•													
Skim milk powder			•			•													
Ovoalbumin	•		•	•		•													
Unicellular Protein				•	•	•													
Chickpea flour							•												
Pea isolate								•											
Soya	•	•	•	•	•	•			•		•	•		•	•		•	•	
Carob germ										•									
Ripened Whey														•	•	•			
Fresh whey																	•	•	•

Starch-based: S; starch-based with unicellular protein: S_{up}; starch-vegetable-based: SV; starch-vegetable-based with unicellular protein: SV_{up}; flour-based: F and flour-based with unicellular protein: $F_{up;}$ Chickpea flour; Pea isolate; Soya flour; Carob germ flour; 5 % soya flour and water: 5W; 2.5 % soya flour and water: 2.5W; 0 % soya flour and water: 0W; 5 % soya flour and ripened whey: 5R; 2.5 % soya flour and ripened whey: 2.5R; 0 % soya flour and ripened whey: 0R; 5 % soya flour and fresh whey: 5F; 2.5 % soya flour and fresh whey: 2.5F; 0 % soya flour and fresh whey: 0F.

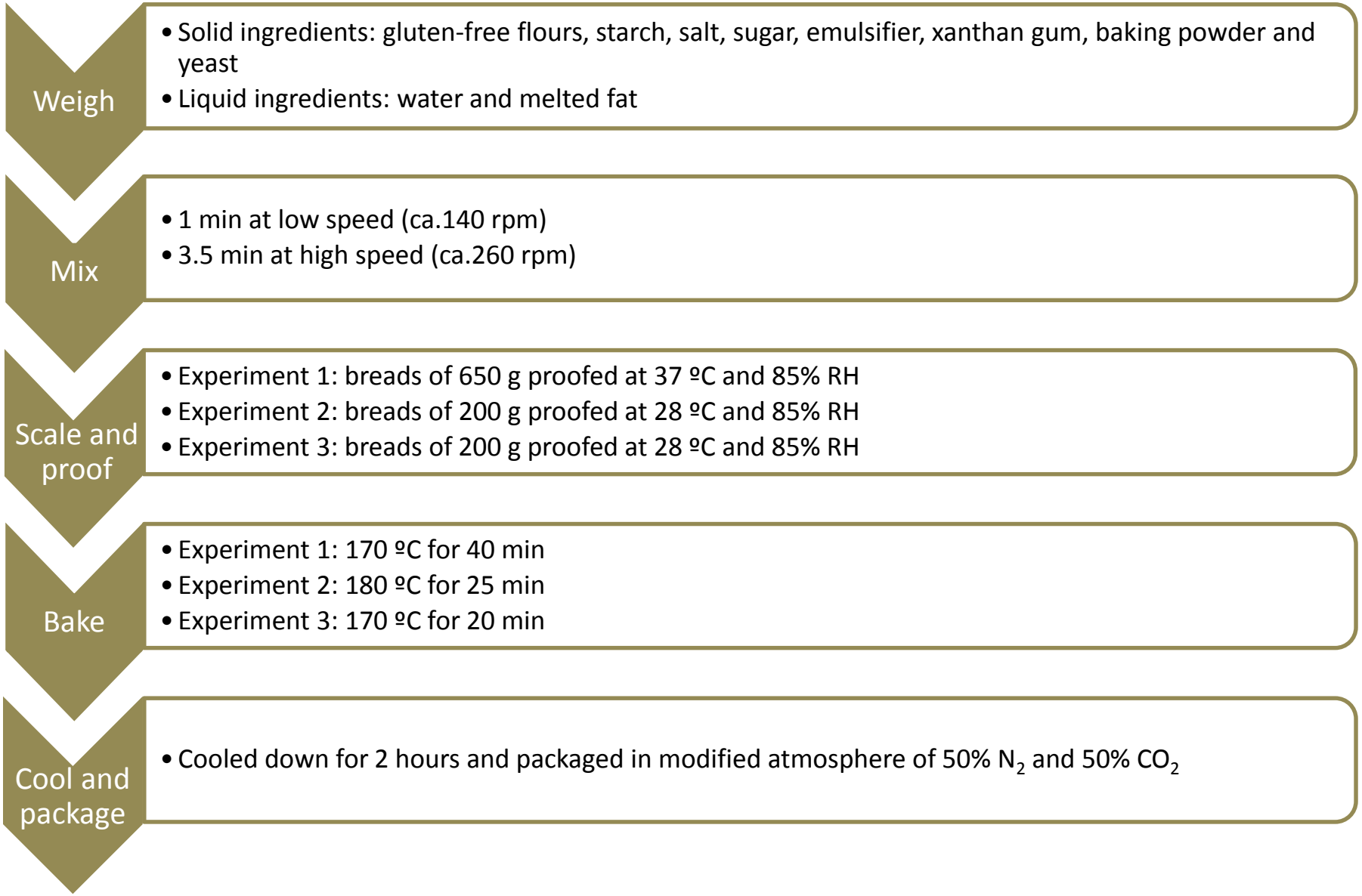

Fig. 1.1 Bread making procedure

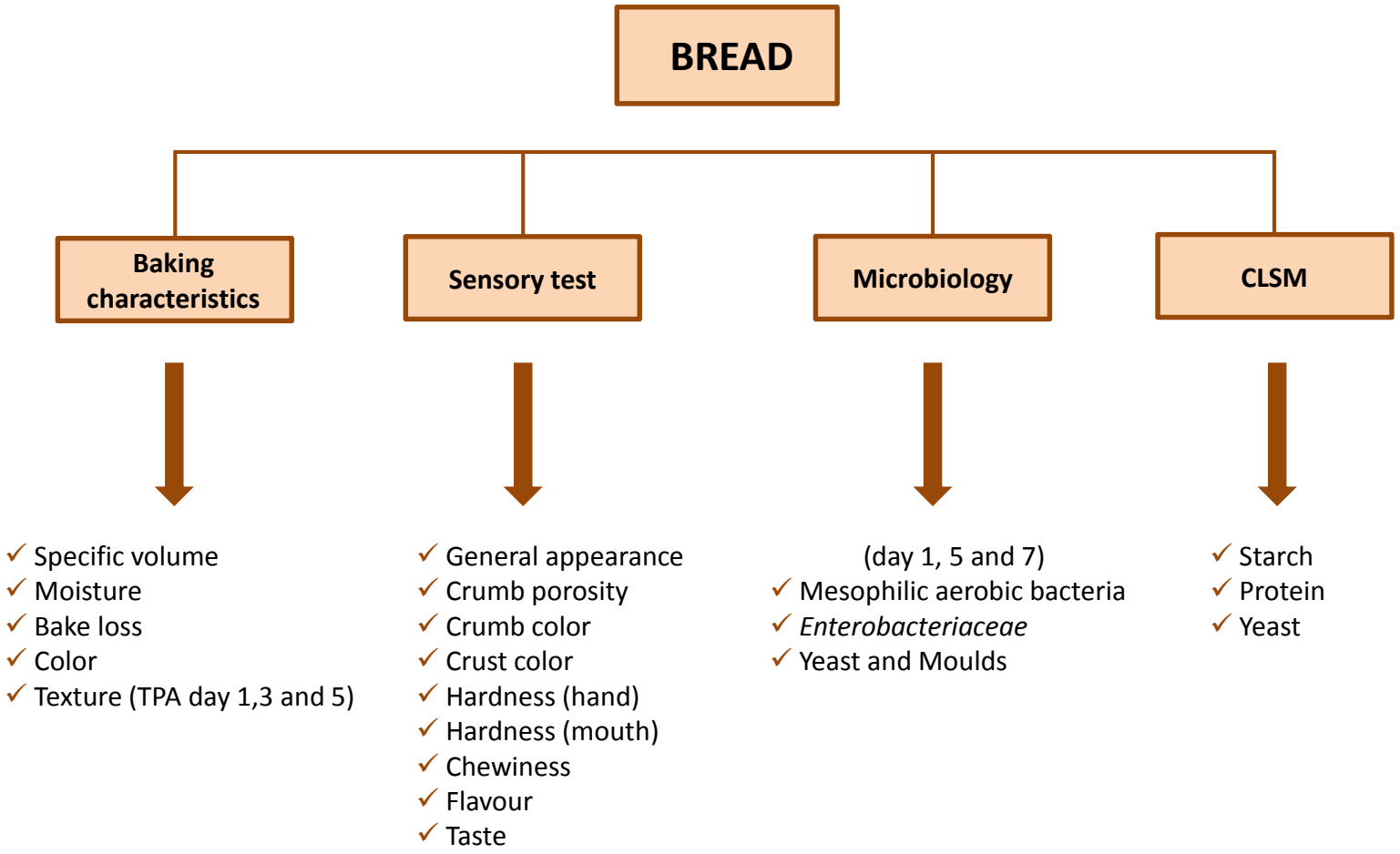

Fig. 1.2 Influence of unicellular protein on gluten-free bread (Experiment 1)

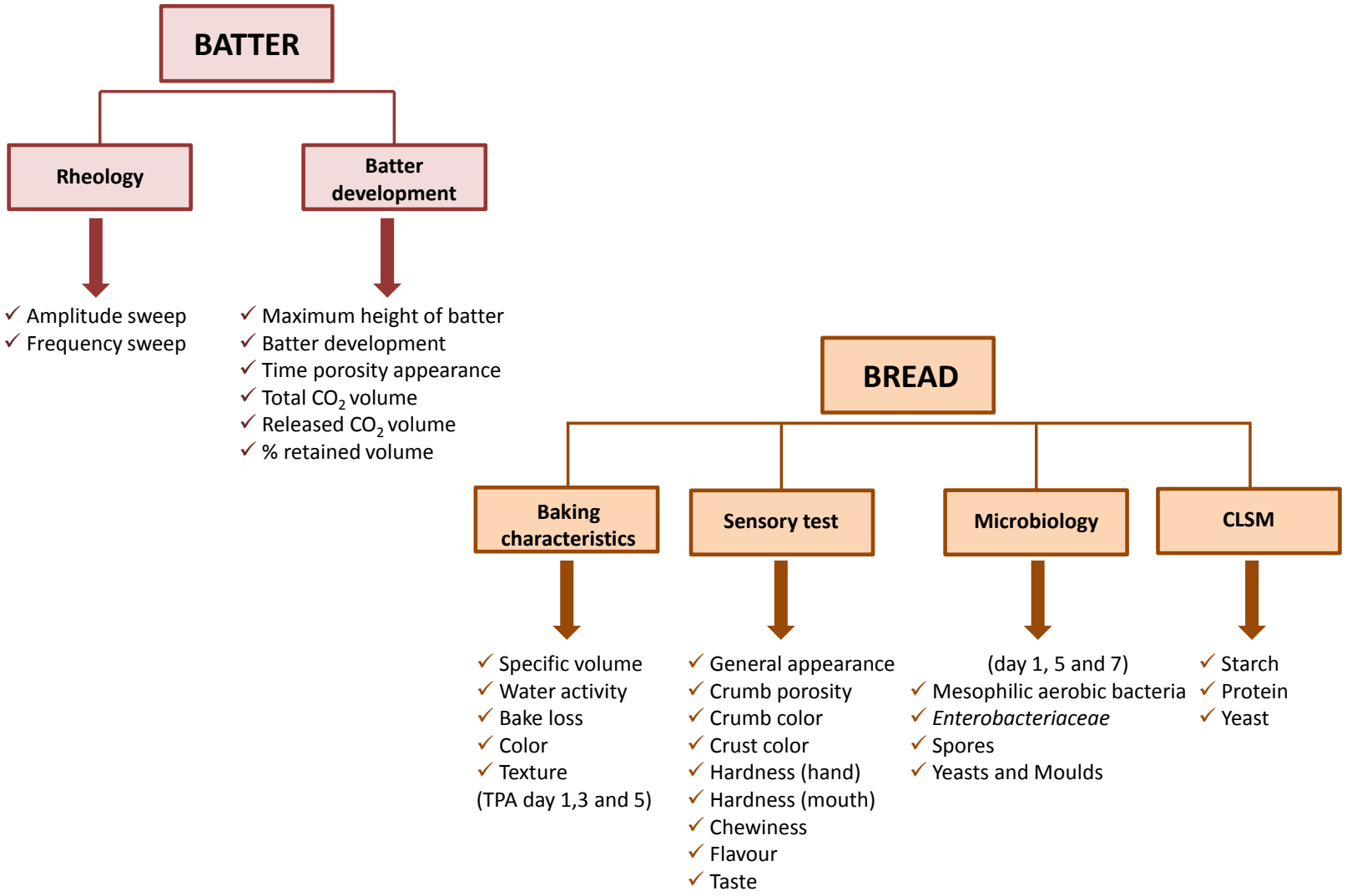

Fig. 1.3 Effect of legume flours on baking characteristics of gluten-free bread (Experiment 2)

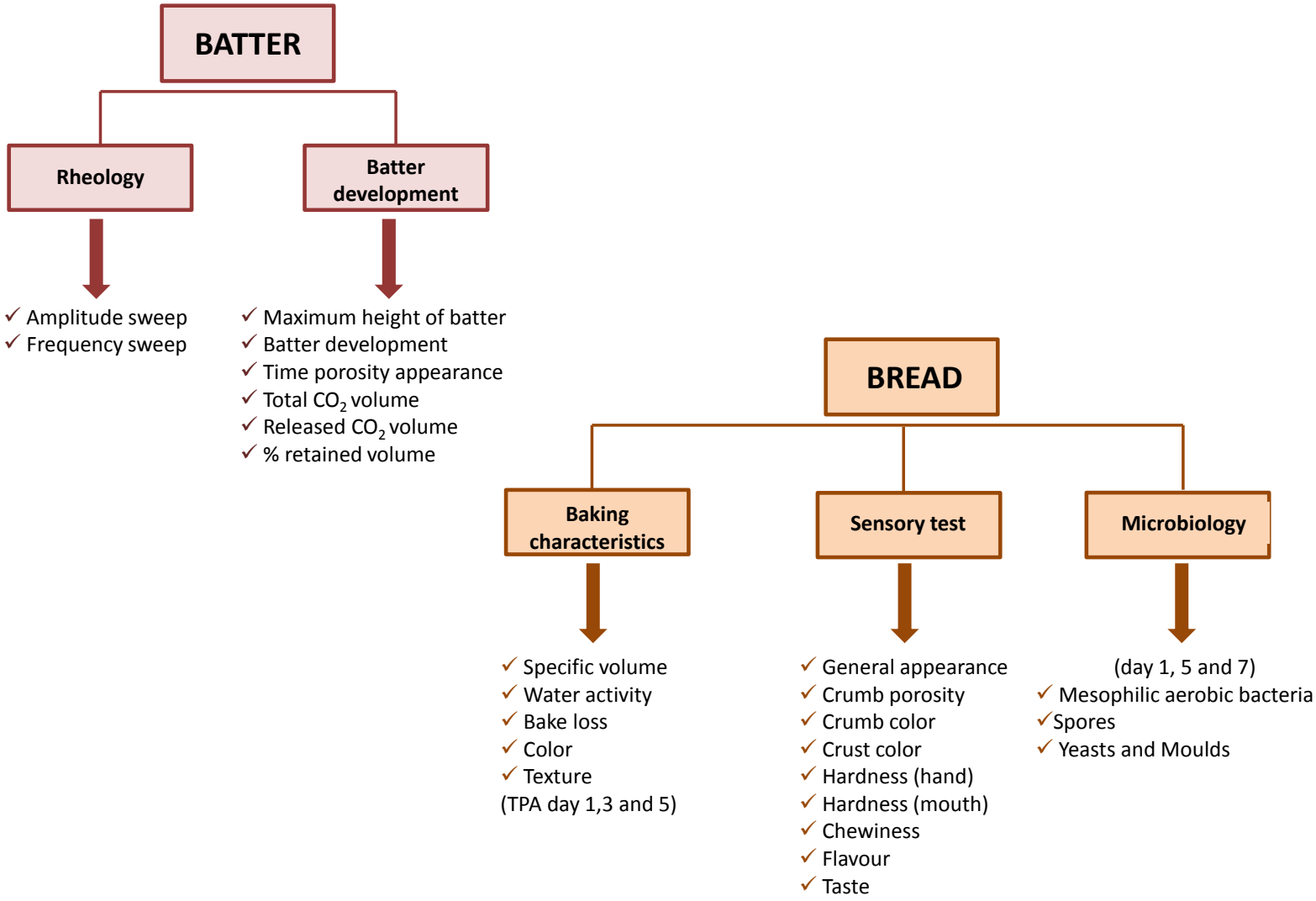

Fig 1.4 Liquid whey as an ingredient to formulate gluten-free bread (Experiment 3)

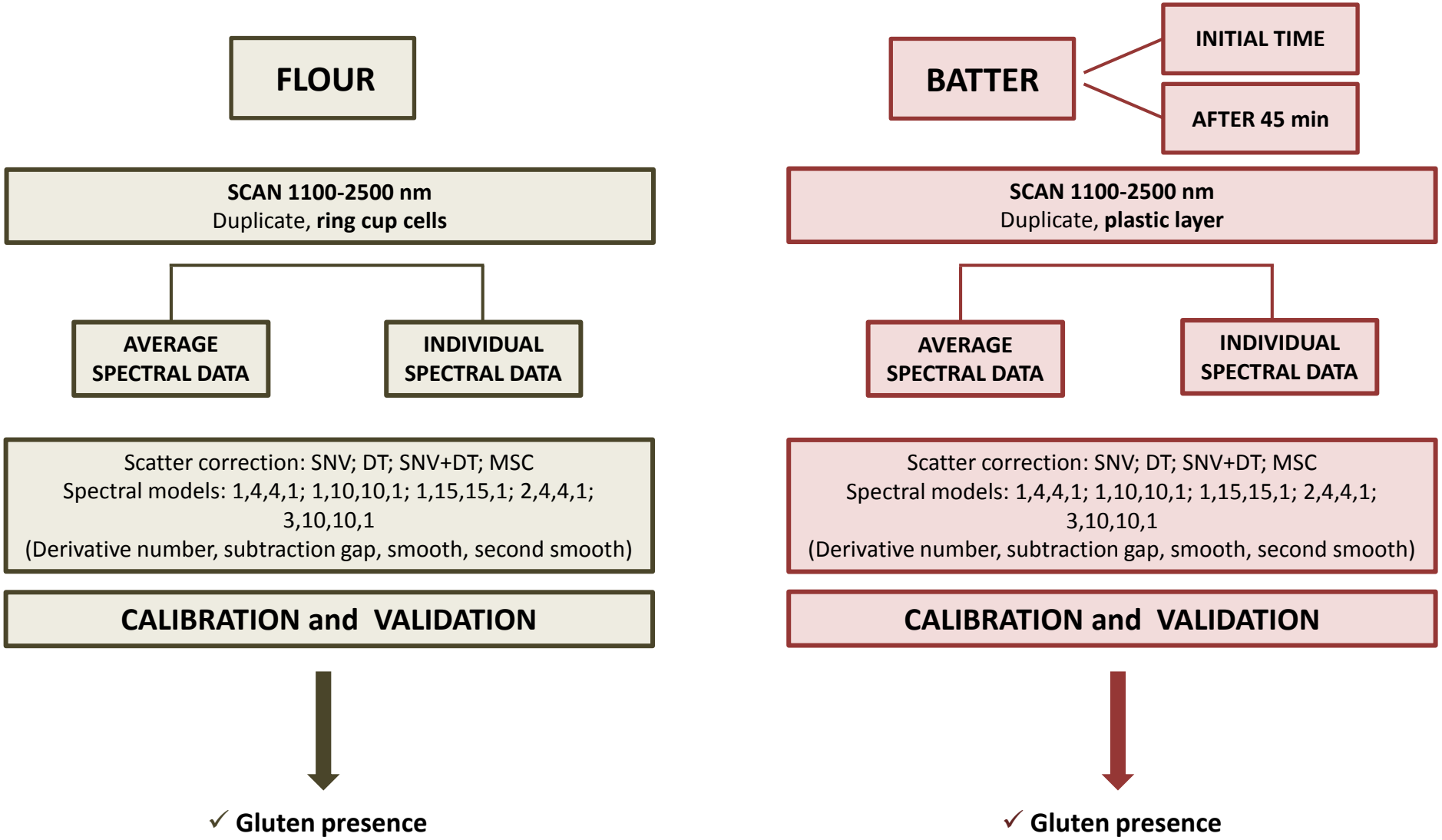

Fig. 1.5 Detection of low-level gluten content in flour and batter by near infrared reflectance spectroscopy (Experiment 4)

Chapter 2

Background

2.1 General Introduction

Wheat, rice and maize are the most widely consumed food grains in the world. Wheat, the most extensively grown crop, is immensely diverse with more than 25.000 different cultivars developed by plant breeders worldwide. It is used in food for many purposes and in such different ways like bakery, pasta and noodles. It is also used as an ingredient in processed food. Gluten is the main structural protein in wheat and other cereals like rye and barley. It plays an important role in bakery products since it provides the dough with its adequate viscoelastic properties.

A few decades ago, an old gluten-related disease became apparent, celiac disease (CD). In the last 10 years, deep research in this field has been performed. Apart from CD, two new forms of gluten reactions have been described recently: wheat proteins allergy and, gluten sensitivity, an immuno-mediated reaction not followed by anti-TG autoantibodies or other autoimmune comorbidities (Sapone et al., 2012).

The current treatment for CD is a strict life-long gluten-free (GF) diet, where cereals from the grass tribe *Triticeae*, e.g. wheat, barley and rye, must be avoided (Dicke, 1951;

Green and Jabri, 2003). Oats belong to a separate grass tribe (*Aveneae*) and are nowadays considered harmless for the majority of CD patients (Janatuinen et al., 2002; Hogberg et al., 2004).

The fact that bread is considered one of the main foods in the diet of Mediterranean countries combined with the low quality of the commercial available GF breads, aimed this research. The main objective of this thesis was to develop and optimize gluten-free bread formulations for celiac patients.

2.2 Celiac Disease

2.2.1 History

CD is an autoimmune disorder caused by the ingestion of gluten in susceptible patients (Fasano and Catassi, 2001). An English physician and pediatrician, Dr. Gee, provided the first complete modern description of CD back in 1888. He observed that children in ages between one and five started to present diarrhea, abdominal distension and failure to thrive, which are the most important symptoms of CD in children (Vogten and Peña, 1987). He proposed that the only cure, if available, was by means of diet but, like many others afterwards, he failed to recognize the dietary factor behind this chronic condition. During the first half of the past century, it was generally agreed that the treatment for CD was rest and diet.

It was not until 1941 that Willem-Karel Dicke published his findings linking CD with a wheat-free diet. In his thesis, published in 1950, he described the growth curves and symptoms of five children who clinically improved when wheat, rye and oat flour were omitted from their diet, and who relapsed when these flours were added again, proving that components from these grains caused CD**.** Together with Anderson et al. (1952), he later identified gluten as the harmful protein in CD. A few years later, Dicke, and Van de Kamer and Weijerers, showed that gliadin was the toxic compound causing CD (Van de Kamer et al., 1953).

The small bowel mucosal abnormalities typical of CD were first described by Paulley (1954). He studied small bowel biopsy specimens taken from CD patients undergoing laparotomy and noted that broad villi and chronic inflammatory cell infiltrate in the intestinal mucosa. Two years later, peroral intestinal biopsy equipment was developed, facilitating the histological diagnosis of CD based on typical small bowel mucosal

villous atrophy and crypt hyperplasia (Shiner, 1956)**.** It is now well known that one of the major protein components of wheat, rye and barley, the storage proteins named gluten, are the components that cause CD, and a lot of progress has been made in identifying the causes and symptoms of the disease.

2.2.2 Pathogenesis

Although the pathogenesis of CD has been deeply studied in the last few years, it is yet not fully understood. The genes linked to CD encode the human leukocyte antigens (HLA) HLA-DQ2 and HLA-DQ8, but it is known that the genetic component is clearly not sufficient to account for the development of CD, and its pathogenesis involves interactions among environmental, genetic, and immunological factors (Kagnoff, 1992).

Human dietary gluten is poorly digested in the upper gastrointestinal tract because of its high proline content. Gluten has many immunogenic peptides identified as α-gliadins, γ-gliadins and glutenins (Molberg et al., 1998), which are resistant to degradation by gastric, pancreatic and intestinal brush-border membrane proteases and remains undigested in the intestinal lumen. Transglutaminase 2 (TG2) is an enzyme in the intestine, found both at the brush border and just below the epithelium, which catalyzes the deamidation of specific glutamine residues of gliadins, and forms a negatively charged complex that fits into HLA-DQ2 or HLA-DQ8 and activates T cells. Briefly, the immune reaction generates antibodies against TG2 and gliadin, and promotes an inflammatory reaction in the upper small intestine which causes villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis.

There are several hypothesis to explain the trigger of early CD, as the induction of secretion of zonulin, which modulates the permeability of intercellular tight junctions (Gujral et al., 2012); or a situation of stress, infection or inflammation that would induce a decrease of zinc level in the intestinal wall, which, in turn, would activate TG2 (Stenberg et al, 2008). Recently, several studies have pointed different viral infections as triggers for the development of CD (Stene et al., 2006; Abadie et al., 2011).

2.2.3 Symptomatology

The clinical manifestations of CD markedly vary depending on the age of the patient, the duration and extent of the disease, and the presence of extraintestinal pathologic conditions. In addition to the classical gastrointestinal form, a variety of other clinical manifestations of the disease have been described, including atypical and asymptomatic forms.

Infants and young children present diarrhea, abdominal distention, and failure to thrive. However, vomiting, irritability, anorexia, and constipation are also common. Older children often present extraintestinal manifestations, such as short height, neurological symptoms, or anemia. Within weeks to months of starting gluten ingestion, weight gain velocity decreases and, finally, weight loss can be observed. Atypical CD is usual in older children where malabsorption is absent. Intestinal or extraintestinal symptoms can be involved, such as enamel defects, recurrent abdominal pain, constipation, short height or delay of puberty.

The major CD manifestation in adults is diarrhea, though this symptom occurs in less than 50% of patients (Rampertab et al., 2006). Other symptoms include anemia, that is mainly due to iron deficiency, though anemia due to nutritional factors and chronic disease may also be present at diagnosis of CD (Harper et al., 2007). Anemia is more frequently in adults than in children. Osteoporosis is another presentation of CD in adults.

Nowadays, there is a vast body of evidence demonstrating that the manifestations of the disease are not always intestinal. Other atypical or extraintestinal symptoms most often associated with CD are dermatitis herpetiformis, depression, epilepsy and infertility. Anyway, it is not always obvious whether these associations are true or coincidental findings and, further, the distinction between atypical symptoms and complications of CD is occasionally ambiguous.

2.2.4 Diagnosis and treatment

With the plethora of symptoms, diagnosis of CD can be very difficult and is often falsely diagnosed as another common bowel disorder, such as irritable bowel syndrome. There are several methods used for CD diagnosis. The types and order of the tests are often determined by visible symptoms (Hopper et al., 2007). The gold standard of

diagnosis remains the small-bowel mucosal biopsy (Fasano, 2003; Bonamico et al., 2004), being always the last step for a diagnose confirmation.

The best approach to diagnose CD appears to be a systematic process of case identification, targeting those patients with signs, symptoms, or conditions associated with CD. Serology testing is the first step in the diagnosis of CD in symptomatic patients. Both immunoglobulin A anti-tissue transglutaminase (TGA) and total serum IgA are performed. The test for total serum IgA is suggested because there is a high prevalence of IgA deficiency in patients with CD (Kumar et al., 2002).

If results of both of these tests are negative, CD is unlikely. However, if the tests are negative but a high clinical suspicion for the disease remains, it is suggested to test for IgG and IgA anti-endomysial (EMA), which is highly sensitive and specific for CD. If results of either TGA, EMA or IgG tests are positive, duodenal biopsy will be conducted to confirm the diagnosis. If results of this test are negative, CD is unlikely (Westerberg et al., 2006). Serological tests should be performed before starting a GF diet, in order to avoid possible altered results (Green and Jabri, 2003). Because more than 98% of people with CD share the major histocompatibility complex II class HLA-DQ2 or HLA-DQ8 genes, the inclusion of HLA typing for these haplotypes is useful, especially in patients with equivocal biopsy results or negative serological tests, or for patients already on a GF diet. People who do not have HLA-DQ2 or HLA-DQ8 are unlikely to have CD (Kaukinen et al., 2002). Therefore, diagnosis of CD is extremely challenging and, although some years ago specific algorithm was proposed to diagnose CD, nowadays, the wide variability of CD-related findings suggests that it is difficult to conceptualize the diagnostic process into rigid algorithms that do not always cover the clinical complexity of this disease. A new method has been proposed defined as the “4 out of 5” rule as the diagnosis of CD is confirmed if at least 4 of the following 5 criteria are satisfied (Catassi and Fasano, 2010):

1- Typical symptoms of CD
2- Positivity of serum CD immunoglobulin A class autoantibodies at high titer
3- HLA-DQ2 or DQ8 genotypes
4- Celiac enteropathy at the small bowel biopsy
5- A response to the GF diet

The unique treatment for people suffering gluten reaction is the strict adherence to a GF diet for their entire life. While GF diet is the only approved treatment, advances in understanding the immunopathogenesis of celiac disease have suggested several types of therapeutic strategies that may augment or supplant the GF diet. Some of these strategies are: decrease of the immunogenicity of gluten-containing grains by manipulating the grain itself; use of oral enzymes to break down immunogenic peptides; prevention of the absorption of immunogenic peptides; prevention of tissue transglutaminase from rendering immunogenic peptides, or inhibiting their binding to celiac disease-specific antigen presenting molecules; limitation of T cell migration to the small intestine; or reestablishment of mucosal homeostasis and tolerance to gluten antigens. Some of these therapies are in human trials and may be available within the next 5-10 years (McAllister and Kagnoff, 2012).

2.2.5 Epidemiology

The awareness and prevalence of CD have changed greatly during the last decades. In the past, CD was considered a strange disorder, affecting around 1:1000 individuals (Greco et al., 1992). However, since 1960, the awareness and detection of the disease increased due to the increase of accuracy in malabsorption tests and pediatric biopsy techniques (Meeuwiss, 1970).

Since then, and especially during the past four decades, many epidemiological studies have been conducted, particularly in Europe, to establish the prevalence of CD. As both genetic (HLA and non-HLA genes) and environmental factors (wheat consumption) are crucial in the pathogenesis of CD, the distribution of these two factors seems to identify the world areas-at-risk (Fasano and Catassi, 2001). Although, until recently, the geographical distribution of CD was mostly restricted to Europe and developed countries with European genetically ascendants, the new serological studies have reported that CD is a global disease with a worldwide distribution (Catassi, 2005). Countries like Brazil or Argentina have shown a great prevalence of CD. It is also found in populations in the Middle East, India and North Africa (Malekzadeh et al., 2005). However, it appears to be a rare disease in Russia and North Asia (Kondrashova et al., 2008).

Nowadays, CD has become the most common well-known disorder affecting almost 1% of the European population (Hogberg et al., 2004; Mustalahti et al., 2010). Not only it is very common in Europe, but also highly underdiagnosed in all countries, even in those with a high knowledge of the disease (e.g. Finland). The iceberg model is often used to gain an epidemiological understanding of CD. The visible tip of the iceberg represents the diagnosed cases. The first part under the surface represents the undiagnosed or silent cases with gluten-induced enteropathy (CD patients found by serological screening). The deepest part represents the genetically pre-disposed individuals without gluten-induced enteropathy, called latent or potential CD, in which it is unknown when or if they will ever develop CD. Mustalahti et al. (2010) found that the ratio between the visible (previously diagnosed) and the overall size (overall prevalence) of the celiac iceberg varies between 6% (Italy) and 24% (Finland).

2.3 Gluten-free market

2.3.1 Overview of the Gluten-free market

The GF products market has greatly increased in the last decades, not only because of the increasing number of celiac patients (due to a more accurate diagnosis) but also because of a high demand from of non-celiac patients who perceive that GF diet can help in treatment of disorders such as autism, chronic fatigue, schizophrenia, attention deficit disorder, multiple sclerosis, migraine and fertility problems. Moreover, with celebrities endorsing GF and wheat-free diets as a weight loss regimen, more people are taking interest in GF products, and including them as part of their lifestyles (Packaged Facts, 2011). As a result of the increasing market, companies are expanding their offer with a high number of snacks and beverages designed to appeal consumers on a sensory level. The research made during the last decade and the high information about GF ingredients has allowed companies to innovate using alternative grains and new ingredients that previously were considered unsuitable for celiac consumers. North America is the most developed market, with more than one in ten food products launched in 2009 containing a GF claim. Packaged Facts (2012) estimated that the U.S. market for GF foods and beverages will reach 4.2 billion in 2012, for a compound annual growth rate of 28% for the 2008-2012 period. A double digit growth is forecasted in the German (+11.1%) and UK markets (+10.4%) between 2009 and 2014.

Although considerable progress has been made in GF products development, their limited quality and quick staling still represent a problem. As the niche of market is still small, there is very low product rotation, thus, long shelf-life is required for GF products. This is difficult to achieve, particularly in breads, due to the inherent characteristics of the product. Hence, in many cases, the quality of GF products is not acceptable when they arrive to celiac population. In addition, GF products are, on average, five times more expensive than their wheat counterparts and, although in some European countries (UK or Italy), celiac patients receive an economic aid from the government, in others, such as Spain, do not.

Growing awareness, acknowledgement, and prevalence of celiac disease, food allergies, and related disorders will, in turn, promote more accurate diagnoses of conditions that may be alleviated by GF lifestyles. The trend adopted by friends and family to consume GF diets to support celiac patients is among the factors stimulating continuing expansion in this market. In addition, the increase in the offer of GF foods and beverages higher in quality, more readily available, and more consumer-friendly in terms of packaging and convenience, have raised sales. While growth rates will moderate over the next five years, Packaged Facts (2012) projects that U.S. sales of GF foods and beverages will exceed $6.6 billion by 2017.

2.3.2 Legislation

The standard for gluten and other regulations concerning GF products can be found in the Codex Alimentarius. Three of the committees working under the Codex Alimentarius Commission have specific importance with regard to GF foods. These are: 1) the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), 2) the Codex Committee on Food Labelling and 3) the Codex Committee on Methods of Analysis and Sampling.

The first standard for GF foods was the 118-1981 Codex Standard. At that time, no specific methods were available for gluten detection, and the maximum gluten level allowed in GF products was 0.05 g of total nitrogen per 100 g dry matter.

During the past 30 years, new methods based on the immunological detection of gluten proteins have been developed and revision of the standard has become necessary. The first draft of the revised standard is from 1996 (Codex Alimentarius, 1997). At that

time, the maximum gluten levels were discussed. After some approaches, a two-level model of GF labeling was decided, one level was suggested for products that are naturally GF (20 ppm) and, a second, for processed products specially formulated or prepared to meet the dietary needs of people intolerant to gluten (200 ppm). In the CCNFSDU meeting in 2006, the gluten threshold for products rendered GF was lowered from 200 ppm to 100 ppm. Moreover, oats were moved from the list of prohibited grains to the list of cereals for which use may be determined at the national level.

In 2007, the standard was given a new name, the *Codex Standard for foods for special dietary use for persons intolerant to gluten*. At that time, CCNFSDU continued the discussion concerning the thresholds for gluten, but also about the labels and the role of oats (Codex Alimentarius 2008). In 2008, the new standard was finally released. One of the key issues is that oats are considered GF; however, the gluten content of oats should not exceed 20 ppm. The other key issue is the establishment of two categories: GF products and products with very low gluten content. GF products are the ones previously labeled as naturally GF, and products with very low gluten content are those rendered GF (deglutinized products). The gluten content of GF products should not exceed 20 ppm, whereas products with very low gluten content should not exceed 100 ppm. The method for the determination of gluten should be the enzyme-linked immunoassay (ELISA) R5 Mendez Method. The Codex Standard was used as a basis for EU regulation (Commission Regulation (EC) No 41/2009) concerning GF foods, which was adopted at the beginning of 2009 and became mandatory by January 2012. All members of the European Union are therefore obliged to follow this new revised standard concerning the composition and labeling of foods suitable for persons intolerant to gluten.

2.4. Gluten-free bread

2.4.1 The role of gluten in bakery products

Gluten comprises the major storage proteins that are deposited in the starchy endosperm cells of the developing grain. They form a continuous proteinaceous matrix in the cells of the mature dry grain and form a continuous viscoelastic network when flour is mixed with water to form the dough. Gluten proteins belong to the glutelin and prolamin

classes and contribute to 80-85% of the total wheat protein content. Wheat gluten contains two major proteins: glutenin and gliadin. Glutenin is responsible of the gluten elastic properties while gliadin contributes primarily to gluten viscous properties (Mills et al., 1990; Ciaffi et al., 1996).

Glutenins are present in high molecular weight polymers stabilized by disulphide bonds that are considered to form the 'elastic backbone' of gluten. However, there are glutamine-rich repetitive sequences comprising the central parts of the high molecular weight subunits, which also form extensive arrays of interchain hydrogen bonds that may contribute to the elastic properties via a 'loop and train' mechanism (Shewry et al., 2002). Gliadins provide viscous character and strain hardening to wheat gluten. These proteins are formed by a non repetitive domain rich in α-helix structure and a heterogeneous repetitive domain rich in β-reverse turns. Both proteins give the viscoelastic properties needed to allow the formation of a thin gas-retaining film and a stretchable, extensible and coagulable protein-starch matrix in yeast-leavened bakery products (Gallagher et al., 2004; Soares et al., 2012).

During bread making, a complex foam system is produced with three ingredients: flour, salt and water. An appropriate combination of these components together with proper kneading, proofing and baking makes possible to obtain a solid matrix of a continuous phase of gelatinized starch and a continuous gluten network that encloses the starch granules and fiber fragments, resulting in a proper crumb structure (Durrenberger et al., 2001). This process is not possible in GF breads, where the lack of gluten originates batters rather than doughs and where a complex formulation including gums, starches, baking powder and different types of protein sources are required to try to mimic the viscoelastic properties of gluten with the objective of developing acceptable GF breads (Haque and Morris, 1994; McCarthy et al., 2005; Lazaridou et al., 2007).

Non yeast-leavened GF products such as cakes, muffins or cookies are easier to obtain because the lack of viscoelasticity of their batters is not an issue, as gas is generated during baking and immediately retained. Moreover, the extended use of egg and/or dairy ingredients contributes to the viscoelastic properties in these final products.

2.4.2 Gluten-free bread production

The production of GF breads differs significantly from standard wheat bread production. Although the same general steps are carried out, times and conditions usually change considerably.

Traditionally, wheat dough is mixed, bulk fermented, divided/molded, proofed and finally baked. Usually, GF breads have liquid batters that make difficult to follow a wheat bread process. Since GF batters form a weak, unstable and porous matrix, mixing and proofing times are shorter than in wheat bread. Moore et al. (2004) developed a procedure to manufacture GF breads with modified conditions, especially during mixing, moulding and proofing steps. This method was successfully applied by other researchers (Schober et al., 2005; Renzetti et al., 2008) in further studies on GF breads (Fig. 2.1).

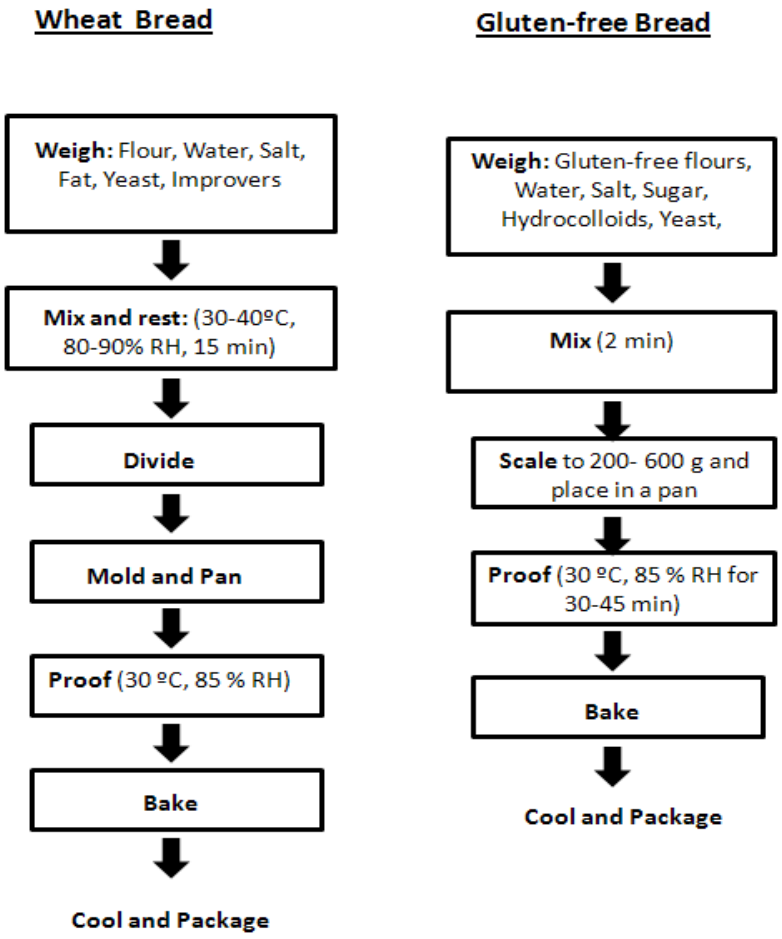

Fig. 2.1 Wheat and GF baking process adapted from Arendt et al. (2008)

2.4.3 Gluten-free bread characteristics

In GF breads, the lack of gluten is counterbalanced by complex formulations based in GF flours, starches, and hydrocolloids with the aim of reproducing the viscoelastic properties of wheat dough. However, GF breads are usually still of poorer quality than wheat breads.

Removal of gluten and, consequently, the lack of a strength protein matrix able to expand and retain gas, results in weak batters with high permeability to carbon dioxide and big difficulties to maintain the structure, which decreases the volume of the baked good (Stauffer, 1998). The absence of gluten also impairs the water holding capacity of the breads, which show an early crumbly structure and a quick staling. In addition, due to the common use of starches in formulations and the short proofing times, GF breads are pale and have poor flavor and taste (Arendt et al., 2002). Commercial GF breads are mainly starch-based and, therefore, lack fibre, vitamins and nutrients, characteristics that worsen the already nutritionally unbalanced diet of CD sufferers who strictly adhere to a GF diet (Mariani et al., 1998).

2.5 Gluten-free bread ingredients

The increasing number of diagnosed celiac patients and the poor quality of GF products has encouraged researchers to investigate new ingredients and technologies that reproduce gluten properties and improve the quality of GF baked goods. The development of GF bakery has involved the use of starches, gums, GF flours (rice, soya, buckwheat, oats, chickpea, and corn), animal and vegetable protein supplements, and alternative technologies, such as sourdough fermentation or high hydrostatic pressure processing. The following section reports the main ingredients used in the manufacture of GF products, including those used in regular bakery such as salt or yeast; and the main sources of protein that replace gluten, particularly focusing on the ingredients tested in this thesis.

2.5.1 Common Gluten-free ingredients

● **Water** is one of the main constituents of bread and GF bread dough. It allows the solubilization, rehydration and interaction of the ingredients and also plays an important

role in the major physical (e.g. expansion of bubbles) and chemical changes (e.g. starch gelatinisation) that occur during bread making. Water content and its distribution govern textural properties such as crumb softness, crust crispness and shelf-life (Wagner et al., 2007).

● **Salt** is a source of low levels of sodium in wheat dough, which helps obtaining and easily workable dough, by promoting interactions among gluten strands (Danno and Hoseney, 1982).

Salt plays other purposes in bread such as flavor enhancement, modulation of yeast growth and fermentation rate, development of crust color and preservation by limiting the growth of undesirable bacteria during bread fermentation (Salovaara 1982; Quílez et al., 2006).

● **Yeast** or baker's yeast *(Saccharomyces cerevisiae)* is a living organism belonging to the fungi kingdom. Yeast is the leavening agent in bread that transforms sugars into carbon dioxide and ethanol during fermentation. The trapped carbon dioxide makes the dough rise while the alcohol evaporates during baking. During long fermentations, yeast also produces organic substances that contribute to the development of bread flavor. Shorter fermentation times in GF products (due to its weak batter structure) prevent yeast to fully develop organic substances, thus, it only acts as a leavening agent.

● **Baking powder** is a dry chemical leavening agent used in bakery to increase the volume and lighten the texture of baked goods. It works releasing carbon dioxide into the batter or dough through an acid-base reaction at oven temperatures (around 80ºC), causing bubbles in the wet mixture to expand and, thus, leavening the mixture. Baking powder is usually used in non leavened baked goods such as cookies or muffins. In the GF unstable batter, it helps to increase volume and obtain a softer texture by releasing carbon dioxide during oven baking.

● **Starch** has been used for many years in the food industry for its unique functional properties as a thickener, gel forming agent, filler, etc. In bakery products, starch contributes to the texture, appearance, and overall acceptability of cereal-based foods (Ward and Andon, 2002). Starch is the main component of the dry substance of bread and plays an important role in establishing its structure and mechanical properties.

Its role is even greater in the case of GF bakery, where the elastic wheat protein is replaced with mixtures of different hydrocolloids. The addition of starch to GF

formulations improves batter consistency during mixing and enhances the softness of the crumb. Starch gelatinization is crucial in GF formulation because of its ability to trap air bubbles improving the gas holding capacity of the batter. Many studies have been performed in last years for its inclusion in GF bread formulations, including corn, rice, potato or cassava starch, which are the most suitable for GF bread development.

● **Shortening** contributes to the final bakery products flavour and palatability. Butter, margarine, vegetable oil or shortening are the fats most commonly used in bakery. Moreover, in GF breads, shortening has an important role on gas cell stabilization. During baking, fat crystals melt and the fat-liquid interface of the absorbed shortening provides a source of extra interfacial material that allows bubble formation and expansion and prevents collapse (Brooker, 1993).

● **Emulsifiers** are substances that possess both lipophilic and hydrophilic properties that reduce the surface tension between two normally immiscible phases so that they are able to form an emulsion (Krog, 1977; Flack, 1985; Dziezak, 1988). The unstable structure of GF bread batter made of starch, water and other hydrocolloids is incapable of supporting foam, so it requires additional surface active molecules that form a film around the bubbles and stabilizes them against coalescence and collapse.

Emulsifiers commonly used in bakery products are mono- and diglycerides of fatty acids. They enhance the structure by either increasing dough strength or crumb softness. Some emulsifiers (e.g. sodium stearoyl lactylate) may exhibit both functional properties.

The softening effect is related to the ability of mono- and diglycerides to form complexes with amylose, which are insoluble in water and lack a role in the formation of the gel during bread making. Then, upon cooling, the amylose would neither re-crystallize or contribute to the staling of the bread crumb (Stampfli et al., 1996).

● **Sugar** adds flavor to the bread and also provides a starter for yeast fermentation. It assists in the incorporation of air cells during creaming and in the formation of a good crumb structure, flavor and texture. Sugar also retains moisture, prolongs freshness and promotes caramelisation and Maillard reactions that result in desirable crust browning and color (Nip, 2007).

● **Hydrocolloids** are high molecular weight compounds consisting of hydrophilic long chains with colloidal properties that are capable of controlling both the rheology and texture of aqueous systems throughout the stabilization of emulsions, suspensions and

foams, and are also capable of gel formation in water-based systems; e.g. highly viscous suspensions or solutions with low content of dry substances (Diezak 1991; Hoefler, 2004). They are derived from seeds, fruits, plant extracts, seaweeds or microorganisms, being of polysaccharide or protein nature (Norton and Foster, 2002) and their functionality depends on its source, extraction process, original chemical structure and modifications, used doses, and interactions with other food polymers and ingredients (Anton and Artfield 2008; Huettner and Arendt 2010).

Hydrocolloids are used in a variety of food applications as thickening, stabilizing or gelling agents (Rosell et al., 2007). The bakery industry has a high demand for hydrocolloids for different purposes. Obviously, main bread hydrocolloid is starch, which is part of the flour and acts as a filler and a gelling agent (as already discussed in section 3.4.1), but other hydrocolloids, particularly gums, are being used. Guar gum has been employed for improving the volume and texture of frozen dough bread (Ribotta et al. 2004b), while utilization of hydroxypropyl methyl cellulose (HPMC) has been used to soften bread crumb, increase bread volume, improve sensory characteristics and extend shelf-life (Bárcenas and Rosell 2005; Collar et al. 1998). Similar functions have been attributed to HPMC when it was studied in frozen breads (Bárcenas and Rosell 2006).

The use of hydrocolloids with thickening and stabilizing properties such as arabic gum, carboxymethyl cellulose, guar gum or xathan gum seems suitable for gluten-free breads as the lack of gluten in GF batters requires the development of complex matrices with sufficient viscoelastic properties for holding both the carbon dioxide released during fermentation and the matrix structure expansion along baking. A simple starch batter consists in a suspension of starch granules and yeast cells in water, with small amounts of dissolved salt and sugar. When gas is incorporated during mixing, bubbles are suspended, which may be enlarged during fermentation. If gums are used, they act as thickeners, increasing the viscosity of the liquid phase and preventing bubble coalescence, and also keeping the starch and yeast from settling (Bemiller, 2008).

Table 2.1 Main studies involving hydrocolloids in gluten-free bread

Reference	Hydrocolloid	Main ingredients
Kulp et al., 1974	Xanthan gum	Wheat starch
Nishita et al., 1976	HPMC, guar gum	Rice flour
Acs et al., 1997	Xanthan gum, guar gum, locust bean gum	Corn starch
Kang et al., 1997	HPMC, locust bean gum, guar gum, carrageenan, xanthan gum	Rice flour
Gambus et al., 2001	Pectin, guar gum	Potato starch, corn starch, corn flour
Cato et al., 2004	HPMC, CMC, guar gum	Rice flour, potato starch
Kobylanski et al., 2004	HPMC	Corn starch, cassava starch
Lopez et al., 2004	Xanthan gum	Rice flour, corn starch, cassava starch
Moore et al., 2004	Xanthan gum, konjac gum	Rice flour, dairy-based proteins
Ribotta et al., 2004	Gelatin	Rice flour, cassava flour, soybean flour
Sivaramakrishnan et al., 2004	HPMC	Rice flour
Ahlborn et al., 2005	Xanthan gum, HPMC	Rice flour, milk proteins, egg proteins
McCarthy et al., 2005	HPMC	Rice flour, potato starch, skim milk
Schober et al., 2005	Xanthan	Sorghum
Korus et al., 2006	Guar gum, pectin	Corn starch, potato starch, inulin, fructooligosaccharides
Lee and Lee, 2006	HPMC	Rice flour
Moore et al., 2006	Xanthan gum	Rice flour, potato starch, corn flour

HPMC: hydroxypropil methyl cellulose; CMC: sodium carboxyimethyl cellulose

Table 2.1 (cont.) Main studies involving hydrocolloids in gluten-free bread

Reference	Hydrocolloid	Main ingredients
Lazaridou et al., 2007	CMC, pectin, agarose, xanthan gum, β-glucan	Rice flour, corn starch, sodium caseinate
Lorenzo et al., 2009	HPMC, xanthan gum, guar gum, HPMC	Corn starch, cassava starch
Mezaize et al., 2009	CMC, guar gum, HPMC, xanthan gum	Rice flour
Sciarini et al., 2010	Carragenan, alginate, xanthan gum, CMC, gelatine	Rice, corn and soya flour
Summu et al., 2010	Xanthan gum, guar gum	Rice
Turabi et al., 2010	Xanthan gum, guar gum, locust bean gum, κ-carrageenan	Rice flour
Andersson et al., 2011	HPMC, high β-glucan oat bran	Zein starch
Crockett et al., 2011a	HPMC, xanthan gum	Cassava starch
Moreira et al., 2011a	Agar, HPMC, xanthan gum	Chesnut flour
Moreira et al., 2011b	Arabic gum CMC, guar gum, tragacanth gum	Chesnut flour
Peressini et al., 2011	Xanthan gum, propylene glycol alginate	Rice and buckwheat flour
Preichardt et al., 2011	Xanthan gum	Rice and corn flour
Sabanis et al., 2011	HPMC, xanthan gum, k-carrageenan, guar gum	Corn starch, rice flour
Sciarini et al., 2012a	Xanthan gum, CMC	Rice flour
Sciarini et al., 2012b	Xanthan gum, CMC, alginate, carrageenan	Rice flour, cassava starch, full fat soya flour
Moreira et al., 2013	Guar gum, HPMC and tragacanth gum	Chesnut flour and chia flour

HPMC: hydroxypropil methyl cellulose; CMC: sodium carboxyimethyl cellulose

All types of hydrocolloids have been considered as gluten replacers in GF breads (Toufeili et al., 1994; Gallagher et al., 2004; Lazaridou et al., 2007; Schober et al., 2008; Sciarini et al., 2010; Sabanis and Tzia, 2011). However, HPMC and xanthan gum are the most suitable gums for GF breads, regardless of the ingredients included in the formula (Anton and Artfield, 2008). Main studies involving hydrocolloids in gluten-free breads are summarized in Table 2.1.

2.5.2 Animal protein sources

Animal protein sources and, particularly, dairy and egg proteins, have been widely used in bakery industry for many purposes. Main studies including these two proteins in wheat bread and gluten-free bread are reported in Table 2.2a and Table 2.2b.

● **Egg proteins.** Besides being a unique source of nutrients, egg provides components with different functional properties that have become essential ingredients in many food products, such as mayonnaises, noodles, cakes, etc. Egg is especially effective as a foaming agent, mainly because the ovalbumin and phospholipids from egg yolk act as emulsifiers (Mine, 2002; Kioseeoglou, 2003).

Table 2.2a Animal protein sources (egg proteins)

Reference	Egg ingredient	Main ingredients
Toufelli et al., 1994	Ovalbumin	Corn flour, corn starch
Kobylañski et al., 2004	Ovalbumin	Corn starch, cassava starch
Moore et al., 2004	Whole egg	Corn starch, cassava starch, rice flour, soya
Ahlborn et al., 2005	Whole egg	Rice flour, tapioca flour, potato and corn starch
Miñarro et al., 2010	Ovalbumin	Rice, buckwheat and soya flour, corn starch
Crockett et al., 2011b	Ovalbumin	Rice flour and cassava starch

In bakery, egg components allow air incorporation into the matrix, promote foam stability during kneading and proofing, and take part in gel formation during oven baking. Although egg is not usually added into wheat bread formulations, its inclusion in GF breads is more common. Moore et al. (2004) studied different GF formulations and observed a continuous protein network similar to gluten in breads containing egg.

The authors also found that formulations containing egg had higher initial hardness which was compensated by a lower staling rate during shelf-life. Kato et al. (2006) observed that addition of egg improved foam development due to the formation of an excellent quality viscoelastic network.

Although egg is a valuable ingredient for GF bread formulation, its main disadvantages come from its allergenicity, economic cost and safety risks.

● **Dairy proteins.** Milk and milk proteins (used in various forms as cheese whey, buttermilk, skim milk powder, sodium caseinate, etc) have been commonly used in bakery, due to their technological and nutritional properties.

Main serum milk proteins (globulins and albumins) are soluble in water and have a high hydration capacity. For this reason, their inclusion in bakery products results in a stronger and stable dough (Bilgin et al., 2006). Moreover, their structure allows the formation of networks capable of retaining water and, thus, slowing staling and extending shelf life of these products.

In GF breads, the absence of gluten network causes a quick loss of moisture which may generate a crumbly structure. Dairy proteins water holding and network formation capacities delay the staling process and allow prolonged moisture retention in these products (Kenny et al., 2000; Gallagher et al., 2003b). In addition, dairy proteins improve dough adherence, but not the energy needed to handle it (Güemes et al., 2009), which is a desirable effect in GF batters, which are usually difficult to process due to their liquid behaviour.

When dairy proteins are added in form of whey or skim milk powder, crust darkening via Maillard reaction is induced due to lactose presence. This darkening effect is desirable in GF breads as their colour is lighter than their wheat gluten counterparts. In addition, milk contributes to a better taste and flavor (Gallagher, 2003b).

In general, the incorporation of dairy proteins in GF products has been reported to be beneficial in nutritional and technological terms. However, it may be detrimental in cases of lactose intolerance which is high prevalent (50%) among celiac patients (Murray, 1999).

Table 2.2b Animal protein sources (dairy proteins)

Reference	Dairy ingredient	Main ingredients
Erdogdu-Arnoczky et al., 1996	Whey powder, casein and non-fat dry milk	Wheat flour
Yousif et al., 1998	Concentrated acid whey	Wheat flour
Kenny et al., 2000	Whole and skim milk powder, sodium caseinate, hydrolysed casein, whey protein	Wheat flour
Crowley et al., 2002	Casein hydrolysates	Wheat flour
Gallagher et al., 2003a	Sweet whey, demineralised whey, fresh milk solids, spray dried milk solids, spray dried skim milk, casein and protein isolate	Gluten-free flour, wheat starch
Gallagher et al., 2003b	Milk protein isolate	Gluten-free flour, rice starch
Moore et al., 2004	Skim milk powder	Rice flour, corn starch
Sánchez et al., 2004	Milk powder	Corn starch, cassava starch,
Gallagher et al., 2005	Whey protein concentrate and sodium caseinate	Wheat flour
Bilgin et al., 2006	Pasteurized whey and buttermilk	Wheat flour
Rantamaki et al., 2006	β-lactoglobulin, protein concentrate, α-lactalbumin and skim milk powder	Wheat flour
Indrani et al., 2007	Whey protein concentrate	Wheat flour
Divya and Jayaraj, 2009	Cheese whey	Wheat flour
Güemes et al., 2009	Whey	Wheat flour
Nunes et al., 2009	Sodium caseinate, milk protein isolate, whey protein isolate and whey protein concentrated	White rice flour, potato starch
Visentín et al., 2009	Cheese whey concentrate	Wheat flour
Secchi et al., 2011	Sweet ovine whey powder	Sweet and bitter almonds
Van Riemsdijk et al., 2011	Whey protein particles	Wheat starch

2.5.3 Vegetable protein sources

2.5.3.1 Cereals

● **Maize** (*Zea mays*), also known as corn, is one of the world leading cereal grains along with rice and wheat. Maize provides approximately 15% of the world protein and 20% of the world calories, and is a dietary staple for more than 200 million people (Nus and Tanumihardjo, 2010).

Maize is a rich source of energy provided by highly digestible carbohydrates, high protein content composed of essential amino acids, free oil and good quantity of trace minerals (Martin and Leonard, 1967). It contains about 72% starch, 10% protein, 4% lipids and 7.3% of fiber. Typical yellow maize contains several important vitamins such as Vitamin A (as provitamin A or carotenoids), and vitamin E (as tocopherols), but lacks vitamin B12. These predominant fat-soluble vitamins found in maize play important roles as antioxidants, among other functions (Kurilich and Juvik, 1999). Starch is the primary carbohydrate and kernel constituent in maize, totaling about 72% dry weight. Sugars range from 1% to 3%, with sucrose as the chief component and maltose, glucose, fructose, and raffinose in trivial amounts (Mertz, 1970; Boyer and Shannon, 1987). The ratio of amylose to amylopectin is ordinarily 25/75, although it can be altered by genetic modifications. Corn starch is one of the main components used in GF bread formulations, it absorbs up to 45% of the water and is considered an inert filler in the continuous matrix of the dough (Bloska, 1990).

● **Rice** (*Oryza sativa*) is one of the most important foods in human diet, and one of the most extended cereal crops (9% of the total cultivated soil). In fact, rice has probably fed more people in history than any other crop. Even today, rice grains sustain two-thirds of the world population (Rosell and Marco, 2008). Rice is one of the leading food crops in South East Asia, including India, where its production is much higher than that of wheat (Sivaramakrishnan et al., 2004).

Rice flour is colourless, has soft taste, hypoallergenic properties, low levels of sodium and easily digestible carbohydrates. Because of these properties, rice flour is the most suitable cereal to make GF products (Gujral and Rosell, 2004; Lopez et al., 2004).

● **Oats** (*Avena sativa*) is an important cereal crop throughout the world, which is primarily utilized as livestock feed. However, in the last few years human consumption has increased progressively due to its high nutritional properties (soluble fibres,

proteins, unsaturated fatty acids, vitamins, minerals and phytochemicals (Flander et al., 2008). The health effects of oats are mainly based on the total dietary fibre and particularly, β-glucan content, which can help lower blood cholesterol, glucose and insulin concentrations (Welch, 1995).

Moreover, recent studies show that oats can be tolerated by most people suffering from CD (Hoffenberg et al., 2000; Janatuinen et al., 2002; Kupper, 2004). Although oat proteins lack viscoelastic properties, their inclusion in GF products improves the nutritional quality of these starch-based breads that would otherwise lack fibre, vitamins and micronutrients.

● **Millet** refers to different species within a single genus, or even, different genus. They are simply cultivated cereal grasses that have small kernels and are grouped together solely on this basis. There are many different millets, some of which are closely related, like proso millet or common millet (*Panicum miliaceum*) and little millet (*Panicum sumatrense*), and some of which are not, particularly, finger millet or african millet (*Eleusine coracana*) and teff (*Eragrostis tef*), which belong to a different tribe.

Millets have the potential to add variety to our diet and may have useful health promoting properties, particularly antioxidant activity. They have recently been included in different food products that are commercially available and suitable for celiac patients (Taylor et al., 2006).

● **Sorghum** (*Sorghum bicolor*) is one of the world most important cereal crops. The top three sorghum producers in 2010 were the United States, Mexico and India (FAO, 2012). In most of Africa and Asia, sorghum is an important human food, whereas in Europe, Unites States and Australia it is used primarily as animal feed. Sorghum contains various phenolic compounds, such as flavonoids and tannins, phytochemicals that have gained interested due to their antioxidant activity, cholesterol-lowering properties and other potential health benefits (Hahn et al., 1983; Hahn et al., 1984; Kaluza et al., 1988; Waniska et al., 1989; Rey et al., 1993; Simontacchi et al., 2003; Kamath et al., 2004).

Recently, sorghum has been included in a number of products such as breads (Schober et al., 2005 and 2007), tortilla chips (Rooney and Waniska, 2000), cookies (Morad et al., 1984), and noodles (Kunetz et al., 1997; McDonough et al., 1997; Suhendro et al., 2000). Sorghum GF nature allows its inclusion in GF bakery but some disadvantages

like high gelatinization temperature or the tendency to form coarse grits, i.e. a sandy mouth-feel, has limited its application in GF products (Schober et al., 2008).Table 2.3 shows a list of studies including cereals used to produce or enrich bakery products.

Table 2.3 Cereals used to produce or enrich bakery products

Reference	Cereal	Main ingredients
Simoncelli and Faustalinta, 1947	Millet flour	Wheat flour
Gromeley and Morrissey, 1993	Oat flour	Wheat flour
Rao et al., 1997	Sorghum flour	Wheat flour
Carson et al., 2000	Sorghum flour	Hard wheat flour
Hugo et al., 2003	Malted sorghum flour	Sorghum flour
Cato et al., 2004	Rice flour	Rice flour, potato starch, wheat
Shober et al., 2005	Sorghum flour	Sorghum flour, corn starch
Moore et al., 2006	Rice flour	Potato starch, corn flour
Flander et al., 2007	Oat flour	White wheat flour
Diowksz, et al., 2008	Maize starch and flour	Wheat, potato starch
Kawka et al., 2008	Oat bran	Wheat flour
Pruska-Kędzior et al., 2008	Maize flour, maize starch, rice flour	Maize flour, rice flour, buckwheat flour, maize starch
Renzetti et al., 2008	Brown rice and sorghum	Brown rice, buckwheat, corn, oat, sorghum, teff
Ferreira et al., 2009	Sorghum flour	Rice flour, corn starch
Renzetti et al., 2009	Sorghum flour	Buckwheat, corn, teff
Brites et al., 2010	Maize flour	Maize flour
Demirkesen et al., 2010	Rice flour	Chesnut flour
Huettner et al., 2010	Oat flour	Oat flour
Onyango et al., 2010	Sorghum flour	Cassava starch, sorghum and egg white
Schamne et al., 2010	Maize flour	Rice flour, cassava starches
Torbica et al., 2010	Rice flour	Buchkwheat flour
Vallons et al., 2010	Sorghum flour	Sorghum flour
Huettner et al., 2011	Oat flour varieties	Oat flour
Nikolic et al., 2011	Rice flour	White and brown rice flour

Table 2.3 (cont.) Cereals used to produce or enrich bakery products

Reference	Cereal	Main ingredients
Onyango et al., 2011	Sorghum flour	Cassava, maize, potato or rice starch, sorghum
Rajiv et al., 2011	Finger millet flour	Wheat flour
Serrem et al., 2011	Sorghum flour	Deffated soya flour, wheat flour
Sakac et al., 2011	Rice flour	Buckwheat flor
Saha et al., 2011	Finger millet flour	Wheat flour
Alaunyte et al., 2012	Teff flour	Wheat flour
Angieloti and Collar, 2012	Oat, millet and sorghum flour	Wheat flour
Hager et al., 2012	Oat, rice, sorghum, maize flour	Wheat and wholemeal wheat
Maekinen and Arendt, 2012	Oat malt	Wheat flour
Han et al., 2012	Rice flour	Wheat flour
Peymanpour et al., 2012	Oat flour	Wheat flour
Phimolsiripol et al., 2012	Rice flour	Rice flour and rice bran
Velazquez et al., 2012	Sorghum flour	Corn starch
Yousif et al., 2012	Sorghum flour	Wheat flour
Schoenlechner et al., 2013	Millet composite flour	Wheat flour
Sangwan and Dahiya, 2013	Sorghum flour	Wheat flour

2.5.3.2 Pseudocereals

From the botanical point of view, amaranth, quinoa, and buckwheat are dicotyledoneous plants and, thus, they are not cereals (which are monocotyledoneous), but since they produce starch-rich seeds that are usually processed into flour, they are called pseudocereals.

Whole pseudocereal grains are rich in a wide range of compounds e.g. flavonoids, phenolic acids, trace elements, fatty acids and vitamins with known beneficial effects on human health (Li and Zhang, 2001; Tomotake et al., 2007; Gorinstein et al., 2008; Kalinova and Radova, 2009). Pseudocereals are not often used in bread making but they can be useful in dietotherapy of CD (Thompson, 2001). A summary of the studies focused in pseudocereals in bakery and gluten-free bakery can be seen in Table 2.4.

● **Buckwheat** (*Fagopyrum esculentum*) is a traditional crop in Asia and Europe. In recent years, buckwheat has received increasing attention because of its nutritional properties, as it is a dietary source of protein and amino acids, vitamins, starch, dietary fiber, essential minerals and trace elements (Steadman et al., 2001; Bonafaccia et al., 2003; Skrabanja et al., 2004). Phenolic compounds are also found in abundance in buckwheat, including rutin, orientin, vitexin, quercetin, isovitexin, kaempferol-3-rutinoside, isoorientin, and catechins (Dietrych-Szostak and Oleszek, 1999). In comparison to most frequently used cereals, buckwheat has been reported to possess high antioxidant activity mainly due to its high rutin content (Kreft et al., 2006).

The rising interest in functional products have stimulated studies on the enrichment of wheat-based products with buckwheat flour in order to increase the nutritional value of final products (Chillo et al., 2008; Lin et al., 2009).

Buckwheat high quality nutritional profile and GF nature have promoted its study as a potential raw material to enrich GF bakery products (Alvarez-Jubete et al., 2009) since they are frequently made from refined GF flour or starch and are rarely enriched or fortified. As a result, many GF cereal foods do not contain the same levels of B-vitamins, iron and fiber as their gluten-containing counterparts. Apart from its nutritional quality, buckwheat has higher water binding capacity (109.9 %) than wheat and corn starch and, also, higher gelatinization temperature, peak and set back viscosity, characteristics that are beneficial in GF bread making.

● **Amaranth** (*Amaranthus* sp.) has been consumed throughout history particularly by the Inca, Maya and Aztec civilizations, which used it as a staple food. The amaranth grain is an excellent source of high quality protein and lipids (Bodroza-Solarov et al., 2008), with higher content of minerals (calcium, potassium and phosphorus) and dietary fiber as compared to cereal grains (Whittaker and Ologunde, 1990). Amaranth protein is rich in lysine, which is usually deficient in cereal grains (Becker, 1989). Nowadays, interest in amaranth has increased and is used to enrich different commercial foodstuffs everywhere and the substitution level of wheat flour in bread and pasta products is between 10–20%. Amaranth nutritional properties and GF profile provide potential to enhance GF products (Schoenlechner et al., 2010).

● **Quinoa** (*Chenopodium quinoa*) is a GF seed crop traditionally cultivated in the Andean region of South America for several thousand years. Quinoa is considered as a

Table 2.4 Pseudocereals used in wheat and gluten-free breads

Reference	Pseudocereal	Main ingredients
Lorenz and Coulter, 1991	Quinoa flour	Wheat flour
Moore et al., 2004	Buckwheat flour	Corn starch, cassava starch, rice flour, soya flour
Park and Morita, 2005	Non germinated quinoa flour	Wheat flour
Capriles et al., 2008	Amaranth flour	Wheat flour and corn starch (cake)
Pruska-Kdzior et al., 2008	Buckwheat flour	Maize flour, maize starch, potato starch and rice flour
Renzetti et al., 2008	Buckwheat flour	Buckwheat flour
Wronkowska et al., 2008	Buckwheat flour	Wheat and corn starches
Alvarez-Jubete et al., 2009	Amaranth, quinoa and buckwheat	Rice and potato starch
Gambus et al., 2009	Amaranth and buckwheat flour	Corn flour, potato starch
Mariotti et al., 2009	Amaranth flour	Corn starch
Mezaize et al., 2009	Buckwheat flour	Rice flour, corn flour, corn starch and potato starch
Alvarez-Jubete et al., 2010	Amaranth, quinoa and buckwheat flour	Rice and potato starch
de la Barca et al., 2010	Amaranth	Amaranth flour
Sedej et al., 2010	Whole and refined buckwheat flour	Whole and refined buckwheat flour
Torbica et al., 2010	Buckwheat flour	Rice flour
Wronkowska et al., 2010	Buckwheat flour	Corn starch
Angioloni and Collar 2011	Buckwheat flour	Wheat flour
Filipčev et al., 2011	Buckwheat flour	Wheat flour

Table 2.4 (cont.) Pseudocereals used in wheat and gluten-free breads

Reference	Pseudocereal	Main ingredients
Kupra-Kozak et al., 2011	Buckwheat flour	Potato and corn starch
Sakac et al., 2011	Buckwheat flour	Rice flour
Chlopicka et al., 2012	Buckwheat flour	Wheat flour
Burluc et al., 2012	Buckwheat flour	Wheat flour
Hager et al., 2012	Buckwheat flour	Buckwheat flour
Torbica et al., 2012	Buckwheat flour	Rice flour (cookies)

multipurpose agricultural crop. The seeds may be utilized for human food, in flour products and in animal feedstock because of its high nutritive value. It is an important source of proteins of high biological value, carbohydrates with low glycemic index, vitamins (thiamine, riboflavin, niacin, and vitamin E), and minerals (magnesium, potassium, zinc, and manganese). It is also rich in phytosterols and omega-3 and -6 fatty acids. In addition, it has techno-functional properties such as solubility, water-holding and gelation capacity and, emulsifying and foaming ability, which allow diversified uses (Abugoch and Lilian, 2009).

The seed is used to prepare different food products including breads, biscuits, cookies, crepes, muffins, pancakes, and tortillas. More recently, attention has been given to quinoa use in GF due to its nutritional and techno-functional properties (Jacobsen 2003)**.**

2.5.3.3 Legumes

Legumes are edible seeds that grow in pods and encompass a wide range of sizes, shapes, and colors. In general, legumes are now recognized as a food choice with significant potential health benefits as they contain high amounts of protein with a good amino acid profile (high lysine content), complex carbohydrates (dietary fibres and resistant starch), important vitamins and minerals (B-vitamins, folates, iron), and antioxidants as polyphenols. Legumes are also GF and have a low glycemic index, a characteristic that benefits people with diabetes, cardiovascular disease and CD (Han et al., 2010) .

Because of the interesting nutritional properties of legumes, many organizations, such as the World Health Organization, encourage their inclusion in the diet. Actually, their incorporation into bakery products has increased in the last few years, as it can be seen in Table 2.5. Apart from their nutritional properties, legume proteins also possess techno-functional properties that play an important role in food formulation and processing. Some functional properties include solubility, water and fat binding capacity, and foaming and emulsifying ability. All these properties have lead technologists to suggest legume flours as an alternative to produce GF products.

● **Soya** (*Glycine max*) is a plant of the legume family native of China, its seeds have been used for centuries in the East and is still an essential food in East countries diet. In

recent years, soya has also become a very common food in the Western world due to its extraordinary nutritional qualities; i.e. it is an excellent source of protein and high quality fat, with low content of saturated fatty acids. Moreover, isoflavones present in soya have been considered beneficial in reducing cardiovascular disease and preventing cancer, among other effects (Zhang et al., 2003). In the last decades, the development of processing techniques and the improvement of the traditional ones, along with the emergence of new varieties, has allowed the production of different soya products and ingredients (soya flour, soya protein isolate, soya protein concentrate, etc.) with a wide range of functional properties (Dubois and Hoover, 1981).

In bakery, soya has been used to improve wheat flours by increasing the content of lipoxygenase and phospholipids that enhance the mechanical behavior of the dough (Ribotta et al., 2004). There are conflicting results regarding the inclusion of soya in wheat bread: a 1-3% addition of soya flour resulted in whiter breads and slowed aging (Maga, 1975), while larger amounts (6-8%) led to breads with less volume and more firmness than those made without soya (Conforti and Davis, 2006). Previously, Knorr and Betschart (1978), observed a weakening effect of gluten structure when other proteins where incorporated in the dough. The lack of interaction between gluten and soya prevents the formation of an elastic matrix, allowing the formation of bread with good qualities (Brewer et al., 1992).

By contrast, in GF breads, besides contributing to protein content increase, soya addition improves its structural properties. Although soya proteins do not generate a protein network such as egg proteins (Moore et al., 2006), they have high affinity for water, increasing the consistency of batters and improving its specific volume (Sanchez et al., 2004; Sciarini et al., 2010). The addition of soya affects GF bread crumb, resulting in a reduction of firmness and in a delay in aging, due to the water absorption capacity of the protein. The high affinity of soya proteins for water results in less available water for starch and, therefore, a decrease in retrogradation rate, since starch retrogradation is controlled by its water content (Zeleznak and Hoseney, 1987; Ryan et al., 2002).

Soya is, therefore, beneficial for GF products quality characteristics. However, the high allergenicity of soya and the associated digestive problems are leading the research of alternative protein sources which may be able to provide gas-holding capacity and bake development without its disadvantages.

● **Carob germ** (*Ceratonia siliqua*) is an evergreen leguminous tree which grows throughout the Mediterranean region, mainly in Spain, Italy, Portugal and Morocco. Its seeds and pods have been traditionally used as a food thickener and sweetener (Wang et al., 2001). In recent times, carob primary use is in the production of carob bean gum and other food and industrial additives (Wang et al., 2001; Dakia et al., 2007). Carob germ flour is the byproduct of carob gum production (Bengoechea et al., 2008). The germ flour is primarily used as a protein supplement in animal feed and for dietetic supplements for humans (Dakia et al., 2007). However, these proteins have been identified as having viscoelastic properties similar to wheat gluten and have the potential to be used in GF baked goods in order to mimic gluten properties (Feillet and Roulland, 1998; Wang et al., 2001; Dakia et al., 2007; Bengoechea et al., 2008).

● **Chickpea** (*Cicer arietinum*) belongs to the family *Leguminoseae* and originates in Asia. India has the highest rates of consumption of chickpea (90% of the world production) which is processed to *dhal* (splits) or flour and is widely used for the preparation of a number of sweets and snack foods. Spain is one of the major countries that import chickpea. It contains relatively high amounts of protein (23-27%) and lipids (5.8-6.2%) compared to other legumes (Dodok et al., 1993). Its high lysine content makes chickpea an excellent enhancer of protein when combined with cereal proteins, which have a low content in lysine but are rich in sulphur amino acids (Iqbal et al., 2006).

Boye et al. (2010) reported that, due to its specific content of amino acids, chickpea protein presents high foam expansion and stability values compared to other legumes, such as pea and soya protein. Foam expansion and stability have been found beneficial for GF bread development due to the lack of a strong gluten network.

● **Pea** (*Pisum sativum*) is a legume commonly used in animal feed, being most used for pig feeding in Europe. It is rich in protein and shows a well balanced profile of amino acids, especially a high content of lysine (Schneider-Belhaddad and Kolattukudy, 2000). Pea has become a promising potential protein source in food formulation since, besides their nutritional characteristics, it has good techno-functional properties such as gelling, emulsifying and foaming capacity (Sumner et al., 1981; Bacon et al., 1990; Gueguen, 1991; Bora et al., 1994; Ipsen, 1997; Tomoskozi et al., 2001; Nunes et al., 2006).

Table 2.5 Legumes used in wheat and gluten-free breads

Reference	Legume	Main ingredients
Dalgetti et al., 2006	Fibers isolated from pea, lentil, and chickpea	Wheat flour
Moore et al., 2006	Soya	Potato starch, soya, corn and white rice flour
Nunes et al., 2006	Pea protein isolate	Native maize starch
Utrilla-Coello et al., 2007	Chickpea	Wheat flour
Lodi and Vodovotz , 2008	Soy milk powder and soya	Wheat flour
Marco and Rosell, 2008	Pea isolated	Rice flour
Gómez et al., 2008	Chikpea flour	Wheat flour
Mariotti et al., 2009	Pea isolated	Corn stach
Roccia et al., 2009	Soya protein isolated	Vital wheat gluten
Demir et al., 2010	Chickpea flour	Wheat flour
Han et al., 2010	Pea and chickpea flour and fractions	Pulse ingredients
Sciarini et al., 2010	Soya flour	Corn starch, rice flour
Yamsaengsung et al., 2010	Chickpea flour	White and whole wheat flour
Crockett et al, 2011b	Soya protein isolate	Rice flour, cassava starch
Gómez et al., 2011	Pin milled pea flour	Wheat flour

Table 2.5 (cont.) Legumes used in wheat and gluten-free breads

Reference	Legume	Main ingredients
Baik et al., 2012	Bean and chickpea flour	Wheat flour
De la Hera et al., 2012	Lentil flour	Wheat flour
Gularte et al., 2012	Chickpea, lentil, pea and bean	Rice flour
Miñarro et al., 2012	Pea isolate, chickpea flour, carob germ flour, soya flour	Corn stach
Mohammed et al., 2012	Chickpea flour	Wheat flour
Serpen et al., 2012	Soya flour	Wheat, oat, rye, wheat bran, maize
Smith et al., 2012	Carob germ	Native corn starch
Tsatsaragkou et al., 2012	Carob flour	Rice and carob flour
Yamsaengsung et al., 2012	Chickpea flour	White or whole wheat
Kohajdova et al., 2013	Lentil and bean flour	Wheat flour

It has been suggested as an alternative to soybean protein, although it contains less sulphur amino acids and tryptophan (but more lysine) per unit of protein than soy meal or soya defatted flour (Gatel and Grosjean, 1990).

2.5.4 Unicellular protein

The term unicellular protein is used to describe protein derived from cells of microorganisms such as yeast, fungi, algae and bacteria which are grown on various substrates for synthesis. The dried cells of microorganisms or the whole organism is harvested and consumed as a protein source for human food supplements and animal feeds. Single cell contains between 50-85% crude protein, a high content of amino acids (most are rich in lysine and methionine), vitamins, minerals, and useable energy (Taylor and Senior, 1978; Anupama, 2000).

2.6 Detection of gluten

The consumption of manufactured products carries potential risks assumed by celiac patients, as gluten can be added to a product as an ingredient or additive, or it may contain gluten for technological reasons due to the manufacture process. Therefore, gluten can be present not only in the products made from wheat flour, barley and rye as bread, pasta, cakes and cookies, but also in sausages and meat products, sauces, snacks, sweets, ready-to-eat meals, etc., and even, in certain medications as excipient. Moreover, when GF products are elaborated in a company that also produces wheat products, there are many critical points during manufacture that can lead to GF contamination.

As previously stated, in January 2009, the European Commission published a regulation concerning to the composition and labelling of foodstuffs suitable for persons intolerant to gluten; this regulation indicates that foods may display the term “GF” if the gluten content does not exceed 20 ppm as sold to the final consumer. Consequently, methods for gluten analysis must be sensitive enough to quantify these levels of gluten in foods.

2.6.1 Gluten extraction

One of the critical steps in gluten determination is the extraction of gluten present in food systems. During elaboration, foods are subjected to heat treatments and other processes that can modify the gluten structure. These changes and the heterogeneity of food make difficult to achieve a correct extraction of gluten. The traditional method consists in extracting gluten using a 60% ethanol-water mixture. Extraction efficiency can be improved, especially in heat-processed foods, by adding reducing and denaturing substances to solubilize gluten aggregates produced by heat, followed by the final extraction in ethanol. The solution containing these substances has been patented in Spain under the name of gluten extraction cocktail, with patent number ES2182698. Next, quantification of gluten must be performed by analytical methods.

2.6.2 Immunochemical assays

Nowadays, the method for gluten determination accepted by the Codex Alimentarius Commission is the ELISA sandwich method. It is an immunological test based on the R5 monoclonal antibody recognising potential coeliac-toxic epitopes, which occur repeatedly in gliadins, hordeins and secalins (the prolamin proteins from wheat, barley and rye, respectively). This technique is used in combination with the cocktail extraction solution for an enhanced gluten extraction from heat-treated foods. Due to the great complexity of the prolamin proteins and other ingredients in foods, sometimes these technologies give false positive or false negative results. Moreover, by comparing several ELISA-test kits based on different antibodies, it has been shown that the measured prolamin content varies greatly (van Eckert et al., 2006). Consequently, the development of complementary and alternative non-immunological systems that confirm the results of the immunological methods is of great interest.

2.6.3 Polymerase Chain Reaction (PCR)

PCR is based on the detection of deoxyribonucleic acid (DNA) from the cereal species that contain gluten. Köppel et al. (1998) used PCR combined with agarose gels to detect wheat contamination in oats. Three years later, the first quantitative PCR system to detect simultaneously contamination of wheat, barley and rye in GF food was developed (Dahinden et al., 2001). It was a quantitative competitive PCR or QC-PCR system

(Studer et al., 1998) which also needed agarose gels. In 2003, four individual quantitative real-time PCR systems (Q-PCR) were published for the individual detection of wheat, barley, rye and oats (Sandberg et al., 2003). Although quantitative PCR technology is employed in these four systems, they do not quantify DNA. They only discriminate if the contamination comes from wheat, barley, rye or oats, based on the melting temperature of the amplification products.

Recently, different Q-PCR systems based on TaqMan probes for the detection and quantification of wheat, barley and/or rye DNA, have been described (Hernandez, et al., 2005; Ronning et al., 2006; Zeltner et al., 2009). The main advantage of using probes is that they only produce a fluorescent signal if they have specifically hybridized with the target DNA sequence. In this way, primer dimers or other unspecific amplification products do not produce a signal. Nevertheless, these systems are less sensitive than others and are not suitable for GF food analysis, in which even small levels of contamination can produce serious problems to celiac patients.

A new quantitative real-time PCR system (Q-PCR) for reliable and rapid quantification of wheat DNA in GF food and in raw materials has been developed and optimised. This is a highly specific and sensitive system which presents a quantification limit of 20 pg DNA/mg. In addition, it is a robust PCR system which allows the analysis of wheat DNA even when very different substances contained in foods could interfere with the results. However, due to the different food manufacturing processes, it has not been possible to establish a correlation between the amount of DNA and the prolamin content in the analyzed foods. Nevertheless, this Q-PCR system represents a very useful tool as a non-immunological technique to confirm the presence of wheat in foods not only for celiac patients but also for individuals with wheat allergy (Mujico et al., 2011).

2.6.4 Mass spectroscopy

Mass spectrometry is an analytical technique used to measure the molecular mass of chemical or biological compounds, their structural data and deduct identification from them. The analysis, characterization and quantification of cereal grain gluten peptides and proteins is complicated as protein composition, profile and natural sequence fluctuations of grains are in a constant state of flux, due to variances in cultivars, grow areas, climate and the emergence of genetic engineering. Gluten protein composition

also changes during processing stages of manufacturing. A specific and sensitive liquid chromatography–mass spectrometry (LC–MS) has been developed for the quantitative detection of trace levels of immunogenic gluten marker peptides from complex mixtures, such as food samples which can be considered complementary to the ELISA assay. Studies show how a direct enzymatic digestion-LC–MS/MS method releases the physiologically relevant marker peptides, then detects and quantifies them, in a wide variety of native and processed foods in order to form a correlation between the quantities of the gluten marker peptides in the digested food and a known gluten adverse reaction (Sealey-Voyksner et al., 2010).

This versatile methodology allows both cooked and native food to be analyzed for the presence of wheat gluten. Nevertheless, this system does not detect prolamins below 20–25 mg/kg and is therefore not appropriate for confirming the ELISA results in food samples with low prolamin levels.

2.6.5 Near Infrared Spectroscopy (NIRS)

Near Infrared (NIR) light is defined as the wavelength region from 730 to 2500 nm, lying between the visible light with shorter wavelengths and the infrared with longer wavelengths. When a sample is irradiated, light is absorbed selectively according to the specific vibration frequencies of the molecules present and gives rise to a spectrum. The NIR region is characterized by overtone and combination bands of fundamental vibrations occurring in the mid infrared. All organic bands have absorption in the NIR region, whereas minerals may only be detected in organic complexes and chelates or indirectly by their effect on hydrogen bounds (Shenk et al., 1992).

The determination of water was the first application of near infrared spectroscopy (NIRS) in the analysis of food. Calibrations have been developed for estimating the water content of a wide range of food including barley, bread and cheese. However, the most important application in food was the determination of protein as it gave the milling industry the opportunity to screen deliveries of wheat. The use of NIRS for quality control of cereals is well established in the literature (Osborne, 2000) and has been introduced successfully as a rapid technique for grain (Miralbés, 2003; Scholz et al., 2007), flour (Paulsen et al., 2003; Miralbés, 2004; Baslar and Ertugay, 2011), dough (Alava et al., 2001; Kaddour and Cuq, 2011; Sinelli et al., 2008) and bread (Osborne et

al., 1984; Sørensen, 2009). In wheat flours, protein fractions (gliadin and glutenin) have been predicted using NIRS technique (Wesley et al., 2001). In addition, rapid infrared spectroscopy methods have been successfully used for detection of adulteration of a wide range of complex food products, including oils, milk and wheat (Ozen et al., 2003; Cocchi et al., 2006; Kasemsumran et al., 2007). Norris (2009) proposed a simple method using multiple calibrations on a single near infrared scan to detect adulteration in food products.

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Chapter 3

Related papers

Paper 1

3.1 Influence of unicellular protein on gluten-free bread characteristics

B. Miñarro, I. Normahomed, B. Guamis, M. Capellas

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ORIGINAL PAPER

Influence of unicellular protein on gluten-free bread characteristics

B. Miñarro · I. Normahomed · B. Guamis · M. Capellas

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Abstract The objective of this work was to study the characteristics of three gluten-free bread formulations and the effect of the inclusion of unicellular protein. Bread recipes were starch-based, starch-vegetable-based and flour-based and the same recipes with added unicellular protein. Flour-based breads had lower specific volume values (2.2–2.3 cm^3/g) than starch-based breads (3.1–3.3 cm^3/g). Starch-based and starch-vegetable-based formulations with unicellular protein showed less bake loss (18.3 and 17.8%) than their counterparts (21.1 and 19.6%) probably due to increased water retention caused by unicellular protein. Flour-based recipes resulted in the firmest crumb, mainly caused by the high content in dietary fiber. The addition of unicellular protein resulted in a darker crumb color, and significant differences were also found in crumb color because of ovalbumin addition. Confocal scanning laser microscopy results showed a more compact microstructure in flour-based recipes compared with starch-based and starch-vegetable-based formulations. Starch-vegetable-based formulations without unicellular protein were the most preferred by consumers, followed by starch-vegetable-based formulations with added protein. Main differences detected by consumers were related to texture attributes. No major changes in shelf-life could be attributed to differences in formulation.

Keywords Celiac disease · Gluten-free bread · Starch-based formulations · Unicellular protein

B. Miñarro · I. Normahomed · B. Guamis · M. Capellas (✉)
Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), CeRTA, XiT, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
e-mail: marta.capellas@uab.cat

Abbreviations

CLSM	Confocal laser scanning microscopy
F	Flour-based
F_{up}	Flour-based with unicellular protein
S	Starch-based
S_{up}	Starch-based with unicellular protein
SV	Starch-vegetable-based
SV_{up}	Starch-vegetable-based with unicellular protein

Introduction

Celiac disease is an inflammatory disease of variable severity which results from sensitivity to ingested dietary gluten in genetically susceptible people [1]. The reaction to gluten ingestion by patients with celiac disease is inflammation of the small intestine, leading to malabsorption of several important nutrients including iron, folic acid, calcium and fat-soluble vitamins [2]. The symptoms of celiac disease vary widely, and the only way to prevent them is a lifelong gluten-free diet.

Gluten is the main structure-forming protein in wheat flour giving to dough its elastic and extensible properties. Due to these gluten characteristics, the formulation of gluten-free products is a big challenge [3]. Currently, there are some companies producing different gluten-free products, such as breads, pizzas, biscuits, and so on. However, most of them present poor mouthfeel, crumbly structure, difficulties in CO_2 retention and, consequently, low volume.

The global poor quality of gluten-free products and the increasing number of diagnosed patients with celiac disease along with the appearance of other food intolerances and allergies (because of new serological screening tests) [4] have lead technologists to investigate new ingredients and formulations to obtain gluten-free products as similar as possible to wheat products.

In recent years, some studies have been done by different authors, mainly involving the approach of incorporation of starches, dairy proteins and hydrocolloids into a gluten-free flour base, to try to mimic the properties of gluten and result in improved structure, mouthfeel, acceptability and shelf-life [5–11].

We have been developing gluten-free recipes testing different ingredients and their concentrations to improve the quality of gluten-free products. About 40 preliminary trials have been done, resulting in the recipes included in this study. Flour-based recipe includes three gluten-free flours (rice, buckwheat and soy) and cornstarch, while starch-based recipe is a cornstarch-based formulation with low content in buckwheat and soy flour. Starch-vegetable-based formulation is also free from egg and dairy proteins to avoid other known food intolerances and allergies. One of the ingredients tested is unicellular protein, obtained from yeast cells, which is considered to have a special interest because of its nutritive value which could contribute to balance the diet of patients with celiac disease as it contains approximately 50% of high-value protein, 37–40% of carbohydrates, low lipid content and high content of phosphorus (1.8%) [12–14].

The objective of this work was to study the characteristics of three gluten-free formulations and the effect of the inclusion of unicellular protein in them. We have studied their physico-chemical characteristics, consumer acceptance and shelf-life.

Materials and methods

Materials

Ingredients used for bread formulations were cornstarch (Agrana Maisita, Gemünd, Austria), white rice flour (Farines Estapé, Barcelona, Spain), soya flour (Farines Estapé), granulated buckwheat flour (Do-IT Bv, Barneveld, The Netherlands), white sugar (Azucarera Ebro SL, Madrid, Spain), skim milk powder (Reny Picot, Paris, France), iodized refined salt (Mercadona, Barcelona, Spain), baking powder (Panreac Química SA, Barcelona, Spain), shortening (Puratos, Barcelona, Spain), ovalbumin (Disproquimia, Barcelona, Spain), xanthan gum (Degussa Texturant Systems, Paris, France), baker's yeast (Mercadona), esters of mono and diglycerides of fatty acids as emulsifiers (Degussa Texturant Systems), unicellular protein from *Saccharomyces cerevisiae* (Uxafarma, Barcelona, Spain) and tap water.

Baking procedure

Six formulations were prepared, namely starch-based (*S*), starch-vegetable-based (SV), flour-based (*F*), starch-based with unicellular protein (S_{up}), starch-vegetable-based with unicellular protein (SV_{up}) and flour-based with unicellular protein (F_{up}). Ingredients for each formulation and their concentrations are listed in Table 1. Moisture (%) of rice, buckwheat, cornstarch and soya are respectively 12.0, 11.7, 12.0 and 9.0, and protein content (N% × 6.25) 6.6, 13.2, 0 and 38.3. Ingredients were weighed and mixed (Sammiv Gio, Gipuzkoa, Spain), adding first the dry ingredients, followed by yeast, shortening and water at 25 °C ± 1. Kneading was done during 1 min at low speed (ca. 140 rpm) and 3.5 min at medium speed (ca. 260 rpm). After homogenization, two tins (10 × 10 × 20 cm) of each recipe with 650 g of batter each were proofed at 37 °C and 85% RH for 45 min. Baking process was carried out in a convection oven (GPG, Barcelona) at 170 °C for 40 min. Breads were cooled down at room temperature for 2 h.

Table 1 Ingredients and amounts used in gluten-free bread formulations

Ingredients (%)[a]	*S*	SV	*F*	S_{up}	SV_{up}	F_{up}
Cornstarch	92	92	30	92	92	30
Flour	7.5[b]	7.5[b]	70[c]	7.5[b]	7.5[b]	70[c]
Sugar	5.4	5.4	6	5.4	5.4	6
Shortening	4.6	4.6	4	4.6	4.6	4
Yeast	4.6	4.6	6	4.6	4.6	6
Skim milk powder			1			1
Ovoalbumin	3		3	3		3
Baking powder	2.3	2.3	2.5	2.3	2.3	2.5
Xanthan gum	1.8	1.8	1	1.8	1.8	1
Salt	1.8	1.8	2.5	1.8	1.8	2.5
Emulsifier	2.3	2.3	5	2.3	2.3	5
Water	110	110	90	110	110	90
Unicellular protein				4.6	4.6	4

[a] Relative amounts in percent over flour plus starch weight basis (%)

[b] Buckwheat and soya (1:1)

[c] Rice, buckwheat and soya (4:2:1)

Evaluation of bread

Loaf volume was measured in duplicated by the method of displacement of millet seeds [15]. Specific volume was calculated using the formula: specific volume (cm^3/g) = volume (cm^3)/weight (g). The initial batter weight and the weight of bread after cooling was measured, and the bake loss was calculated using the formula: Bake loss (%) = (initial weight of batter − weight of bread after cooling) × 100/initial weight of batter.

Texture profile analysis of the crumb was performed on three slices taken from the center of each loaf. The slices were obtained by cutting the bread transversely using a ruler and bread knife to obtain uniform slices of 2.5 cm

thickness. Texture profile analysis was carried out using a TA-TX2 texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a 25-kg load cell and a 20-cm-diameter aluminium cylindrical probe. Probe speed was set to 2 mm/s to compress the center of the bread crumb to a 60% of its original height. Time between compressions was 5 s. Parameters studied were hardness, the peak force of the first compression; cohesiveness, the area of work during the second compression divided by the area of work during the first compression; and springiness, spring back of the product after it has been deformed during the first compression, measured at the downstroke of the second compression.

Crust and crumb color of bread samples were measured with a Hunter Lab colorimeter miniScan XTE (Hunter Associates Laboratory INC, Reston, Virginia, USA). CIE L^*, a^* and b^* values were measured with an illuminant of D65 and a standard observer of 10°. L^* represents the lightness with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser. Chromatic components are represented by a^* and b^* axes. Positive values of a^* are red and negative values are green, whereas positive values of b^* are yellow and negative values are blue. ΔE^* (total color difference) is calculated as $\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$. Crust color was measured on four different zones of the top of the whole bread. Crumb color was measured on four equidistant points to the center of each slice.

Bread moisture was determined according to the AOAC approved method 935.36 [16].

Confocal laser scanning microscopy

Bread recipes were prepared as indicated, but safranin (Merck, Darmstadt, Germany) was added at a rate of 0.002% in a flour plus starch weight basis. The dye was solubilised in the water before mixing to ensure homogeneous distribution. After baking, a small piece of the sample (around 1 mm) was taken from the center of the crumb, placed onto a welled slide and dyed with a phosphate buffer solution of 0.04% propidium iodide (Sigma, Madrid, Spain) for 45 min. The objective of this double dye was to stain protein network and yeast separately. The sample was immersed in oil and covered with a glass coverslip. A confocal laser scanning microscope Leica TCS SP2 AOBS (Leica, Heidelberg, Germany) was used with a 40× oil immersion objective to acquire fluorescence images (λ_{exc} 488 nm, λ_{em} 605–640 nm) of a number of optical sections by scanning the sample vertically with a slice interval of 1 μm.

Sensory evaluation

A consumer test was carried out with 39 people, all regular bread consumers. One day after baking, samples were sliced and placed in codified recipients using three digits randomly. Each sample was given to each tester, and one sample of the whole bread of each formula was shown to evaluate the external appearance. Consumers evaluated different bread attributes using a intensity scale of seven points. A preference test between samples was also performed following the method described by Kramer et al. [17]. Samples were ranked in order of preference. To obtain the ranking of each sample, each rank position (1–4) was multiplied by the number of consumers that had selected it, and the sum of the rankings of each sample was calculated. Low values in rank sum of samples indicated that the sample had mainly been ranked in first order of preference.

Microbiological analysis

The microbiological quality of the different breads was assessed by enumerating the following microorganisms: Aerobic mesophilic bacteria, counted on Plate Count Agar medium (Oxoid, Basingstoke, Hampshire, UK). One microliter of sample diluted in peptone water (Oxoid) was pour plated and incubated for 48 h at 30 °C; *Enterobacteriaceae*, on Violet Red Bile Glucose Agar medium (Oxoid) and pour plated and incubated for 48 h at 37 °C; Yeasts and molds, on Rose-Bengal Chloramphenicol Agar Base medium (Oxoid) and pour plated and incubated at 20 °C for 7 days [18]. Detection limit was 10 cfu/g of bread for all microorganisms studied.

Experimental design and statistical analysis

Three independent trials were conducted to determine physico-chemical characteristics of the six formulations mentioned. In each experiment, two breads from each recipe were prepared and three slices from each were analyzed 2 h after cooling. Analyses performed were volume, moisture, texture and color.

For confocal laser scanning microscopy (CLSM), three independent experiments including the dye in the formulation were prepared.

For shelf-life studies, two independent experiments were conducted. Following the procedure mentioned before, breads were packaged in a modified atmosphere of N_2 and CO_2 at 50:50. Microbiology, texture, color and moisture were analyzed at 0, 7 and 15 days of storage at room temperature.

Results were analyzed by analysis of variance using the general linear models procedure of Statistical Analysis System (SAS, 8 version). Tukey test was used for comparison of sample data. Evaluations were based on a significant level of $p < 0.05$.

Results and discussion

Baking characteristics

The specific volume was lower ($p < 0.05$) in F than in S and SV recipes (Table 2). Differences in bread volume can also be observed in Fig. 1. Generally, volume depends on gas formation, which depends on many factors, such as yeast, fermentable sugars, baking powder and pH, and gas retention, which is affected by fiber content. The non-endosperm components (germ, bran and epicarp hairs), which are present at 15% in buckwheat, are known to be responsible for producing the low volume and dense crumb texture of whole breads. Some reasons explain this effect, like the dilution effect of the non-endosperm components on the gluten-forming proteins, the restriction of water available for gluten hydratation and development, or the physical disruption of starch-gluten matrix [19–23]. According to Moore et al. [24], this negative effect of fiber on volume can be assumed to be even worse when the structure is not stabilized by gluten. Moreover, Gallagher et al. [25] reported that specific volume decreases with the decrease in water addition, which agrees with our results, as *F* doughs contain less water than *S* and SV. Although it is known that egg protein improves batter stability [26, 27], we could not find significant differences ($p > 0.05$) on volume due the addition of ovalbumin neither with unicellular protein.

Moisture of all breads falls within the usual range for this type of products (40–45%) [28]. No significant differences ($p > 0.05$) were found between samples. Regarding to bake loss, *F* and F_{up} had lower values, because of the low initial % of water and the high % of fiber contained in buckwheat flour, which absorbs water and helps to its retention during baking. In *F* breads, the lower specific volume and bake loss correlate with a higher crumb firmness or hardness compared with *S* and SV recipes (Fig. 2a). Moreover, this increase in hardness in *F* breads may be explained by the inclusion of egg and dairy protein in these recipes, which have structure-building properties.

The presence of unicellular protein in S_{up} and SV_{up} resulted in breads with lower bake loss (%) and a higher hardness than their counterparts, *S* and SV. This may be explained because the unicellular protein functionality, which tends to retain water and consequently, decreases bake loss. Probably, this effect was masked in F_{up} recipe by the high water retention due to buckwheat fiber. No significant differences in texture could be detected between *S* and SV formulations.

Table 2 Baking characteristics of breads

	S	S_{up}	SV	SV_{up}	*F*	F_{up}
Loaf specific volume (cm^3/g)	3.06 ± 0.1^{a}	3.14 ± 1.5^{a}	3.3 ± 0.2^{a}	3.09 ± 0.1^{a}	2.26 ± 0.2^{b}	2.21 ± 0.1^{b}
Moisture (%)	42.12 ± 1.4^{a}	41.09 ± 0.8^{a}	44.57 ± 2.4^{a}	42.91 ± 1.6^{a}	42.09 ± 1.1^{a}	40.99 ± 2.8^{a}
Bake loss (%)	20.09 ± 0.9^{a}	$18.32 \pm 1.2^{b,c}$	$19.57 \pm 1.4^{a,b}$	17.75 ± 1.6^{c}	13.87 ± 0.7^{d}	14.69 ± 1.0^{d}

Mean value ± standard deviations of three replicates. Values labeled with a different letter in the same row are significantly different ($p < 0.05$)

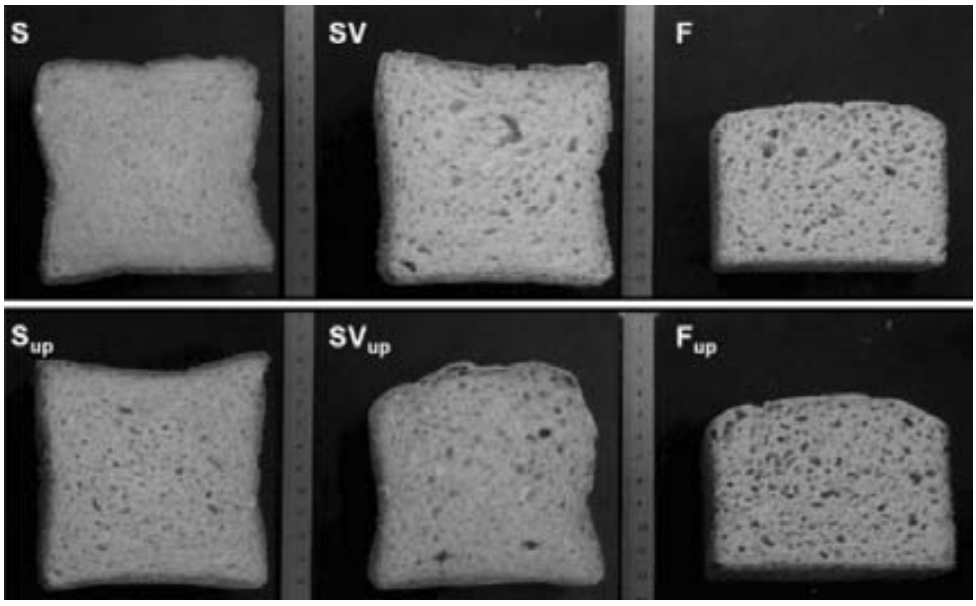

Fig. 1 Digital images of different breads. Starch-based: *S*; starch-based with unicellular protein: S_{up}; starch-vegetable-based: SV; starch-vegetable-based with unicellular protein: SV_{up}; flour-based: *F* and flour-based with unicellular protein: F_{up}

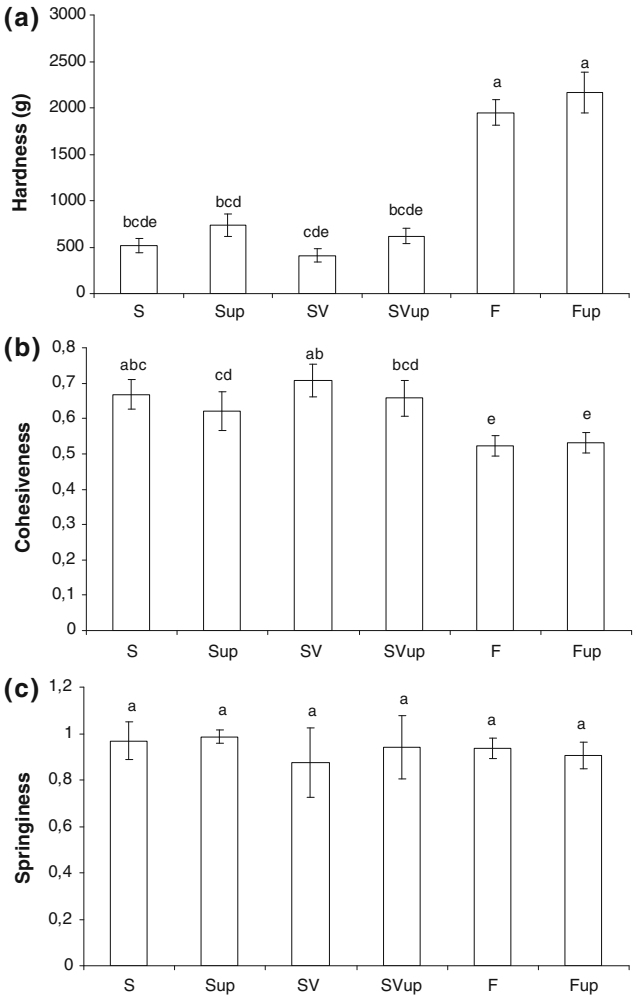

Fig. 2 Crumb texture values. **a** Hardness, **b** cohesiveness, **c** springiness of starch-based: *S*; starch-based with unicellular protein: S_{up}; starch-vegetable-based: SV; starch-vegetable-based with unicellular protein: SV_{up}; flour-based: *F* and flour-based with unicellular protein: F_{up}. Mean values ± standard deviations of three replicates. Mean values with *different letters* are significantly different ($p < 0.05$)

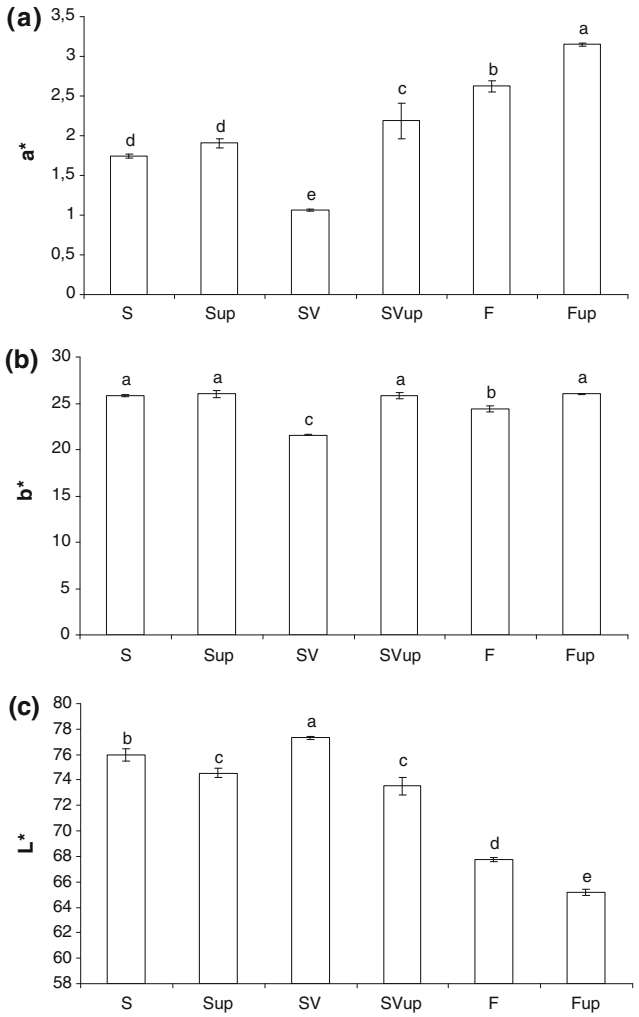

Fig. 3 Crumb color values. **a** a^*; **b** b^*; **c** L^* of starch-based: *S*; starch-based with unicellular protein: S_{up}; starch-vegetable-based: SV; starch-vegetable-based with unicellular protein: SV_{up}; flour-based: *F* and flour-based with unicellular protein: F_{up}. Mean values ± standard deviations of three replicates. Mean values labeled with *different letters* are significantly different ($p < 0.05$)

Moore et al. [24] indicated that hardness was generally higher in formulations with egg protein than without it, reflecting the stronger structure caused by ovalbumin, but we could not clearly detect this effect with the addition of a 3% of protein. *F* recipes showed less cohesiveness than *S* and SV, mainly due to the effect of the fiber in the formulation. On the other hand, recipes with unicellular protein showed less cohesiveness than their counterparts. Cohesiveness (Fig. 2b) is correlated negatively to the rate of breakdown in the mouth and easy separation in the hand. No differences were found between springiness of different samples (results not shown).

Crumb color of different bread recipes varied considerably (Fig. 3). On the one hand, *F* breads were different ($p < 0.05$) from *S* and SV, with ΔE values ranging from 8.37 to 10.30 and differences were mainly due to L^* and a^* values. Color is an important attribute of baked products, and it contributes to consumer preference. It depends on factors such as formulation or baking conditions. F formulations were expected to be darker than the others because of the high content in buckwheat flour, an ingredient which has an initial darker color compared with other flours and starches used in this work (Fig. 3c). On the other hand, breads containing unicellular protein were darker than the same formulation without this ingredient (ΔE values ranging from 1.47 for S recipes to 6 for SV recipes), due to the color of the protein itself ($L^* = 65$, $a^* = 6$ and $b^* = 21$). Moreover, significant changes can be attributed to the addition of ovalbumin, as SV is the formulation that shows a lighter color, with the highest differences in all color parameters measured. The addition of unicellular protein to SV recipe would mask the absence of ovalbumin.

Crust color did not vary between formulations (results not shown). During baking, high temperature and loss of water in bread surface results in crust formation, where Maillard reaction is extensive. Melanoidins develop during the final stages of Maillard reaction in crust, being responsible for the brown color formation. The darker color in crust would have masked color differences attributed to different ingredients which where observed in our bread crumb. L^* values were between 55 and 60, according to the results obtained by Gallagher et al. [6]. This author pointed that the darkening of the crust color is desirable, as gluten-free breads tend to have lighter crust color than wheat breads.

CLSM

To obtain a better understanding of the different bread structures, confocal microscopy was used. Common structures can be observed in all samples: protein network, in green color, characterized by continuous associated strands; yeast cells, as globular yellowish bodies; and green diffused spots, more difficult to observe, embedded in protein network that would be gelatinized starch granules.

F breads (Fig. 4) showed denser microstructure than *S* and SV recipes. This was expected due to the high protein content in *F* formulations, and it also agrees with the low specific volume and firm texture obtained in these recipes. *F* and *S* recipes showed continuous structures similar to gluten, but not SV recipes, due to the absence of ovalbumin and milk powder. On the one hand, Jonah et al. [26] found that proteins such as egg albumen were able to link starch granules together and Moore et al. [24] revealed continuous film-like protein structures similar to wheat gluten when observed by confocal laser microscopy, when egg protein was added. On the other hand, dairy proteins are capable of forming networks and have good swelling properties [25]. Although the addition of unicellular protein to the different initial formulations seems to not cause negative effects on volume, it is clear that it interferes with the protein network, acting like bran particles and behaving only as filler, retaining its form. The presence of relatively large and non-reactive particles could induce network instability.

Sensory analysis

Sensory analysis was conducted with S and SV recipes. F formulations were not included in the experiment because of the strong different appearance.

The most relevant differences between *S* and SV breads were found in texture parameters, and no significant differences were detected in flavor intensity, salty taste, sweet taste and aroma (Table 3). SV formulation obtained the highest significant scores in crumb elasticity and sponginess, and it also was the bread that obtained the best rank in the preference test (Table 4). It is supposed that these differences are due to the lack of ovalbumin and unicellular protein in this recipe, resulting in a lighter mouthfeel. As it was also reflected by color parameters, testers found formulations with unicellular protein darker than the ones without protein.

Shelf-life study

Microbiological counts are shown in Table 5. *Enterobacteriaceae* were not detected in any sample at day 0. At day 7, they were not detected in any sample except S and F, which presented about 3 and 2 log units, respectively. Mesophilic bacteria counts at day 0 were about 1 and 2 log units, except for SV recipe where no growth was detected. Their counts at day 7 increased between 3 and 7 log units. It is known that bacterial spoilage of bread is mainly caused by *Bacillus* spp, which is usually present in raw ingredients (e.g., flour, sugar and yeast), survives the baking process, germinates upon cooling, growths under both aerobic and anaerobic packaging conditions and causes an alteration known as ropiness, characterized by a discolored bread crumb, due to the protein degradation during growth of the bacteria [29]. In our bread, we observed ropiness alteration from day 10 of storage. Yeast and mold counts were between 1.5 and 1.8 log units at day 0 except for SV samples, where no counts were detected and increased around 1 log unit at day 7. At day 7, no macroscopic signs of mold spoilage were detected, but at day 10 of storage, all samples showed macroscopical signs of mold growth. Fresh baked bread is free of molds and their spores, due to the thermal inactivation during baking, so baked bread was contaminated during cooling and packaging process. It is estimated that 1 g of flour may contain 8,000 mold spores [30], so weighing and mixing of ingredients may have helped to increase mold contamination in the processing room, the same where breads were cooled.

Taking into account the high counts of mesophilic bacteria at day 7, compared with values from commercial flours—maximum of $1x10^6$ CFU/g—and of baked goods—10^2 to 10^3 CFU/g—[31, 32] along with the macroscopical signs of mold spoilage at day 10 of storage, we decided not to follow with the shelf-life analyses at day 15 of storage as it was initially planned. Anyway, high counts at few days of storage were expected due to bread formulations, which contain a high % of water and no preservatives.

Color changes during 7 days of storage were detected. L^* values decreased in S_{up} samples and increased in *F* and F_{up} (results not shown). a^* values were different for S_{up}, SV and SV_{up}, while in b^* values, no differences were detected except for SV breads.

Texture changes during storage showed a similar trend in all samples (results not shown). Hardness

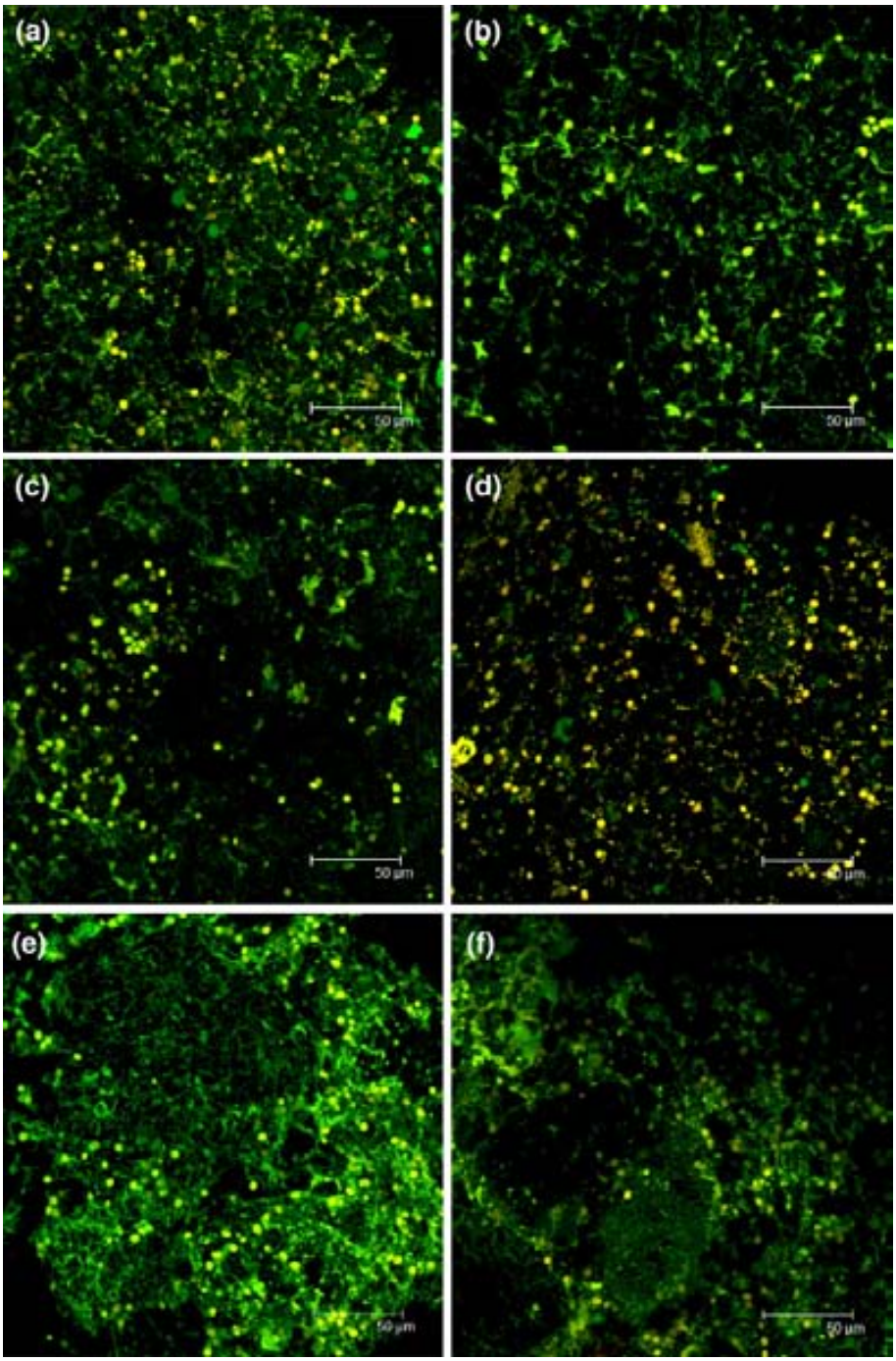

Fig. 4 Confocal laser scanning micrographs (40 magnification) of: **a** starch-based: *S*; **b** starch-based with unicellular protein: S_{up}, **c** starch-vegetable-based: SV, **d** starch-vegetable-based with unicellular protein: $SV_{up,}$ **e** flour-based: *F* and **f** flour-based with unicellular protein: $F_{up.}$ Protein network, in *green color*; yeast cells, as globular *yellowish* bodies

increase was only significant in SV recipes, which were the ones that had lower initial values (364.13 g) of this parameter. Moore [24] found that egg protein formulations where higher in firmness than non-egg protein formulations, but the increase in firmness over time was less, due to the essential behavior of ovalbumin in foam and emulsion stabilization, delaying staling. This agrees with our results, as the formulation without ovalbumin suffered a marked increase in hardness (609 g) after 1 week of storage. Cohesiveness decreased considerably in all samples, indicating that all breads were more brittle and fracturable at first bite at day 7 than at day 0. Springiness was not significantly different except for samples *S* and $SV_{up.}$

No differences were found in moisture within the 7 days of storage, except for SV which showed a slight decrease, probably due to an initially higher content compared with other breads (results not shown).

Table 3 Sensory evaluation of the breads by consumers test

	S	S_{up}	SV	SV_{up}
Sponginess	3.7 ± 1.6^{b}	3.67 ± 1.4^{b}	5.5 ± 1.7^{a}	4.4 ± 1.6^{b}
Elasticity	$3.7 \pm 1.4^{b,c}$	3.3 ± 1.4^{c}	5.3 ± 1.5^{a}	4.3 ± 1.6^{b}
Moisture	$4.01 \pm 1.5^{a,b}$	3.3 ± 1.23^{b}	4.9 ± 1.4^{a}	3.7 ± 1.5^{b}
Crumbliness	$3.6 \pm 1.5^{a,b}$	4.6 ± 1.45^{a}	3.4 ± 1.9^{b}	4.5 ± 1.56^{a}
Flavor intensity	$3.89 \pm 1.5^{a,b}$	3.6 ± 1.5^{b}	4.7 ± 1.7^{a}	$3.9 \pm 1.5^{a,b}$
Sweet taste	3.78 ± 1.3^{a}	3.5 ± 1.3^{a}	4.2 ± 1.6^{a}	3.9 ± 1.32^{a}
Salty taste	3.8 ± 0.8^{a}	4.12 ± 1.3^{a}	3.7 ± 1.2^{a}	3.6 ± 0.9^{a}
Aroma intensity	4.1 ± 1.3^{a}	4.6 ± 1.3^{a}	4.6 ± 1.3^{a}	4.6 ± 1.4^{a}
Crust brightness	4.01 ± 1.5^{a}	4.3 ± 1.4^{a}	3.6 ± 1.9^{a}	4.1 ± 1.7^{a}
Crust color	3.8 ± 1.1^{b}	4.8 ± 1.1^{a}	3.0 ± 1.7^{b}	5 ± 1.3^{a}

Mean values ± standard deviations of 39 consumers. Values labeled with a different letter in the same row are significantly different ($p < 0.05$)

Table 4 Preference test

Rank position	*S*		S_{up}		SV		SV_{up}	
	A	B	A	B	A	B	A	B
1	3	3	1	1	23	23	10	10
2	11	22	5	10	7	14	12	24
3	13	39	10	30	1	3	10	30
4	8	32	19	76	6	24	3	12
Rank sum of the samples		96^{b}		117^{a}		64^{c}		76^{b}

Values labeled with a different letter in the same row are significantly different ($p < 0.05$)

A, number of judgements; B, rank sum of the respective rank position (number of judgements × respective rank position)

Table 5 Microbiological counts during storage at room temperature (Log CFU/g)

	Enterobacteriaceae		Mesophilic bacteria		Yeasts and molds	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
S	ND	3.1 ± 0.0	1.3 ± 0.1	8.2 ± 0.0	1.8 ± 0.0	2.2 ± 0.3
S_{up}	ND	ND	2.3 ± 0.1	8.1 ± 0.0	1.5 ± 0.5	2.4 ± 0.2
SV	ND	ND	ND	6.1 ± 0.1	ND	2.8 ± 0.8
SV_{up}	ND	ND	2.1 ± 0.1	5.7 ± 0.4	1.8 ± 0.5	2.5 ± 0.0
F	ND	2.3 ± 0.0	1.8 ± 0.0	6.2 ± 0.1	1.5 ± 0.2	2.8 ± 1.2
F_{up}	ND	ND	2.0 ± 0.1	8.3 ± 0.0	1.6 ± 0.1	1.6 ± 0.2

Mean values ± standard deviations of two replicates

ND, not detected

Conclusions

It is possible to formulate gluten-free starch-based breads with good baking characteristics, while the use of high fiber content in the recipe leads to breads with low specific volume and hard crumb.

The inclusion of unicellular protein to gluten-free starch-based breads provides formulations with low bake loss. However, recipes with unicellular protein are less preferred by consumers than the same recipes without it, probably due to texture characteristics.

No remarkable changes in shelf-life seem to be caused by differences in formulation, although ovalbumin addition could contribute to the delay of staling.

Further chemical, nutritional and rheological research is needed to complete the application study of unicellular protein on gluten-free breads.

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Paper 2

3.2 Effect of legume flours on baking characteristics of gluten-free bread

B. Miñarro, E. Albanell, N. Aguilar, B. Guamis, M. Capellas

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Effect of legume flours on baking characteristics of gluten-free bread

B. Miñarro [a], E. Albanell [b], N. Aguilar [a], B. Guamis [a], M. Capellas [a,*]

[a] Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), CeRTA, XiT, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

[b] Grup de Recerca en Remugants (G2R), Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain

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ABSTRACT

The objective of this work was to study the characteristics of four gluten-free bread formulations and the possibility of substituting soya protein with other legume proteins. Four bread recipes were prepared with chickpea flour, pea isolate, carob germ flour or soya flour. Carob germ flour batter structure was thicker compared with the other batters, probably due to the different protein behaviour and the residual gums present in carob germ flour. However, carob germ flour bread obtained the lowest specific volume values (2.51 cm^3/g), while chickpea bread obtained the highest (3.26 cm^3/g). Chickpea bread also showed the softest crumb. Confocal scanning-laser microscopy results showed a more compact microstructure in carob germ flour bread compared with soya and chickpea formulations. Chickpea bread exhibited the best physico-chemical characteristics and, in general, good sensory behaviour, indicating that it could be a promising alternative to soya protein.

1. Introduction

Celiac disease is an inmuno-mediated enteropathy triggered by the ingestion of gluten in susceptible patients (Kennedy and Feighery, 2000). The reaction to gluten by celiac patients leads to the malabsorption of several important nutrients such as iron, folic acid, soluble vitamins, etc. Gluten is the main structural forming protein of most baked products contributing to the elastic, cohesive and viscous characteristics of dough and its replacement presents technological problems such as poor quality mouthfeel, crumbly structure, and a quick staling. Celiac disease is nowadays the most common lifelong dietary disorder worldwide, affecting around 1% of the European population and highly underdiagnosed in all countries (Mustalahti et al., 2010). For this reason, there is an increasing interest in gluten-free products, mainly involving the incorporation of starches, different animal and vegetable sources of proteins, and hydrocolloids into a gluten-free flour base, with the aim of mimicking the properties of gluten to achieve an improved structure, mouthfeel, acceptability and shelf-life (Lazaridou et al., 2007; Moore et al., 2006; Nunes et al., 2009; Renzetti et al., 2008; Ribotta et al., 2004). Our research group has developed a formulation which contains soya protein and offers good baking and sensory characteristics (Miñarro et al., 2010). However, the high allergenicity of soya (*Glycine max* L.) and the associated digestive problems, are leading to more research into alternative protein sources which may be able to provide gas-holding capacity and bake development.

Legumes are important sources of food proteins. They contain high amounts of lysine, leucine, aspartic acid, glutamic acid and arginine, and provide well balanced essential amino acid profiles when consumed with cereals and other foods rich in sulphur-containing amino acids and tryptophan. Apart from their nutritional properties, legume proteins also possess functional properties that play an important role in food formulation and processing (Boye et al., 2010; Dakia et al., 2007; Roy et al., 2010). The functional properties of legume proteins such as chickpea flour (*Cicer arietinum* L.), pea protein isolate (*Pisum sativum* L.), and carob germ flour (*Ceratonia siliqua* L.) have been used in the preparation and development of bakery products, soups, extruded products and ready-to-eat snacks. The benefits associated with legumes together with the increase of celiac patients have led some to suggest their study as an alternative to common flours for preparing gluten-free products. Carob germ flour gluten-like properties have been reported and studied by some authors (Bengoechea et al., 2008; Bienenstock et al., 1935; Feillet and Roulland, 1998; Plaut et al., 1953; Rice and Ramstad, 1950; Smith et al., 2010; Wang et al., 2001), however, few studies have been carried out on its behaviour in baked goods.

The aim of this research was to study the characteristics of four gluten-free formulations prepared with legume protein sources

* Corresponding author. Tel.: +34 935811446; fax: +34 935811397.
E-mail address: marta.capellas@uab.cat (M. Capellas).

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(chickpea flour, pea protein isolate, soya flour and carob germ flour) to develop new formulations that could substitute soya flour.

2. Materials and methods

2.1. Raw materials

Ingredients used for bread formulations were: corn starch (Syral Iberia SAU, Zaragoza, Spain), white sugar (Azucarera Ebro S.L., Madrid, Spain), iodized refined salt (Sal Costa S.A., Barcelona, Spain), baking powder (Panreac Química S.A., Barcelona), shortening (Puratos, Barcelona), xanthan gum (Degussa Texturant Systems, Paris, France), baker's yeast (Mercadona, Tavernes Blanques, Spain), esters of mono and diglycerides of fatty acids as emulsifiers (Degussa Texturant Systems) and tap water. Protein sources used were: chickpea flour (21.34% protein) from El Granero Integral S.L. (Madrid); carob germ flour (43.17% protein) from Danisco Cultor España S.A. (Valencia, Spain); pea protein isolate (80.74% protein) from Sofralab S.A.S. (Magenta, France); and soya flour (38.14% protein) from Trades, S.A. (Barcelona). The Dumas method (AOAC, 2003) with a Leco analyser (Leco Corporation, St. Joseph, MI) was used for N determination. Protein content (%) was calculated as N × 6.25.

2.2. Baking procedure

Ingredients for each recipe and their concentrations are listed in Table 1. The amount of protein source in each formulation was adjusted to obtain the same final protein % in bread (1.5%). Ingredients were weighed and mixed (Sammic S.L. BM5, Gipuzkoa, Spain), adding first the dry ingredients, followed by yeast, shortening and water at 25°C ± 1. Kneading was performed for 1 min at low speed (level 1 out of 5) and 3.5 min at high speed (level 3 out of 5). After homogenization, twelve tins (5 × 8 × 14 cm) of each recipe, each one containing 200 g of batter, were proofed at 28 °C and 85% RH for 45 min. The baking process was carried out in a deck oven (Sveda Dahlen, Fristad, Sweden) at 180 °C for 25 min. Breads were cooled at room temperature for 2 h. Bread samples were packed in a modified atmosphere of 50% N_2 and 50% CO_2 and stored for 5 days. Twelve loaves were obtained from each formulation. Three independent batches of the four formulations were prepared for this study.

2.3. Batter analysis

2.3.1. Rheofermentometer evaluation

The Chopin Rheofermentometer (Villeneuve-la-Garenne, France) was used to measure batter height and CO_2 development. As previous research with gluten-free batters has not been reported, the technique was optimized for this type of sample. The best reproducibility and optimum results were obtained when batters were fermented for 3 h with 500 g of weight. Batters were prepared as mentioned in 2.2. Parameters measured were: maximum height of batter development, time of porosity appearance, total volume CO_2, volume CO_2 released, retained volume; % retention: retained volume/total volume, and weakening coefficient: % of decrease after 3 h.

2.3.2. Fundamental rheology

For rheological measurements, batters were prepared as in 2.2. without yeast addition. A controlled stress and strain rheometer Haake Rheo Stress 1 (Thermo Electron Corporation, Karlsruhe, Germany) was used. Samples were examined using a parallel plate system, which consisted of a 35 mm diameter corrugated probe. A circulating Haake SC100 bath (Thermo Electron Corporation) with an accuracy of ±0.1 °C was used to set the system at 30 °C. When thermal equilibrium was set, the batter sample was loaded onto the corrugated plate and the probe was lowered to a gap of 2 mm. After removing excess batter, the exposed edges were covered with water to avoid desiccation. A rest period of 5 min was allowed to enable the sample to recover from the stress due to preparation. To ensure that all measurements were carried out within the linear visco-elastic region, amplitude sweeps were performed in the range of strain 0.01–10%. Based on these results, oscillation stresses were selected. Frequency sweep tests were performed at frequencies between 0.1 and 10 Hz with a target strain of 5×10^{-4} (0.05%). Rheological properties, such as storage modulus (G') and loss modulus (G''), were calculated using the manufacturer software (Rheoplus, Anton Paar, Germany). All measurements were performed in triplicate.

2.4. Bread analysis

2.4.1. Baking evaluation

Loaf volume was measured by the method of displacement of millet seeds (Griswold, 1962). Specific volume was calculated using the formula: specific volume (cm^3/g) = volume/weight. Initial batter weight and weight of bread after cooling were measured and bake loss was calculated using the formula: Bake loss (%) = (initial weight of batter − weight of bread after cooling) × 100/initial weight of batter.

Texture profile analysis of the crumb was performed on three slices taken from the center of each loaf. The slices were obtained by cutting the bread transversely using a cutter to obtain uniform slices of 12.5 mm thickness. Two slices of 12.5 mm were stacked together to obtain a uniform slice of 25 mm. Texture profile analysis was carried out using a TA-TX2 texture analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell and a 35 mm diameter aluminium cylindrical probe. Probe speed was set to 1.7 mm/s to compress the centre of the bread crumb to 40% of its original height. Time between compressions was 5 s. Parameters studied were: hardness (peak force of the first compression), cohesiveness (area of work during the second compression divided by the area of work during the first compression) and springiness (spring back of the product after it has been deformed during the first compression, measured at the downstroke of the second compression).

Crust and crumb colour were measured with a Hunter Lab colorimeter miniScan XTE (Hunter Associates Laboratory INC, Reston, Virginia, USA). CIE L^*, a^* and b^* values were measured with an illuminant of D65 and a standard observer of 10°. L^* represents the lightness, with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser. Chromatic components are

Table 1
Ingredients used in gluten-free bread formulations (g).

Ingredient[a]	Chickpea flour	Pea isolate	Soya flour	Carob germ flour
Corn starch	1132	1201.6	1165.8	1178.8
Sugar	70	70	70	70
Baking powder	30	30	30	30
Shortening	60	60	60	60
Baker's yeast	60	60	60	60
Salt	30	30	30	30
Xanthan gum	24	24	24	24
Emulsifier	24	24	24	24
Water	1260	1260	1260	1260
Chickpea flour	94	–	–	–
Pea isolate	–	24.4	–	–
Soya flour	–	–	60.2	
Carob germ flour	–	–	–	47.2

[a] Ingredients expressed in baker's percentage: corn starch + source of protein: 100%; sugar: 5.70%; baking powder: 2.45%; shortening: 4.89%; baker's yeast: 4.89%; salt: 2.45%; xanthan gum: 1.95%; emulsifier: 1.95%; water: 102.77%.

represented by a^* and b^* axes. Positive values of a^* are red and negative values are green, whereas positive values of b^* are yellow and negative values are blue. Crust color was measured on four different zones of the top of the whole bread. Crumb color was measured on four equidistant points to the center of each slice.

2.4.2. Sensory evaluation

A consumer test was carried out with 66 volunteers, 40 females and 26 males, between 20 and 60 years old, recruited among university staff and postgraduate students. They were regular bread consumers and were used to participate in sensory analyses of different foods. One day after bread making, samples were sliced and placed in recipients randomly codified with three digits. A slice of each formulation was given to every consumer, and a sample of the whole bread of each formula was shown to evaluate the external appearance. All samples were presented at the same time. Consumers evaluated different bread attributes using an intensity scale and a preference scale of 9 points. Finally, subjects were asked to indicate the most and the least preferred sample.

2.4.3. Confocal scanning laser microscopy

After baking, breads were dyed with 2% of concanavalin (Invitrogen, Barcelona), 0.2% Cell Mask (Invitrogen, Barcelona) and 10^{-9}% of Rhodamine B (Sigma, Madrid). The objective of this triple dye was to separately identify starch, yeast and protein, respectively. A small piece of the sample (around 1 mm) was taken from the centre of the dyed crumb, immersed in oil in a welled slide, and covered with a glass cover slip. A confocal laser-scanning microscope Olympus LX81 was used with a 40× oil immersion objective to acquire fluorescence images (λexc 488 nm, λem 605–640 nm) of a number of optical sections by scanning the sample vertically with a slice interval of 1 μm.

2.4.4. Microbiological analysis

To count aerobic mesophilic bacteria, 1 ml of decimal dilutions of bread sample in peptone water (Oxoid) was pour plated on Plate Count Agar medium (PCA, Oxoid, Basingstoke, Hampshire, UK) and incubated for 48 h at 30 °C. To assess spore counts, decimal dilutions in Ringer solution were subjected to heat shock at 80 °C for 10 min and pour plated in PCA. For *Enterobacteriaceae*, dilutions were pour plated on Violet Red Bile Glucose Agar medium (Oxoid) and incubated for 48 h at 37 °C. Yeasts and moulds were counted on Sabouraud with cloranfenicol (Oxoid) after incubation at 30 °C for 5 days. Microbiological analyses were performed on the three batches of bread at day 1, 5 and 7 of storage.

2.5. Statistical analysis

Analysis of variance (ANOVA) was performed using the General Linear Models procedure of Statistical Analysis System (the SAS System for Windows, version 9.1; SAS Institute, Cary, NC, USA). Either LSD or Tukey's tests were used for comparison of sample data, and evaluations were based on a significance level of $P < 0.05$.

3. Results and discussion

3.1. Batter evaluation

The frequency sweep curves of gluten-free batters are shown in Fig. 1. Frequency sweep shows changes in viscous and elastic behaviour with the rate of application of strain or stress, while the amplitude of the signal is held constant. For all the formulations, in the whole range of frequencies, G′ was greater than G″ and both increased with increasing frequencies, suggesting a viscoelastic behaviour. However, the difference between G′ and G″ was more

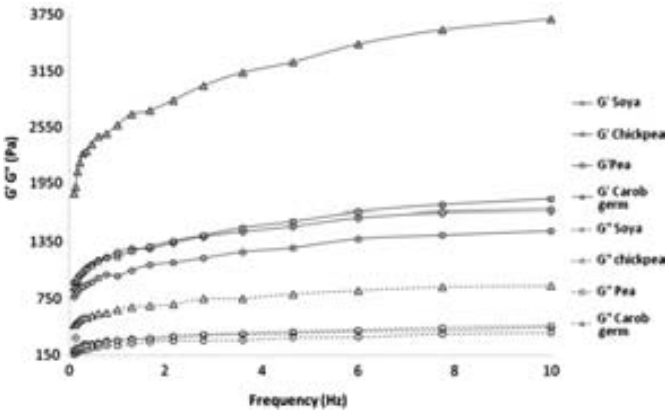

Fig. 1. Rheological properties of legume flour gluten-free batters (▬G′, –G″, ◆ Soya flour, ■ Pea isolate, ▲Carob germ flour, ● Chickpea flour).

evident in carob germ batters, indicating a thicker structure compared to chickpea, pea and soya batters. It has been stated that carob germ protein shows significant potential in gluten-free foods because, besides being safe for celiac patients, it has rheological similarities with gluten (Bengoechea et al., 2008; Bienenstock et al., 1935; Feillet and Roulland, 1998; Plaut et al., 1953; Rice and Ramstad, 1950; Smith et al., 2010; Wang et al., 2001). Some of the mentioned authors have reported that hydrated caroubin (carob germ protein) is capable of forming a network, though weaker than the gluten one. Feillet and Roulland (1998) observed that carob germ protein amino acid content differed from gluten, showing a lower cysteine content in carob germ protein compared to gluten. Smith et al. (2010) studied the composition and molecular weight distribution of carob germ proteins, reporting that, although they contain high molecular weight disulfide-bonded polymers, the lower molecular weight distribution of the polymeric proteins of carob germ flour in comparison with wheat flour may explain their different behaviour. Furthermore, these authors reported no measurable amounts of prolamin in carob germ flour. Hence, carob germ flour network forming ability would account for the different behaviour of carob germ batters in comparison with the other legume flours of this study.

Moreover, carob germ flour contains a high amount of fibre, as reported by Bengoechea et al. (2008), who measured 24% of total fibre when characterizing it. It is probable that carob germ flour contains remaining gums (galactomannan) from the endosperm, which could contribute to increase its batter elastic modulus. The rise in dynamic elastic modulus of gluten-free batters due to addition of hydrocolloids has been reported by Lazaridou et al. (2007) and actually, all gluten-free breads contain hydrocolloids to improve batter behaviour and baking characteristics. In carob germ batter, the effect of the possible remaining gum from carob germ flour could be added to the xanthan gum effect, the hydrocolloid shared by all batters of this study.

Gas production and retention was not significantly different in any of the formulations studied (data not shown), but carob germ batter showed the lowest ($p < 0.05$) batter height (55.10 ± 6.33 mm) after 3 h of development. This result agrees with the low volume of carob germ flour bread, which is discussed in 3.2. Heights of chickpea, pea and soya batters were similar (64.60 ± 1.28, 62.16 ± 4.14 and 66.63 ± 1.38 mm, respectively).

3.2. Bread evaluation

Baking characteristics of legume flour breads are shown in Table 2. No significant differences were observed in bake loss and water

activity values of the four breads analysed. Water activity and bake loss are important parameters related to bread quality because they are highly related with the firming process in starch-based systems. Specific volume of chickpea flour bread was the highest, while carob germ bread showed the lowest volume. These differences can also be perceived in the slice pictures (Fig. 2). Loaf specific volume is considered as one of the most important criteria in evaluating bread quality since it provides quantitative measurements of baking performance (Tronsmo et al., 2003). Boye et al. (2010) reported that, due to its specific content of amino acids, chickpea protein presents a higher foam expansion and stability values compared to pea and soya protein. This functionality could explain the higher volume of chickpea bread observed in our study. The presence of emulsifiers also affects bread volume (Gómez et al., 2004; Stampfli et al., 1996). Added proteins act as emulsifiers by forming a film or skin around oil droplets, preventing structural changes such as coalescence or creaming (Boye et al., 2010). In this study, the high volume of chickpea bread could be related to the good emulsifying stability index of chickpea protein, as reported by Paredes-López et al. (1991).

Plaut et al. (1953) observed a decrease in volume in breads which included carob germ, when they were compared with wheat bread. In the same way, Feillet and Roulland (1998) found a decrease in extensibility and swelling index when carob germ was added at 1% to wheat dough. In our study, 1.5% protein from carob germ flour resulted in a significant decrease of carob germ bread volume compared with chickpea, pea and soya breads. Although the reported ability of hydrated carob germ protein to form a network structure, the use of carob germ flour in our study did not result in a network able to expand as much as the other formulations, when proofed or baked. As discussed previously, Smith et al. (2010) reported that prolamins were not detected in carob germ protein. As the extensibility of the gluten network is attributed to the prolamin fraction, its lack in carob germ flour would account for the low volume of carob germ bread. Furthermore, volume impairment may also be due to an excess of hydrocolloid content due to residual gum content present in carob germ flour, as carob gum and xanthan gum can form a gel when heated (BeMiller, 2008), which would not be able to expand.

Crumb texture values are shown in Table 2. Carob germ bread had the highest values of hardness during the 5 days of storage, while chickpea bread presented the lowest ones. After 3 and 5 days of storage, the initial differences in hardness were maintained. Chickpea bread hardness correlates negatively with its loaf specific volume, agreeing with the observations of Gómez et al. (2008) and Sabanis and Tzia (2011) who also observed a negative correlation between crumb hardness and loaf volume. Some authors (Moore et al., 2004; Sanchez and Osella, 2002) have reported an improvement in gluten-free breads when soya was added to starch based formulations. They found that soya inclusion enhanced the crumb, bread volume, absorption properties and in general overall bread score. In our study, the greatest softening effect was obtained when chickpea flour was added, with soya bread being the second softest formulation. Cohesiveness, a parameter negatively correlated to the rate of breakdown in the mouth and easy separation in the hand, decreased significantly in all breads throughout the 5 days of storage. Carob germ bread was the most cohesive during the five days of the study. Differences in springiness were only observed at day 5. Soya bread showed the highest springiness value, which was significantly different from chickpea bread, with the lowest value.

Crumb colour differences between formulations were larger than crust colour changes. As the crumb does not reach temperatures as high as the crust, the Maillard reaction and caramelisation do not occur. For this reason, crumb colour was more affected by the colour of the protein source added and hence, carob germ bread showed the darkest crumb, while pea bread crumb was the lightest (L^* = 77.40 ± 6.82 and L^* = 73.52 ± 5.44 respectively). The yellowish effect of carob germ also rendered negative a^* values in the crumb of the corresponding bread (results not shown).

3.3. Sensory analysis

Sensory evaluation of the fresh bread was performed by regular bread consumers using a hedonic scale of nine points for different attributes (Table 3). General appearance of gluten-free formulations was acceptable except for the carob germ formulation. The highest score for general appearance was obtained by soya bread. Regarding crumb porosity, carob germ formulation was marked as the less open (or with the more compact structure), probably affected the general appearance and consumer preference. This

Table 2
Baking characteristics and texture parameters of legume flour gluten-free breads.

Baking characteristics	Chickpea flour	Pea isolate	Soya flour	Carob germ flour
Bake loss[a]	$11.78^{a} \pm 1.45$	$11.63^{a} \pm 1.24$	$11.31^{a} \pm 1.23$	$12.06^{a} \pm 1.41$
Specific volume[b]	$3.26^{a} \pm 0.09$	$2.77^{b} \pm 0.08$	$2.76^{b} \pm 0.08$	$2.51^{c} \pm 0.09$
Water activity	$0.98^{a} \pm 0.00$	$0.98^{a} \pm 0.00$	$0.98^{a} \pm 0.00$	$0.98^{a} \pm 0.00$
Hardness[c]				
Day 1	$475.7^{c,z} \pm 105.8$	$574.6^{b,z} \pm 103.4$	$569.8^{b,z} \pm 78.5$	$694.8^{a,z} \pm 99.6$
Day 3	$1187.9^{c,y} \pm 196.7$	$1589.7^{b,y} \pm 287.1$	$1498.1^{b,y} \pm 226.5$	$1972.7^{a,y} \pm 316.7$
Day 5	$1520.5^{c,x} \pm 271.2$	$1859.7^{b,x} \pm 244.3$	$1961.2^{b,x} \pm 466.8$	$2459.2^{a,x} \pm 407.3$
Cohesiveness				
Day 1	$0.55^{b,x} \pm 0.02$	$0.55^{b,x} \pm 0.01$	$0.56^{ab,x} \pm 0.01$	$0.57^{a,x} \pm 0.01$
Day 3	$0.30^{c,y} \pm 0.02$	$0.32^{bc,y} \pm 0.01$	$0.33^{ab,y} \pm 0.03$	$0.34^{a,y} \pm 0.02$
Day 5	$0.27^{b,z} \pm 0.02$	$0.28^{b,z} \pm 0.01$	$0.28^{b,z} \pm 0.01$	$0.30^{a,z} \pm 0.01$
Springiness				
Day 1	$0.91^{a,x} \pm 0.16$	$0.95^{a,y} \pm 0.01$	$0.94^{a,x} \pm 0.03$	$0.93^{a,x} \pm 0.04$
Day 3	$0.94^{a,x} \pm 0.03$	$0.97^{a,x} \pm 0.03$	$0.92^{a,x} \pm 0.12$	$0.94^{a,x} \pm 0.10$
Day 5	$0.92^{bc,x} \pm 0.09$	$0.95^{ab,xy} \pm 0.05$	$0.97^{a,x} \pm 0.04$	$0.96^{ab,x} \pm 0.06$

[a–c]Values labelled with a different letter in the same row are significantly different ($P < 0.05$).
[x–z]Values labelled with a different letter in the same column are significantly different ($P < 0.05$).
[a] %.
[b] cm^3/g.
[c] g.

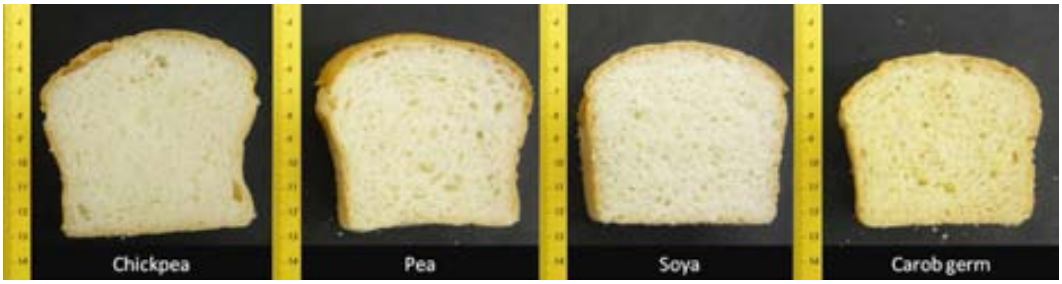

Fig. 2. Digital images of legume flour gluten-free breads.

compact structure also influenced sensory and instrumental hardness values. Despite chickpea bread receiving the highest specific volume values, consumers identified its crumb porosity as being lower than soya and pea bread. However, chickpea, pea isolate and soya bread crumb porosities were equally accepted by panellists. Consumers found significant differences in hardness by mouth between chickpea and the rest of the breads, which was clearly detected as being the softest, although no differences were detected in acceptability values of this parameter. Differences in colour were just noticed in the crumb, where carob germ bread was identified as the more yellowish one, and pea bread as the whitest one (agreeing with the CIE L^*, a^* and b^* values obtained). The yellowish colour of carob germ bread crumb received the lowest acceptability value. This bread also received the lowest score in chewiness, together with pea bread. No significant differences were found in bread flavour and taste scores.

The crumb colour, hard texture and compact structure, as well as general appearance scores seem to be the reasons that lead consumers to indicate carob germ bread as the least preferred (6.06%). Chickpea and soya bread were, in general, the best evaluated in terms of acceptance. Chickpea bread, despite its outstanding softness, was only chosen as preferred by 16.66% of panellists. Soya and pea breads, although not standing out in individual parameters, were the most preferred, being chosen by 54.54% and 21.21% of panellists, respectively. These results could be explained taking into account consumers' comments, which attributed a neutral taste to soya bread, while chickpea taste was identified by some of them.

3.4. Confocal scanning laser microscopy (CSLM)

To gain a deeper insight into the effect of different protein sources, CLSM was used to investigate the microstructure of bread crumb (Fig. 3). Either due to the low concentration of protein in bread or the lack of network formation, protein cannot be observed in the images. Microscopy of carob germ breads showed a compact structure without spaces between starch granules that agrees with the physico-chemical results obtained. The soya formulation showed a dispersed structure of granules, while chickpea and pea breads showed a more homogeneous structure. These formulations resulted in an open structure able to incorporate gas, which accounts for their volume and textural characteristics.

Table 3
Sensory attributes of legume flour gluten-free breads.

Sensory atributes	Chickpea flour intensity[a]	acceptability[b]	Pea isolate intensity	acceptability	Soya flour intensity	acceptability	Carob germ flour intensity	acceptability
General Appearance		$6.41^{x} \pm 1.65$		$6.34^{x} \pm 1.51$		$6.52^{x} \pm 1.38$		$4.30^{y} \pm 2.05$
Crumb porosity	$4.30^{b} \pm 1.68$	$6.50^{x} \pm 1.68$	$5.61^{a} \pm 2.03$	$6.15^{xy} \pm 1.66$	$5.36^{a} \pm 1.86$	$6.17^{xy} \pm 1.29$	$3.55^{c} \pm 2.05$	$5.59^{y} \pm 2.13$
Crumb color	$3.83^{b} \pm 1.64$	$6.63^{x} \pm 1.50$	$2.81^{c} \pm 1.34$	$6.63^{x} \pm 1.51$	$3.42^{b} \pm 1.30$	$6.86^{x} \pm 1.17$	$8.37^{a} \pm 1.14$	$3.59^{y} \pm 2.35$
Crust color	$6.01^{a} \pm 1.62$	$6.47^{x} \pm 1.43$	$6.04^{a} \pm 1.68$	$6.87^{x} \pm 1.23$	$6.48^{a} \pm 1.44$	$6.76^{x} \pm 1.32$	$6.69^{a} \pm 1.49$	$6.41^{x} \pm 1.59$
Hardness (by hand)	$3.61^{c} \pm 1.84$	$6.33^{x} \pm 1.59$	$5.26^{a} \pm 2.12$	$5.58^{y} \pm 1.61$	$4.12^{bc} \pm 1.93$	$6.25^{xy} \pm 1.60$	$4.80^{ab} \pm 2.07$	$5.78^{xy} \pm 1.51$
Hardness (mouth)	$2.78^{b} \pm 1.67$	$5.96^{x} \pm 1.86$	$4.69^{a} \pm 1.87$	$5.87^{x} \pm 1.44$	$4.37^{a} \pm 2.05$	$6.34^{x} \pm 1.55$	$5.11^{a} \pm 2.04$	$5.53^{x} \pm 1.80$
Chewiness	$2.90^{b} \pm 1.59$	$5.80^{x} \pm 2.00$	$4.16^{a} \pm 1.94$	$5.43^{y} \pm 1.51$	$3.76^{a} \pm 1.60$	$6.00^{x} \pm 1.61$	$3.96^{a} \pm 2.07$	$5.59^{y} \pm 1.66$
Flavour	$5.53^{a} \pm 1.63$	$5.93^{x} \pm 1.86$	$4.69^{b} \pm 1.81$	$5.33^{y} \pm 1.60$	$5.44^{a} \pm 1.69$	$5.89^{x} \pm 1.45$	$5.35^{a} \pm 1.37$	$5.56^{x} \pm 1.73$
Taste	$5.63^{a} \pm 1.36$	$4.66^{y} \pm 1.96$	$5.18^{a} \pm 1.48$	$5.33^{y} \pm 1.79$	$5.15^{a} \pm 1.40$	$6.50^{y} \pm 1.53$	$5.53^{a} \pm 1.37$	$5.29^{y} \pm 1.62$

[a–c] Values of intensity labelled with a different letter in the same row are significantly different ($P < 0.05$).
[x–y] Values of acceptability labelled with a different letter in the same row are significantly different ($P < 0.05$).
[a] Intensity descriptors: crumb porosity (many cells, few cells); color (less yellowish, more yellowish); hardness (low, high); chewiness (easy to chew, difficult to chew); flavour (low intensity, high intensity); taste (less salty, more salty).
[b] Acceptability descriptors: left of the scale "dislike extremely"; right of the scale "like extremely".

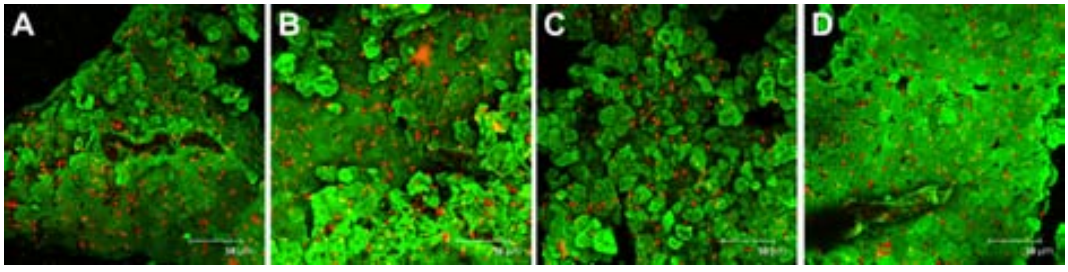

Fig. 3. Confocal scanning laser microscopy images of legume flour gluten-free breads (Chickpea flour (a); Pea isolate (b); Soya flour (c); Carob germ flour (d)).

3.5. Microbiological analysis

Microbiological analysis of legume flour breads was performed at days 0, 5 and 7 of storage. There were no differences ($P > 0.05$) in mesophilic aerobic bacteria, *Enterobacteriaceae*, spores or yeast and moulds counts of the four formulations. Mesophilic aerobic bacteria were between 2.41 ± 1.70 and 2.88 ± 1.51 at day 1. At day 7, a slight increase could be observed, counts being between 3.94 ± 1.82 and 4.95 ± 2.27. *Enterobacteriaceae* were not detected in any sample during the three points of study. Bacterial spore counts were between 1.03 ± 0.07 and 1.86 ± 0.98 at day 1 and reached counts between 2.24 ± 1.24 and 2.83 ± 1.32 at day 7. It is known that bacterial spoilage of bread is mainly caused by *Bacillus* spp, which is usually present in raw ingredients (e.g., flour, sugar and yeast). It survives the baking process, germinates upon cooling, grows under both aerobic and anaerobic packaging conditions and causes an alteration known as ropiness, characterized by a discolored bread crumb, due to the protein degradation during growth of the bacteria. Ropiness was not detected at day 7, when counts were below 3 log CFU/g of spores, but it could be observed in breads stored for 10 days, which were not analysed. Yeast and mould counts went from *ca.* 1.2–1.8 log CFU/g at day 1 to *ca.* 2.5–3.0 log CFU/g at day 7. Fresh baked bread is free of moulds and their spores, due to the thermal inactivation during baking. Hence, baked bread must have become contaminated during cooling and the packaging process. It is estimated that 1 g of flour may contain 8000 mould spores (Cauvain, 2003), so weighing and mixing of ingredients may have helped to increase mould contamination in the processing room, the same as where breads were cooled.

4. Conclusions

Breads with legume flours showed good physico-chemical characteristics and an adequate sensory profile. Carob germ flour generated batters with good rheological properties, however, its breads generally presented poor characteristics. Chickpea flour and pea isolate breads obtained good results in all parameters studied indicating that these ingredients could be a promising alternative to soya flour. Further studies should be done combining protein sources to optimize gluten-free legume formulations, integrating the good baking characteristics and sensory profile provided by chickpea and soya flours.

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Paper 3

3.3 Liquid whey as an ingredient to formulate gluten-free bread

B. Miñarro, E. Albanell, N. Aguilar, B. Guamis, M. Capellas

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Liquid whey as an ingredient to formulate gluten-free bread

Begoña Miñarro [a], Elena Albanell [b], Núria Aguilar [a], Buenaventura Guamis [a], Marta Capellas[a,*]

[a]*Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), CeRTA, XiT, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.*

[b]*Grup de Recerca en Remugants (G2R), Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.*

***Corresponding author: Tel: + 34 935811446; fax: +34935811397**

E-mail address: marta.capellas@uab.cat (M. Capellas)

Abstract

Three main types of gluten-free corn starch based formulations, according to the liquid ingredient used, were compared: formulations with water, fresh whey and ripened whey. Within each type, three soya levels were evaluated: 0, 2.5 and 5 %.

Formulations with the highest protein content (whey and soya) obtained the more visco-elastic batter structure compared with the other batters studied. The increase in visco-elastic would be mainly related to the water holding ability of whey proteins. Bake loss results were also influenced by whey addition and, in general, ripened whey addition caused less bake loss than fresh whey. Specific volume of bread was affected by soya and whey presence. Bread formulated with ripened whey and 5 % of soya was the one with the most compact structure and lowest volume, while formulation with 0 % of soya and water obtained the highest volume and the softest texture values. Whey addition clearly influenced color, causing the darkening of bread crust. Formulations with water or ripened whey but without soya were the most preferred in sensory test, probably due to the softest texture and color. No significant differences in microbiological counts were observed between the nine formulations studied.

Key words Soya flour · Gluten-free bread · Whey · Dairy ingredients

Introduction

Celiac disease has become a well known food disorder affecting almost 1 % of the population (Fasano and Catassi 2001). The increasing number of celiac patients being diagnosed thanks to more accurate detection methods and, also, the increasing intake of gluten free products by patients who perceive that a gluten-free diet can help in the treatment of other diseases, have led to a big increase in consumption of gluten-free products in the last few years (Sapone et al. 2012).

Gluten in wheat dough is a plastic-elastic substance consisting of gliadin and glutenin. Both proteins together give to wheat dough the elastic and extensible properties able to develop good quality bakery products (Gallagher et al. 2003a). Its lack in gluten-free breads has led technologists to search for ingredients and technologies able to mimic its unique properties. Many studies have been carried out, mainly involving the incorporation of starches, different sources of proteins and hydrocolloids into a gluten-free flour base, which results in improved structure or mouthfeel (Gallagher et al. 2003b; Lazaridou et al. 2007; Nunes et al. 2009a). However, gluten free products still experience big difficulties regarding flavor, color and staling.

Our research group has developed a formulation which contains soya (*Glycine max* L.) protein and offers good baking and sensory characteristics (Miñarro et al. 2010; Miñarro et al. 2012). However, soya associated digestive problems and its high allergenicity, are leading to more research into alternative protein sources, which should be able to improve gas-holding capacity and bake development.

Dairy products have been commonly used in the bakery industry for their nutritional and functional properties. Nutritional properties include protein and calcium content, and among functional properties, the improvement of crust color, bread flavor and crumb structure seem to be the most relevant (Erdogdu-Arnoczky et al. 1996;

Kenny et al. 2000; Secchi et al. 2011; Yousif et al. 1998). Whey protein foams well, creating and stabilizing air bubbles in a liquid and also stabilizing emulsions by forming interfacial films between hydrophobic and hydrophilic food components (Renner and Abd El-Salam 1991). Dairy ingredients have been added in bakery goods in multiple forms such as, milk, milk powder, whey protein isolates or concentrates, or liquid whey.

Liquid whey is the greenish translucent liquid obtained from milk after precipitation of casein during cheese making and, depending on the cheese making process, two main types of whey can be obtained: sweet or fresh whey, obtained after rennet coagulation, with pH values between 6.5 - 6.0; and acid or ripened whey, obtained after cheese making procedures that incorporate lactic acid bacteria, with pH values ranging from 4.5 to 5.5. Until now, liquid whey, has been viewed as one of the major problems of dairy industry. Although big dairy industries process whey for multiple purposes, a high number of small and medium cheese Spanish companies do not transform the whey they generate. Direct utilization of whey could be a good alternative to increase its value and reduce its environmental cost management.

The aim of this research was to study the characteristics of gluten-free bread formulated with fresh or ripened whey instead of water, and the replacement of soya flour from the standard formulation by whey.

Materials and methods

Raw materials

Ingredients used for bread formulations were: corn starch (Syral Iberia SAU, Zaragoza, Spain), soya flour (Trades, S.A, Barcelona, Spain), white sugar (Azucarera Ebro S.L., Madrid, Spain), iodized refined salt (Sal Costa S.A., Barcelona), baking powder (Panreac Química S.A., Barcelona), shortening (Puratos, Barcelona), xanthan gum

(Degussa Texturant Systems, Paris, France), baker´s yeast (Mercadona, Tavernes Blanques, Spain), esters of mono and diglycerides of fatty acids as emulsifiers (Degussa Texturant Systems, Barcelona), tap water, fresh and ripened whey from Formatgeries Mogent (Cardedeu, Barcelona), both obtained from goat's milk cheese production.

Ripened whey had a protein content of 1.23 ± 0.04 %, fat content of 0.20 ± 0.14 % and pH of 5.5 ± 0.5. Fresh whey had a protein content of 1.07 ± 0.03 %, fat content of 0.76 ± 0.11 % and pH of 6.5 ± 0.5.

Baking procedure

Ingredients for each recipe and their concentrations are listed in Table 1. Ingredients were weighed and mixed (Sammie Goo, Gipuzkoa, Spain), adding first the dry ingredients, followed by yeast, shortening, and water or whey at room temperature 20 ± 1 ºC. Kneading was performed for 1 min at low speed (level 1 out of 5) and 3.5 min at high speed (level 3 out of 5). After homogenization, twelve tins (5 x 8 x 14 cm) of each recipe, each one containing 200 g of batter, were proofed at 28 ºC and 85% RH for 45 min. Baking process was carried out in a convection rotational oven (Sveda Dahlen, Fristad, Sweden) at 170 ºC for 20 min. Breads were cooled at room temperature for 2 hours. Bread samples were packed in a modified atmosphere of 50 % N_2 and 50 % CO_2 and stored for 5 days. Twelve loaves were obtained from each formulation. Three independent batches of the nine formulations were elaborated for this study.

Fundamental rheology

For rheological measurements, batters were prepared as baking procedure adding the ingredients at 25 ºC without yeast addition. A controlled stress and strain rheometer Haake Rheo Stress 1 (Thermo Electron Corporation, Karlsruhe, Germany) was used.

Samples were examined using a parallel plate system, which consisted of a 35 mm diameter corrugated probe. A circulating Haake SC100 bath (Thermo Electron Corporation) with an accuracy of ± 0.1 ºC was used to set the system at 30 ºC. When thermal equilibrium was set, the batter sample was loaded onto the corrugated plate and the probe was lowered to a gap of 2 mm. After removing excess batter, the exposed edges were covered with water to avoid desiccation. A rest period of 5 min was allowed to enable the sample to recover from the stress due to preparation. To ensure that all measurements were carried out within the linear visco-elastic region, amplitude sweeps were performed in the range of strain 0.01-10 %. Based on these results, oscillation stresses were selected. Frequency sweep tests were performed at frequencies between 0.1 and 10 Hz with a target strain of 5×10^{-4} (0.05 %). Rheological properties, such as storage modulus (G') and loss modulus (G''), were calculated using the manufacturer software (Rheoplus, Anton Paar, Germany). All measurements were performed at least in triplicate.

Table 1 Ingredients (g) used in the gluten-free bread formulations and baking characteristics of the gluten-free breads studied.

Ingredient (g)	5W	2.5W	0W	5R	2.5R	0R	5F	2.5F	0F
Corn starch	1165.8	1195.9	1226	1165.8	1195.9	1226	1165.8	1195.9	1226
Soya flour	60.2	30.1	-	60.2	30.1	-	60.2	30.1	-
Water	1260	1260	1260	-	-	-	-	-	-
Fresh whey	-	-	-	-	-	-	1260	1260	1260
Ripened whey	-	-	-	1260	1260	1260	-	-	-

Common ingredients (g): sugar, 70; baking powder, 30; baker's yeast, 60; salt, 30; shortening, 60; xanthan gum, 24; emulsifier, 24.

Bread analysis

Baking evaluation

Loaf volume was measured by the method of displacement of millet seeds. Specific volume was calculated using the formula: specific volume (cm^3/g) = volume / weight. Initial batter weight and weight of bread after cooling were measured and bake loss was calculated using the formula: Bake loss (%) = (initial weight of batter - weight of bread after cooling) x 100 / initial weight of batter.

Texture profile analysis of the crumb was performed on three slices taken from the center of each loaf. The slices were obtained by cutting the bread transversely using a cutter to obtain uniform slices of 12.5 mm thickness. Two slices of 12.5 mm were stacked together to obtain a uniform slice of 25 mm. Texture profile analysis was carried out using a TA-TX2 texture analyser (Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell and a 35 mm diameter aluminum cylindrical probe. Probe speed was set to 1.7 mm/s to compress the center of the bread crumb to 40 % of its original height. Time between compressions was 5 s. Parameters studied were: hardness (peak force of the first compression), cohesiveness (area of work during the second compression divided by the area of work during the first compression) and springiness (spring back of the product after it has been deformed during the first compression, measured at the down stroke of the second compression).

Crust and crumb color were measured with a Hunter Lab colorimeter miniScan XTE (Hunter Associates Laboratory INC, Reston, Virginia, USA). CIE L*, a* and b* values were measured with an illuminant of D65 and a standard observer of 10º. L* represents the lightness, with values from 0 (black) to 100 (white), which indicates a perfect reflecting diffuser. Chromatic components are represented by *a** and *b** axes. Positive values of *a** are red and negative values are green, whereas positive values of

*b** are yellow and negative values are blue. Crust color was measured on four different zones of the top of the whole bread. Crumb color was measured on four equidistant points to the center of each slice.

Sensory evaluation

As nine samples were too many samples to be evaluated at a time, consumer tests were carried out in four steps. Three individual consumer tests with 30 regular bread consumers recruited among university staff and postgraduate students used to participate in sensory analyses of different foods were carried out with three blocks of samples. First block included breads with fresh whey: 5F, 2.5F and 0F; second block included breads made with water: 5W, 2.5W and 0W; and third block included breads made with ripened whey: 5R, 2.5R and 0R. With the most preferred sample obtained in the previous sensory tests (5F, 0Wand 0R) a final consumer test was performed with 52 regular bread consumers (30 females and 22 males between 20 and 56 years old).

Each consumer test was performed in the same way. One day after preparation, samples were sliced and placed in recipients randomly codified with three digits. A slice of each formulation was given to every consumer, and a sample of the whole bread of each formulation was shown to evaluate the external appearance. Consumers evaluated different bread attributes using an intensity and an acceptability scale of 9 points. Finally, subjects were asked to indicate the most and the least preferred sample (results are expressed in % of preference).

Microbiological analysis

To count aerobic mesophilic bacteria, 1 ml of decimal dilutions of sample homogenized in peptone water (Oxoid) was pour plated on Plate Count Agar medium (PCA; Oxoid, Basingstoke, Hampshire, UK) and incubated for 48 h at 30 ºC. To assess spore counts, decimal dilutions were subjected to heat shock at 80 ºC for 10 min and pour plated in PCA. Yeasts and moulds were counted on Sabouraud with cloranfenicol (Oxoid) after incubation at 30 ºC for 5 days. Microbiological analyses were performed on three batches of each bread at day 1, 3 and 5 of storage.

Statistical analysis

Data were processed by one-way ANOVA (Statgraphics Inc., Chicago, IL, USA). LSD test was used for comparison of sample data, and evaluations were based on a significance level of $P<0.05$.

Results and Discussion

Batter evaluation

Frequency sweep curves of gluten-free batters are shown in Fig. 1. Frequency sweep shows changes in viscous and elastic behavior with the rate of application of strain or stress, while the amplitude of the signal is held constant. For all the samples, values of G' were greater than G'' (data not shown), with the batters displaying viscoelastic-like behavior under the test conditions.

Formulations that contained the highest % of soya and ripened or fresh whey (5R and 5F), had the highest G' values, exhibiting the most marked viscoelastic behavior. In contrast, 0W formulation, without soya flour and water, showed the most viscous behavior, with the lowest values of G'. The rest of the formulations remained between

the two extreme groups. Thus, addition of whey had a marked effect on rheological properties of batters when combined with soya, as shown by the high G' values of 5R and 5F compared with 5W. However, no significant differences were found when soya was partially or completely removed from whey-containing formulations.

The water holding capabilities of whey and soya proteins, which are extensively reported, (Bilgin et al. 2006; Erdogdu-Arnoczky et al. 1996; Roccia et al. 2009) could be responsible for the increase in batter viscoelasticity of samples containing both ingredients. Moreover, the higher solid content present in whey formulations due to whey solids can also contribute to the increase in elastic modulus. The increase in elastic modulus in gluten-free batters is desirable, as it would contribute to improve batter handling. Some studies have reported the strong effect of whey addition in wheat bread, increasing dough development times and stability (Indrani et al. 2007).

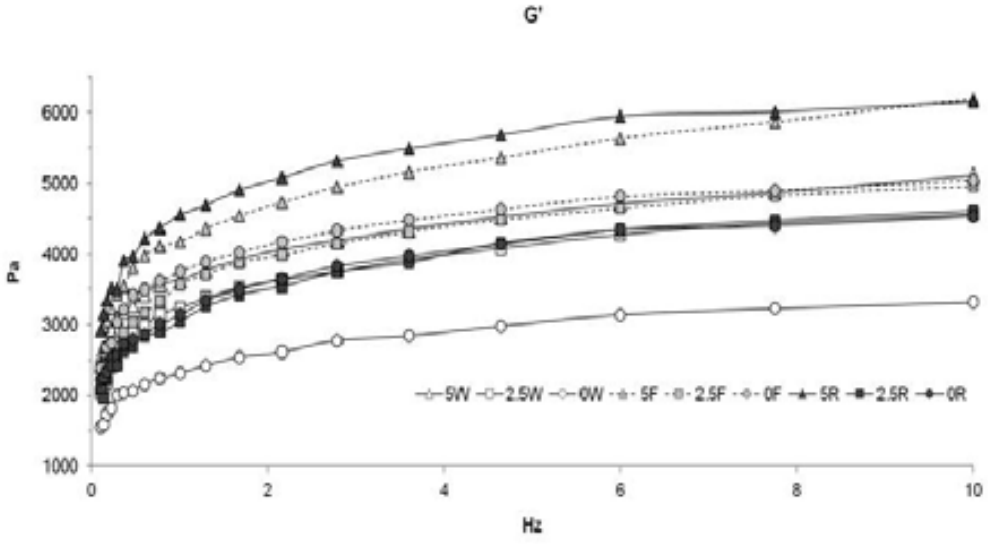

Fig.1 Rheological properties of gluten-free batters

5W: 5 % soya flour and water; 2.5W: 2.5 % soya flour and water; 0W: 0 % soya flour and water; 5R: 5 % soya flour and ripened whey; 2.5R: 2.5 % soya flour and ripened whey; 0R: 0 % soya flour and ripened whey; 5F: 5 % soya flour and fresh whey; 2.5F: 2.5 % soya flour and fresh whey; 0F: 0 % soya flour and fresh whey.

Bread evaluation

Baking characteristics are reported in Table 1. The addition of whey had a significant effect on bake loss, which decreased when fresh or ripened whey were used to substitute water. In general, ripened whey addition caused a higher decrease in bake loss than fresh whey. Soya addition did not affect bake loss values, except in breads with ripened whey. Although no interaction effects between whey and soya addition were observed, breads containing the highest quantity of protein (5 % of soya and ripened whey) showed the lowest bake loss values, while breads made with water showed the highest. In summary, the reported holding ability of whey protein mostly explains bake loss differences between our breads. Divya and Rao (2010), also observed an increase in bake loss after whey incorporation in a wheat bread formulation.

Specific volume was significantly affected ($P<0.05$) by the interaction between soya flour and whey addition (Fig. 2 and Table 2). The combined effect of both ingredients promoted a decrease in bread specific volume, probably caused by a crumb structure modification due to the partial starch substitution, indicating a dilution of the gel formed by starch-gum system as suggested by Sanchez et al. (2004).

A marked effect depending on soya concentration in specific volume was observed comparing 5W, 2.5W and 0W formulations, whose specific volume decreased as soya concentration increased. Whey addition also caused a decrease in specific volume, which can be clearly observed comparing breads without soya (0R; 0F; 0W), as interactions between both ingredients were significant. In wheat breads, Ryan and Brewer (2007) suggested that soya protein was competing for water, not only with gluten proteins, but also with starch. In our breads, in which soya is added to a 2.5-5 %, soya and starch competition for water could also explain the low specific volume values of breads that contain soya. Crockett et al. (2001), found similar results, when adding a

3 % of soya protein into gluten-free breads that also contained hydrocolloids, as the formulations used in our study**.** In contrast, Sciarini et al. (2010), found an increase in specific volume when 10-20 % of soya flour was added to corn flour, in a formulation without other hydrocolloids. These results support the hypothesis that, in complex formulations, soya may interfere with the starch-gum system, rendering breads with low specific volume.

Table 2 Baking characteristics of different gluten-free breads

Formulation	Bake loss (%)	Specific volume (cm^3/g)	Water activity
5W	11.03 ± 1.03^{a}	2.56 ± 0.23^{cd}	0.972 ± 0.005^{a}
2.5W	11.08 ± 0.94^{a}	2.76 ± 0.22^{b}	0.969 ± 0.003^{a}
0W	11.20 ± 0.82^{a}	3.10 ± 0.25^{a}	0.978 ± 0.002^{a}
5F	10.16 ± 0.56^{bc}	2.70 ± 0.14^{cb}	0.969 ± 0.002^{a}
2.5F	10.33 ± 0.67^{b}	2.73 ± 0.19^{cb}	0.968 ± 0.003^{a}
0F	10.23 ± 0.46^{b}	2.77 ± 0.27^{b}	0.978 ± 0.002^{a}
5R	9.45 ± 0.74^{d}	2.41 ± 0.15^{d}	0.974 ± 0.003^{a}
2.5R	10.15 ± 0.57^{bc}	2.61 ± 0.14^{cb}	0.972 ± 0.002^{a}
0R	9.82 ± 0.62^{c}	2.70 ± 0.17^{cb}	0.973 ± 0.002^{a}

5W: 5 % soya flour and water; 2.5W: 2.5 % soya flour and water; 0W: 0 % soya flour and water; 5R: 5 % soya flour and ripened whey; 2.5R: 2.5 % soya flour and ripened whey; 0R: 0 % soya flour and ripened whey; 5F: 5 % soya flour and fresh whey; 2.5F: 2.5 % soya flour and fresh whey; 0F: 0 % soya flour and fresh whey.

$^{a-d}$Values labeled with a different letter in the same column are significantly different ($P<0.05$).

The use of whey or whey proteins in wheat and gluten-free breads results in different effects on bread volume depending on the formulation and type of whey proteins. Recently, Mezaize et al. (2009), observed a significant decrease in bread volume when whey was added at 5 % in a gluten-free formulation. They suggested that proteose-peptone components present in non-heat-treated whey could act as a loaf volume depressant. Oppositely, Nunes et al. (2009b) found that the addition of different whey protein powders (10 %) significantly increased the specific volume of gluten-free bread. In wheat bread, Divya and Rao (2010), found an increase in loaf volume when concentrated whey with 15% or 26% total solids was added. Similar results were also reported by Bishal et al. (1979) and Kadharmestan et al. (1998). Contradictory results were observed by other authors (Gelinas and Lachance 1995; Kenny et al. 2000; Yousif et al. 1998), who reported a decrease in wheat bread loaf volume due to the addition of whey proteins. Although some authors found a significant decrease in water activity due to dairy ingredients addition (Gallagher et al. 2005; Secchi et al. 2011), no significant differences in this parameter were found in our study.

Bread texture is one of the main attributes concerning bakery products, being decisive in consumer choice. A significant interaction between soya and whey inclusion was observed when evaluating hardness values within the 5 days of storage (Fig. 3). At day 1, the increase in soya flour amount caused a significant increase in bread hardness. With regards to whey, only ripened whey addition caused an increase in bread hardness ($P<0.05$). Whey protein and fat content could explain the different results obtained when applying fresh and ripened whey. In general, hardness results agree with specific volume results, as formulations with ripened whey obtained lower volumes and, consequently, higher firmness than fresh whey formulations.

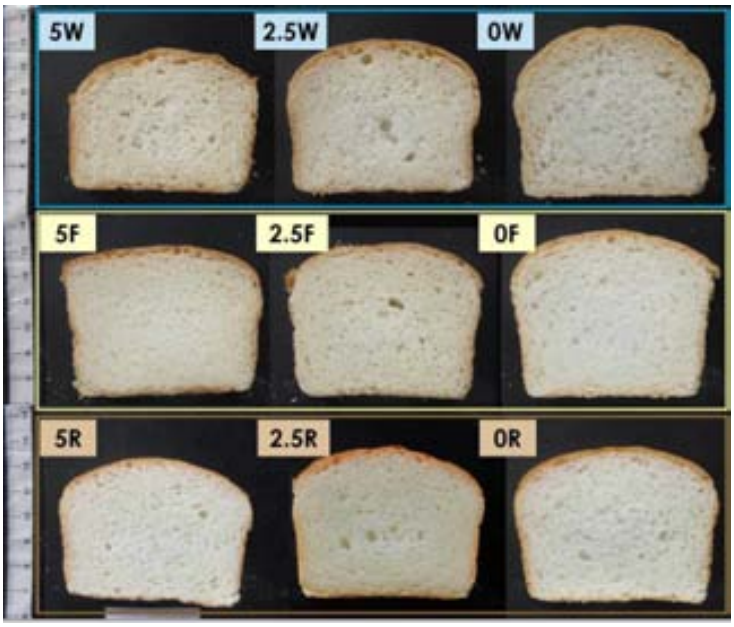

Fig.2 Digital images of gluten-free breads

5W: 5 % soya flour and water; 2.5W: 2.5 % soya flour and water; 0W: 0 % soya flour and water; 5R: 5 % soya flour and ripened whey; 2.5R: 2.5 % soya flour and ripened whey; 0R: 0 % soya flour and ripened whey; 5F: 5 % soya flour and fresh whey; 2.5F: 2.5 % soya flour and fresh whey; 0F: 0 % soya flour and fresh whey.

Over the 5 days of storage, 0W formulation continued being the softest one (Fig. 3). Though results showed that bread firmness increased with soya content, the staling rate (data not shown) was lower in all cases when soya was present. The anti-staling effect of soya can be explained because of the high water holding capacity of soy proteins and because they can interfere in starch retrogradation, as suggested by Ryan et al. (2002), who stated that hydrated soy proteins interact strongly with starch, hindering amylopectin recrystallization during storage and consequently retarding bread staling. Moore et al. (2004) and Sanchez et al. (2002), also found a positive impact in bread crumb texture when soya was added in the formulations. With regard to whey introduction, previous research indicates that the addition of heat-treated dairy

ingredients slowed bread staling and extended its keeping quality (Bilgin et al. 2006). However, contradictory results have been found by other authors who suggest an increase in firmness when dairy ingredients are added to bread formulation (Gallagher et al. 2005; Mezaize et al. 2009).

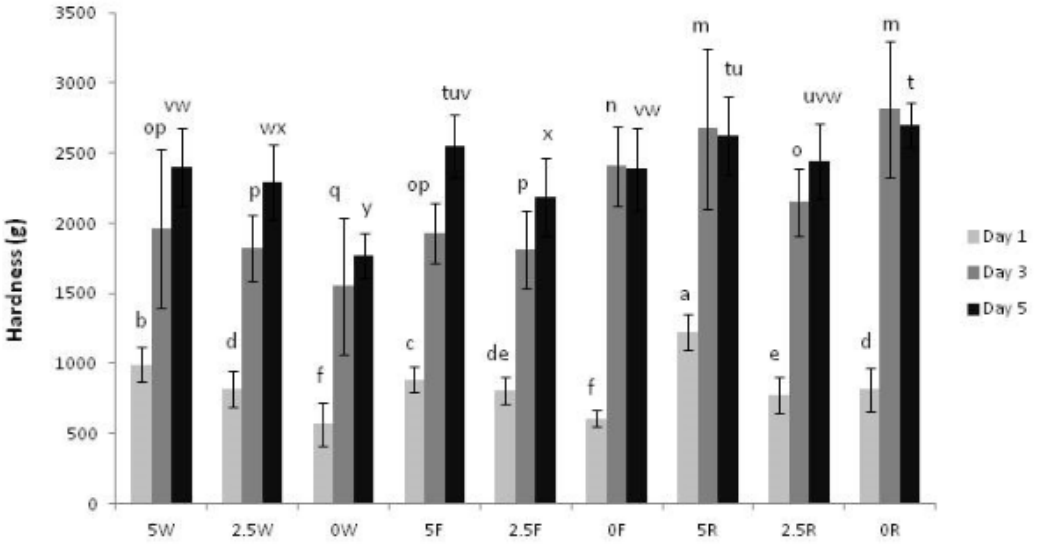

Fig. 3 Hardness (g) of different gluten-free breads

[a-f, m-q, t-z]Values labeled with a different letter in the same day are significantly different ($P<0.05$). 5W: 5 % soya flour and water; 2.5W: 2.5 % soya flour and water; 0W: 0 % soya flour and water; 5R: 5 % soya flour and ripened whey; 2.5R: 2.5 % soya flour and ripened whey; 0R: 0 % soya flour and ripened whey; 5F: 5 % soya flour and fresh whey; 2.5F: 2.5 % soya flour and fresh whey; 0F: 0 % soya flour and fresh whey.

Crumb cohesiveness (data not shown) increased with soya flour amount reduction in the formulation, being significantly different at day 1 and 3 of storage. Breads without soya that included fresh or ripened whey were the most cohesive ones at days 1 and 3. Little differences were found between formulations at day 5. Bread springiness was not significantly affected by whey or soya addition.

Table 3 shows bread crust L* values. Significant interactions were found between soya and whey affecting crust color. The 0W formulation was the lightest formulation because of soya and whey absence, while 2.5F and 5F formulations were the darkest. Whey addition, particularly fresh whey, induced a decrease in bread crust lightness, as well as soya presence due to the soya flour color itself. Darker crust in breads containing whey may be attributed to Maillard and caramelization reactions which occurred more intensively during baking due to higher lactose and protein content, as suggested by Bilgin et al. (2006). Lactose content in fresh whey is expected to be higher than in ripened whey, so this would also explain the differences within breads containing fresh or ripened whey.

Table 3 Crust color characteristics of different gluten-free breads

	L*	a*	b*
5W	55.67 ± 1.18^{a}	13.95 ± 2.10^{d}	38.93 ± 1.78^{cd}
2.5W	58.01 ± 1.56^{b}	13.65 ± 2.08^{d}	39.97 ± 1.85^{b}
0W	63.68 ± 1.48^{a}	12.17 ± 1.90^{e}	42.51 ± 2.10^{a}
5F	53.75 ± 1.98^{e}	18.27 ± 0.68^{bc}	36.85 ± 1.68^{e}
2.5F	52.37 ± 2.40^{f}	$19.05^{ab} \pm 0.68$	36.62 ± 1.69^{e}
0F	57.31 ± 1.73^{bc}	17.73 ± 0.41^{c}	39.52 ± 0.64^{bc}
5R	55.73 ± 2.19^{d}	17.84 ± 2.43^{c}	38.47 ± 1.32^{d}
2.5R	56.47 ± 1.64^{cd}	19.20 ± 1.04^{a}	39.67 ± 1.03^{bc}
0R	58.01 ± 1.84^{b}	18.03 ± 0.72^{bc}	40.45 ± 0.72^{b}

[a-f]Values labeled with a different letter in the same column are significantly different ($P<0.05$). 5W: 5 % soya flour and water; 2.5W: 2.5 % soya flour and water; 0W: 0 % soya flour and water; 5R: 5 % soya flour and ripened whey; 2.5R: 2.5 % soya flour and ripened whey; 0R: 0 % soya flour and ripened whey; 5F: 5 % soya flour and fresh whey; 2.5F: 2.5 % soya flour and fresh whey; 0F: 0 % soya flour and fresh whey.

Sensory analysis

Sensory evaluation of fresh bread was performed by regular bread consumers using a hedonic scale of nine points for different attributes (Table 4). General appearance of gluten-free formulations was acceptable for all formulations. The highest score for general appearance was obtained by 0R bread with a punctuation of 6.58.

Regarding crumb porosity, 5F formulation was identified as the least open (or with the most compact structure). This compact structure can be seen in Fig. 2, and also agrees with the low specific volume of 5F bread compared with 0W and 0R breads. Despite 0W bread obtaining the highest specific volume values, no significant differences were found in general appearance evaluated by consumers between 0W and 0R. Consumers found significant differences in hardness evaluated by hand and by mouth between the three samples. The 5F bread was identified as the hardest, followed by 0W, while 0R bread was distinguished as the softest.

Differences in crust and crumb color were noticed by consumers, which identified 0W bread as the whitest one, while 5F and 0R breads were identified as the most yellow in accordance with the CIE L* values obtained. The yellowish color of 5F and 0R bread crumb is due to whey addition and is also apparent in Fig. 2. Whey addition also affected bread taste and consumers identified 0W breads as sweeter than 0R and 5F breads.

Crumb color and texture, seemed to be the reasons that led consumers to choose 0R and 0W breads as the most preferred (41% and 39 % of acceptance, respectively) while 5F bread was only chosen as the most preferred by a 20 % of consumers.

Table 4 Sensory attributes of gluten-free breads.

Sensory atributes	0R (0% Soya/Ripenned whey)		0W (0% Soya/water)		5F (5% Soya/fresh whey)	
	Intensity*	Acceptability**	Intensity	Acceptability	Intensity	Acceptability
General appearance		5.90± 1.82^{z}		6.58 ± 1.56^{y}		6.38± 1.53^{yz}
Crumb porosity	4.44 ± 2.13^{b}	6.27 ± 1.65^{y}	4.74± 1.76^{b}	6.31± 1.65^{y}	5.98± 1.98^{a}	5.89 ± 1.74^{y}
Crumb color	3.90 ± 1.62^{b}	6.33 ±1.75^{yz}	5.41 ± 1.87^{a}	6.62± 1.25^{y}	3.72 ± 1.84^{b}	5.94 ± 1.53^{z}
Crust color	4.90 ± 1.68^{c}	5.78± 1.74^{z}	7.70± 0.98^{a}	6.68± 1.71^{y}	6.50± 1.36^{b}	6.64± 1.30^{y}
Hardness (hand)	4.37 ± 1.71^{c}	6.09± 1.47^{y}	5.08± 1.68^{b}	6.32± 1.53^{y}	6.44± 1.61^{a}	5.02± 1.83^{z}
Hardness (mouth)	3.44 ± 1.51^{c}	6.12± 1.89^{y}	4.18± 1.79^{b}	6.28± 1.63^{y}	5.66± 1.85^{a}	5.38± 1.68^{y}
Chewiness	3.77 ± 1.88^{b}	6.46± 1.75^{y}	3.88± 1.79^{b}	6.22± 1.64^{y}	5.0 ± 1.95^{b}	5.33± 1.53^{z}
Flavour	5.21± 1.76^{a}	5.76 ± 1.69^{y}	5.88± 1.76^{a}	5.92 ± 1.84^{y}	5.27± 2.05^{a}	5.29 ± 1.87^{y}
Taste	4.88± 1.29^{b}	5.41± 1.61^{y}	6.62±1.35^{a}	5.30± 1.78^{y}	4.71± 1.35^{b}	5.69± 1.73^{y}

$^{a-c}$Values of intensity labelled with a different letter in the same row are significantly different ($P<0.05$).
$^{y-z}$Values of acceptability labelled with a different letter in the same row are significantly different ($P<0.05$).
* Intensity descriptors: crumb porosity (open structure, compact structure); color (less yellowish, more yellowish); hardness (low, high); chewiness (easy to chew, difficult to chew); flavour (low intensity, high intensity); taste (less sweet, more sweet).**Acceptability descriptors: left of the scale “dislike extremely”; right of the scale “like extremely”.

Microbiology results

No differences were found between formulations tested within the five days of storage for the three populations studied. Mesophilic aerobic bacteria and yeast and moulds counts were around 2 logat day 1 and 3. However, at day 5, counts had reached about 5 log. Count of spores was initially low (0.32 ± 0.18,) and did not increase during storage. Although differences could be expected due to different pH of fresh whey, ripened whey and water, the final pH of the corresponding batters (5.54 ± 0.03; 5.53 ± 0.06; 5.66 ± 0.02, respectively) did not significantly differ. Soya flour presence did not influence bread microbiology.

Conclusions

Whey addition allows the production of gluten-free bread with good nutritional and technological properties. In general, breads containing fresh whey showed better baking and physico-chemical characteristics although breads containing ripened whey were the most preferred by consumers. The use of unprocessed cheese whey may help reducing the costs of gluten-free bread formulations and could also be a high quality soya protein substitute, while contributing to increase the value of this cheese by-product.

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Paper 4

3.4 Detection of low-level gluten content in flour and batter by near infrared reflectance spectroscopy (NIRS)

E. Albanell, B. Miñarro, N. Carrasco

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Detection of low-level gluten content in flour and batter by near infrared reflectance spectroscopy (NIRS)

Elena Albanell [a,*], Begoña Miñarro [b], Noemí Carrasco [c]

[a] Grup de Recerca en Remugants (G2R), Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, Campus Bellaterra, 08193 Bellaterra, Barcelona, Spain
[b] Centre Especial de Recerca Planta de Tecnologia dels Aliments (CERPTA), Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[c] NIRproFEED, Onze de Setembre 39, 43480 Vila-seca, Tarragona, Spain

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Dough

ABSTRACT

Near infrared spectroscopy (NIRS) has been used as a valuable tool for quality control in the food industry. The aim of the present study was to investigate the possibility of developing a NIRS calibration for gluten determination in flour and batter, suitable for the analysis of gluten-free food products. Reflectance data was used for calibration based on modified partial least squares (MPLS) regression. Independent prediction equations were developed for flour and for batter. Spectral models using mean spectra of two scans (average spectra), were compared with those using the two individual spectral data. The best model obtained for flour was using the average spectral data ($R^2 = 0.985$; $r^2 = 0.967$) and for batter samples was using the individual spectral data ($R^2 = 0.926$; $r^2 = 0.825$). It is concluded that the application of NIRS methodology can predict accurately the concentration of gluten content in flours and batters, but it should not be considered as a reliable method for determining gluten contamination in gluten-free products.

1. Introduction

Celiac disease is an autoimmune-mediated enteropathy triggered by the ingestion of gluten-containing grains, wheat, rye, oat and barley, in genetically susceptible people. The reaction to gluten ingestion by sufferers of celiac disease is an inflammation of the small intestine leading to the malabsorption of several important nutrients including iron, folic acid, calcium, and fat soluble vitamins (Feighery, 1999). The symptoms of celiac disease vary widely and the only way to prevent them is to exclude gluten-containing cereals from the diet (gluten-free diet).

Gluten is the main structure-forming protein in wheat flour, giving to dough its elastic and extensible properties. Due to gluten functionality, the formulation of gluten-free products is a big challenge (Gallagher et al., 2004). Gluten is present in food products containing cereals mentioned before; however, it may also be present in other food products where gluten is added as a texture agent or as a vegetable source of protein. Moreover, natural gluten free products may contain gluten, due to the cross contamination occurring during the primary production, harvesting and storage of grain and/or during the manufacture of gluten-free food.

Immunochemical analytical methods are currently used to determine gluten in food products. The polymerase chain reaction (PCR), a DNA-based method of high specificity and sensitivity, has been proposed as an effective alternative for the detection of wheat or other gluten-containing cereals (Dahinden et al., 2001). These methods are both laborious and expensive, and near infrared spectroscopy (NIRS) technique could be an alternative. NIRS offers a number of important advantages over traditional chemical methods. It is a physical, non-destructive method, requiring minimal or no sample preparation, no reagents are required, no wastes are produced and several components can be determined simultaneously from a single spectrum with the help of the multivariate calibration process.

The use of NIRS for quality control of cereals is well established in the literature (Osborne, 2000) and has been introduced successfully as a rapid technique for grain (Miralbés, 2003; Scholz et al., 2007), flour (Baslar and Ertugay, 2011; Miralbés, 2004; Paulsen et al., 2003), dough (Alava et al., 2001; Kaddour and Cuq, 2011; Sinelli et al., 2008) and bread (Osborne et al., 1984;

Abbreviations: NIRS, near infrared reflectance spectroscopy; SNV, standard normal variate; DT, detrend; MSC, multiple scatter correction; MPLS, modified partial least squares; SD, standard deviation; SEC, standard error of calibration; SEP, standard error of prediction; RPD, ratio of performance to deviation; RER, range error ratio; R^2, coefficient of determination for calibration; r^2, coefficient of determination for validation.

* Corresponding author. Tel.: +34 935811497; fax: +34 935811494.
E-mail address: elena.albanell@uab.cat (E. Albanell).

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Sørensen, 2009). In wheat flours, protein fractions (gliadin and glutenin) have been predicted using NIRS techniques (Wesley et al., 2001).

Rapid infrared spectroscopy methods have been successfully used in adulteration detection for a wide range of complex food products, including oils, milk and wheat (Cocchi et al., 2006; Kasemsumran et al., 2007; Ozen et al., 2003) and Norris (2009) proposed a simple method using multiple calibrations on a single near infrared scan to detect adulteration in food products.

The objective of the present study was to investigate the possibility of developing a NIRS calibration, as an alternative method for the detection of low-level gluten presence in flours and in different times of batter development, using a NIRS instrument without optical fibre.

2. Materials and methods

2.1. Samples

Due to the inability to find common gluten-free bakery samples which contain natural variations of low and very low gluten levels, it was decided to contaminate commercially gluten-free flours with small amounts of wheat flour. Two commercial gluten-free flour mixes and one corn starch were mixed (adulterated) with two different commercial wheat flours, and in order to obtain more variation, each wheat flour was mixed with one gluten-free flour and with a combination of the gluten-free flours. We obtained 7 different combinations of gluten-free flours, which were mixed with different concentrations of two wheat flours. The manufacture of combinations resulted in 144 samples with a final concentration from 0 to 4.5% (w/w) of wet gluten content (Table 1).

Usually, gluten-free flours produce liquid systems, due to the lack of a gluten network. This is the reason why "batter" is the most common name to refer to gluten-free dough. It must also be mentioned that samples containing just corn starch were not possible to study during fermentation time, due to the impossibility to form a mixing structure able to develop a stable batter.

To prepare the batter ($n = 88$) on a laboratory scale, water at 20 °C (±1 °C) and yeast at 5% concentration were added to the samples. Once mixed, shortening (at 4.7%) was added at the final step of mixing. Shortening make the batters for gluten-free bread more workable and renders the final product more tender and moist. After homogenization, samples were proofed for 45 min at 37 °C. During fermentation the action of yeast results in the production of carbon dioxide and this increases batter volume.

Wet gluten content was determined according to the ICC standard method No. 137 (2001). In wheat flour, there is a plastic–elastic substance consisting of gliadin and glutelin, obtained after washing out the starch from wheat flour dough. Wet gluten was washed and separated from 10 g of flour with 2% chloride buffer using the glutomatic equipment (Perten, Stockholm, Sweden). The determinations were made in duplicate and the differences did not exceed 0.5%.

Fig. 1. Presentation of batter sample for near infrared reflectance measurements.

2.2. NIRS analysis

All samples were recorded from 1100 to 2500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark). Reflectance was recorded in 2 nm steps, which gave 692 data points for each sample, as log (1/*R*) where *R* represented reflected energy. The flour analysis was carried out in duplicate using ring cup cells. In order to make the manipulation of the batter samples easier, instead of using the ring cup cells, the batter samples were covered with a plastic layer (always the same type of plastic) and were scanned as shown in Fig. 1. All measurements were performed by the same operator.

Batter samples were scanned in duplicate at time 0 (initial time) and after 45 min (final time), when the fermentation process had been completed and the batter samples had increased their volume. The batter calibrations were developed using respectively, the initial and final sampling times (0 and 0045), and a calibration derived by combining the two sampling times.

A WinISI III (v. 1.6) software program was employed for spectra data analysis and development chemometric models. Prior to calibration, log 1/*R* spectra were corrected for the effects of scatter using the standard normal variate (SNV), detrend (DT) and

Table 1
Thirty five wet gluten concentrations (w/w) used to obtain the NIRS calibrations. Summary of wet gluten content (%) of flour and batter samples used in calibration and validation sets.

0 ppm	80 ppm	90 ppm	150 ppm	180 ppm	380 ppm	450 ppm	0.08%	0.09%
0.19%	0.23%	0.38%	0.45%	0.58%	0.68%	0.77%	0.90%	0.96%
1.13%	1.15%	1.34%	1.35%	1.53%	1.58%	1.80%	1.92%	2.25%
2.30%	2.68%	2.70%	3.07%	3.15%	3.60%	3.83%	4.50%	

	Calibration set, %				Validation set, %			
	N[a]	Range	Mean	SD[b]	*N*[c]	Range	Mean	SD[b]
Flour	108	0.0–4.5	1.15	1.222	36	0.0–4.5	1.17	1.253
Batter, general	132	0.0–4.5	1.37	1.218	44	0.0–4.5	1.37	1.200
Initial batter	66	0.0–4.5	1.41	1.250	22	0.0–4.5	1.26	1.116
Final batter	66	0.0–4.5	1.41	1.250	22	0.0–4.5	1.26	1.116

[a] Number of calibration samples.
[b] Standard deviation.
[c] Number of validation samples.

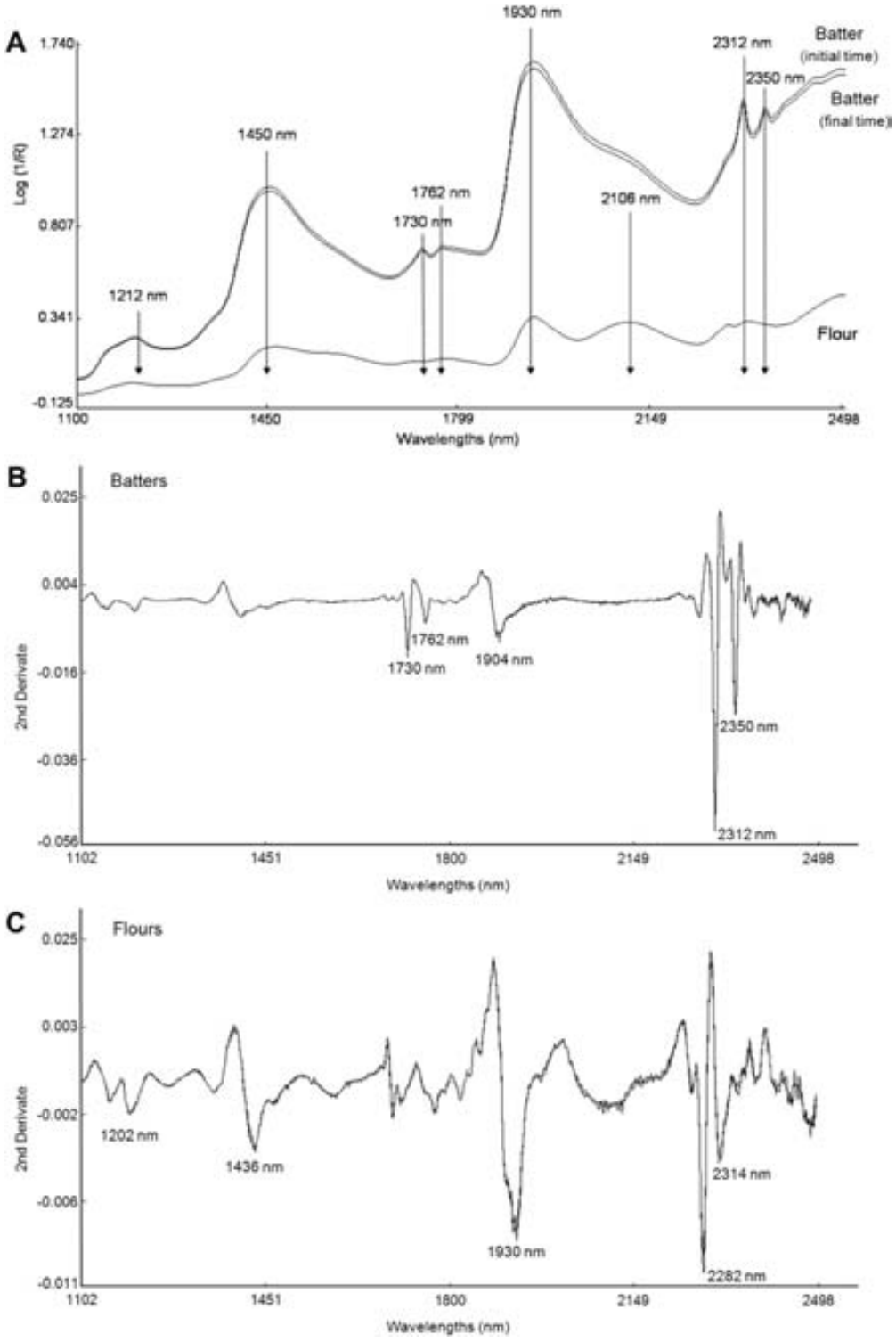

Fig. 2. Near infrared reflectance spectra: (A) Raw spectra of flour and batters. Near infrared second derivate and SNV of the spectra: (B) batters; (C) flours.

multiple scatter correction (MSC) and transformed into first, second or third derivatives using different gap size (nm) and smoothing interval. Modified partial least squares (MPLS) was the regression method used for calibration development and cross-validation was applied to optimize calibration models and to detect outliers.

A total of twenty spectral models for each predicted parameter were developed, resulting from the evaluation of four scatter correction techniques (SNV; DT; SNV + DT; MSC) and five math treatments (1, 4, 4, 1; 1, 10, 10, 1; 1, 15, 15, 1; 2, 4, 4, 1; 3, 10, 10, 1 - derivate number, subtraction gap, smooth, second smooth). Independent calibration equations were generated for flours and for batter samples. In order to find the best possible calibration, the above calibration approach was compared using the mean of two separate scans (average spectral data) or using the individual scans of duplicates (individual spectral data).

Samples used in validation were selected from the total population (25% of the total samples) to represent a full range of composition. Samples in the validation set were not used in the calibration set or vice versa. The performance of the model was determined by the following statistics: standard error of calibration (SEC), standard error of prediction (SEP), coefficient of determination for calibration (R^2), coefficient of determination for validation (r^2), the ratio of performance to deviation (RPD) described as the ratio of standard deviation for the validation samples to the standard error of prediction (SEP), and the range error ratio (RER) described as the ratio of the range in the reference data (validation set) to the SEP (Williams and Sobering, 1996).

To assess the correct detection of gluten presence with the NIR results obtained, a contingency table was performed to compare pared categorical variables (gluten presence vs. gluten-free), and a chi-squared test (without Yates correction) was made.

3. Results and discussion

3.1. Analysis of NIR spectra

Raw NIR spectra obtained for flour and batters are presented in Fig. 2. NIR spectra obtained in batter samples are similar to those previously reported by Alava et al. (2001) and Kaddour et al. (2008) in bread dough mixing; and Bruun et al. (2007) in gluten powder. The NIR spectrum obtained in flour samples was similar to that obtained by Paulsen et al. (2003) in maize starch, and Scholz et al. (2007) in wheat materials. There are few differences between the individual peaks of flours and batters (Fig. 2A). NIR spectra of batters are highly dominated by the water signal, and compared to flour with low moisture content, batters cause much higher baselines and may cause a decrease in the scattering properties. Kaddour et al. (2008), reported that increasing dough water content, induced an increase of water bands and a decrease of the starch and gluten bands due to the dilution effect of water. Bruun et al. (2007) observed changes in the gluten protein absorption bands with increasing moisture content.

During mixing development and during the fermentation process, changes in batter consistency are presented and this can be observed in the raw spectra differences. Changes in raw NIR spectra with mixing time describe changes in specular and diffuse properties of dough and baseline shift evolution caused by dough macroscopic structure modification during mixing (Kaddour and Cuq, 2011).

The following characteristics bands were identified (Fig. 2A): 1212 nm (2nd overtone of the C–H stretch of CH_2 group); 1450 nm (1st overtone of the O–H stretch); the region 1700–1762 nm (1st overtones of C–H stretch of CH_2 and CH_3 group); 1930 nm (a combination of the O–H bend and the stretching band of water); and the bands 2106, 2312 and 2350 nm (combination bands of C–O stretching and bending vibrations) (Fodor et al., 2011; Osborne et al., 1993). Wesley et al. (1998) considered that systematic variation in the peak area at 1200 nm during dough mixing could be associated with moderate starch absorption, while Kaddour et al. (2007, 2008) in bread dough mixing, obtained a broad band centered at 1460 nm and an intense feature centered at 1940 nm assigned to water. Alava et al. (2001) identified the region 1375–1525 nm as a main wavelength contributor to describe dough mixing and suggested that the changes observed for this spectral region relate to changes in starch–water interactions.

The region between 1730–1760 and 2310–2350 nm are affected by numerous overlapping combinations and overtone bands and could be associated with oil structures and probably related with the addition of shortening. The absorption bands at 2106 nm are related with starch and protein content. Liu et al. (2009) in wheat samples, reported that the bands in the 2000–2490 nm regions arise from the combination modes of different vibrations of CH, OH and NH.

When comparing the spectra of flour containing 4.5% gluten, with a gluten-free flour, the gluten presence causes much higher baselines; but the evident effect of gluten content in the baseline variations in the raw spectra was removed with a second derivate transformation (Fig. 2C). A second derivate transformation was performed on the raw spectra to narrow the bandwidths and also remove some of the baseline variations. This transformation made the absorption bands much more evident, overlapping absorbances are separated and the peak resolution is improved, making analysis easier. Fig. 2B and C show the second derivate NIR spectra of flours and batters samples. The same trend absorption bands of major components in the raw spectra were observed. However, the absorption bands at 1730 and 1762 nm in batter samples, and the 1202, 2282 and 2314 nm in flour samples, related with oil, starch and protein content respectively, became clearer than in the raw spectra. Most commercially available gluten-free flours contain a high amount of corn starch (more than 75%), although the protein content is lower than conventional flours, the presence of gluten in flours and batters could be detected in the region 2000–2350 nm.

3.2. Calibration equation development

Ranges, means and standard deviation values of flours and batters used for developed wet gluten calibration are summarized in Table 1. Among the samples used, some of them were selected for calibration by the WinISI software, and the remaining samples (25%) were used for the validation set. The calibration and validation sets covered the same range in all products.

Calibrations by MPLS regression were performed for flours and for batters using the individual spectral data and using the average spectral of the duplicate (Table 2). Different pre-treatments of spectral data were tested for their ability to remove or reduce disturbing effects not related to the chemical absorption of light. The optimal spectral pre-treatment was the first derivate treatment combined with SNV and/or DT. Except in the case of the initial batters, the optimal calibrations were obtained using the mathematical treatment 1, 4, 4, 1 (first derivate; gap = 4; smoothing = 4, second smooth = 1). This mathematical treatment is most commonly used in NIRS food analysis and has been used previously to develop a gluten calibration in commercial wheat flour (Miralbés, 2004) and for starch content in maize flour samples (Paulsen et al., 2003). Nevertheless, Sørensen (2009) developed NIR

Table 2
Calibration and cross validation statistics for determination of gluten content of flour and batter samples by near-infrared analysis.

Calibration set	N^a	Math[b] treatment	Scatter[c] correction	R^2	SEC	SECV	SD/SECV
Individual spectral data							
Flour	216	1, 4, 4, 1	DT	0.982	0.168	0.236	5.21
Batter, general	264	1, 4, 4, 1	SNV + DT	0.926	0.330	0.456	2.66
Initial batter	132	1, 10, 10, 1	SNV + DT	0.931	0.311	0.393	3.10
Final batter	132	1, 4, 4, 1	SNV + DT	0.942	0.298	0.535	2.27
Average spectral data							
Flour	108	1, 4, 4, 1	DT	0.985	0.155	0.267	4.57
Batter, general	88	1, 4, 4, 1	SNV + DT	0.904	0.365	0.530	2.30
Initial batter	44	1, 10, 10, 1	SNV	0.893	0.423	0.613	2.04
Final batter	44	1, 4, 4, 1	SNV + DT	0.942	0.319	0.633	1.97

R^2 = coefficient of determination for calibration. SEC = standard error of calibration. SECV = standard error of cross validation. SD = standard deviation.
[a] Number of spectra.
[b] Designations: derivate order, gap, first smoothing, and second smoothing.
[c] Standard Normal Variate (SNV) and Detrend (DT) transformations.

Table 3
Validation statistics for determination of gluten content of flour and batter samples by near infrared analysis.

Validation set	r^2	SEP	Bias	Slope	RPD	RER
Individual spectral data						
Flour	0.963	0.215	−0.006	1.036	5.72	20.93
Batter, general	0.825	0.514	−0.091	1.019	2.66	8.75
Initial batter	0.873	0.432	0.005	1.097	2.91	7.29
Final batter	0.810	0.539	0.000	1.091	2.34	5.84
Average spectral data						
Flour	0.967	0.215	−0.076	0.992	5.68	20.93
Batter, general	0.767	0.551	−0.035	0.955	2.48	8.17
Initial batter	0.682	0.655	−0.021	0.837	1.92	4.81
Final batter	0.624	0.751	0.096	0.772	1.68	4.19

r^2 = coefficient of determination for validation. SEP = standard error of prediction. SECV = standard error of cross validation. RPD = standard deviation of reference data divided by the SEP. RER = range error ratio.

calibrations for routine determination of dietary constituents of wheat and rye breads using the treatment 1, 10, 10, 1.

The models obtained (Table 2) were satisfactory for the 8 calibrations presented, showing R^2 from 0.893 to 0.985, SEC values from 0.16 to 0.42 and SD/SECV ratio from 1.97 to 5.21. The values obtained were comparable with those reported by Miralbés (2004) in wheat flour samples and NIRS transmittance (R^2 = 0.97, SEC = 0.66).

In batter samples, better results were obtained using individual spectral data than average spectral data. Moreover, scanning samples with a plastic layer seem to be an easy and quick alternative. In flour samples, the best results were obtained when working with average scan data, where the sample population size was smaller and the repeatability was high.

The batter calibration developed using the general model (two sampling times) was more accurate than the calibrations obtained with the initial and final sampling times (Tables 2 and 3), probably due to sample size increases. The statistics obtained exhibited their potential for use with sufficient precision, showing R^2 = 0.93 and 0.90, SEC = 0.33 and 0.37, r^2 = 0.83 and 0.77 and SEP = 0.51 and 0.55 for individual spectral data and average spectral data sets, respectively.

For all models, the mean bias (mean difference between chemical and NIRS values) was near zero and the slopes of the equations did not differ significantly from the unit. The relationship between wet gluten content as determined by traditional chemical analysis and as predicted by NIRS with average spectral data, in flours and batters are illustrated graphically in Fig. 3.

For all calibrations, SEP values were comparable with SEC or slightly higher, because SEP includes both the error associated with chemical analysis and the error associated with the NIRS equipment (Holechek et al., 1982). The accuracy of the calibration can be

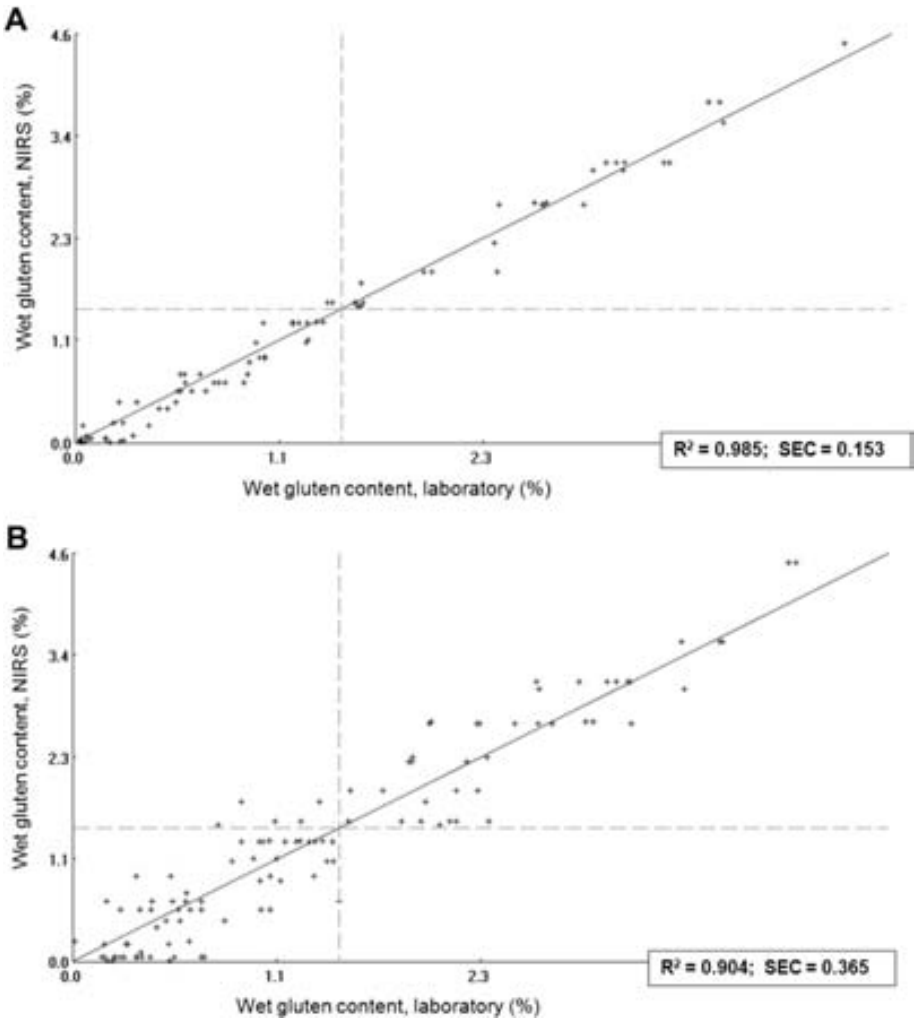

Fig. 3. Relationship between gluten reference method values and near infrared reflectance predicted values in (A) flour samples and (B) batter samples, for the average spectral data calibrations.

evaluated based on the RPD and the RER statistics, and this confirms the high precision of the equations developed, with values larger than the minimum recommended for prediction uses (RER over 10 and RPD over 3) according to Williams and Sobering (1996). For wheat flours in transmittance mode, Miralbés (2004) obtained RPD values of 3.8 and 7.3 for wet gluten and dry gluten content, respectively.

The composition and labeling of foodstuffs suitable for people intolerant to gluten (celiacs) is considered gluten-free if the food contain less than 20 mg gluten per kg and "very low gluten" if less than 100 mg/kg (EU Regulation 41/2009). Contamination of food products by gluten-containing cereals may take place at the stage of flour production or at the stage of the production of the final product. When we analyzed flour and batter samples with a gluten range of 0–4.5% gluten, 84% of the cases were correctly detected (with gluten vs. gluten-free) and the chi-square test confirmed that differences were significant (Xsquare = 40.085 with 1 degree of freedom; $P = 0.0001$). However, if we check the predicted values obtained, we observe that the effectiveness of calibrations decrease when the gluten presence is less than 0.09% gluten (we found false positives and false negatives). Although we obtained good calibrations, it was not possible to clearly detect the absence of gluten at the concentrations currently demanded by the European food and safety laws, because when gluten concentration was between 0 and 100 ppm, the results obtained were considered not quite statistically significant (Xsquare = 3.506 with 1 degrees of freedom; $P = 0.0611$).

4. Conclusion

Good NIRS calibrations for wet gluten content in flour and batter have been developed. In batter samples, our results indicate that NIRS calibrations obtained directly with a plastic layer can be used quickly and accurately. Although the technique can predict accurately the concentration of wet gluten content, it should not be considered as a reliable method for determining gluten content contamination in gluten-free products, and more research on this approach is needed.

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Chapter 4

Results and Discussion

4.1 Introduction

This section discusses the main results obtained from the testing of different ingredients used in the manufacture of gluten-free breads with the aim of optimizing their formula. Each ingredient was selected based on techno-functional, sensory or nutritional potential properties that could improve gluten-free breads in terms of baking characteristics, organoleptic profile and shelf life.

In addition to the gluten-free bread optimization, our research included a study on the application of near infrared reflectance spectroscopy (NIRS) to detect low levels of gluten. The huge increase of gluten-free production in the last fifteen years and the current legislation, which allows a maximum of 20 ppm to label "gluten-free", requires accurate techniques to determine and control gluten levels in foods. NIRS technology has been used in food for many purposes, and we studied its suitability to detect low levels of gluten in flours and batters.

4.2 Influence of unicellular protein on gluten-free bread characteristics

Unicellular protein obtained from yeast cells is of special interest because its nutritional composition may contribute to balance the diet of celiac patients. It contains approximately 50% of high value protein, 37-40% of carbohydrates, low lipid content and high content of phosphorus (1.8 %) (Schulz and Oslage; 1976; Anupama, 2000).

Three groups of formulations were tested with and without the unicellular protein:

- Starch-based with corn starch, buckwheat and soya at low concentration plus ovalbumin; and starch-based with unicellular protein.
- Starch-vegetable-based with corn starch, buckwheat and soya at low concentration; and starch-vegetable-based with unicellular protein.
- Flour-based with rice, buckwheat and soya and low starch concentration plus skim milk powder and ovalbumin; flour-based with unicellular protein.

Unicellular protein inclusion originated gluten-free breads with lower specific volume, lower bake loss, higher hardness and darker crumb color than their counterparts which did not contain the protein. Lower bake loss values observed in formulas containing the unicellular protein revealed that this protein had similar functionality as that of fiber. This effect was probably masked in flour-based formulations, where the higher content of buckwheat also played an important role in water holding ability. Fiber is responsible for producing low volume and dense structure, interfering with the proper formation of the gluten network of baked goods (Pomeranz et al., 1977; Lai et al., 1989). As reported by Moore et al. (2004), this effect is also more evident in gluten-free breads, where the batter is not as stable as in wheat products.

Although egg protein promotes batter stability (Kiosseoglou, 2003; Kato et al., 2006), no significant differences were found in our study in terms of specific volume. In agreement with previous studies (Moore et al. 2004), gluten-free breads containing ovalbumin showed higher initial hardness values, but a lower staling during the shelf life. This delay in staling is probably due to the egg protein capacity to form a continuous film-like protein that is able to link starch granules together.

If formulations without unicellular protein are considered, it can be stated that flour-based formulations originated breads with lower specific volume and higher water

holding ability than starch-based formulations. No significant differences were found between starch-based and starch-vegetable-based formulations in terms of texture.

Crumb color was clearly influenced by unicellular protein, buckwheat and ovalbumin addition. Thus, breads containing unicellular protein were darker than the same breads without this ingredient. Also, flour-based breads with high content of buckwheat showed darker colour than starch-based formulations, while starch-vegetable-based breads were lighter than starch-based breads, due to ovalbumin addition.

Sensory analysis was only conducted with starch-based breads as flour-based breads were discarded because of their low volume and high hardness. Starch-vegetable based bread obtained the highest significant scores in crumb elasticity and sponginess, and was also the most preferred in the preference test. No significant differences were found in flavor intensity, salty taste, sweet taste and aroma.

As a conclusion, it is possible to formulate gluten-free starch-based breads with good baking characteristics, while the high fiber content in the formula leads to breads with low specific volume and hard crumb. The inclusion of unicellular protein allowed obtaining gluten-free starch-based breads with low bake loss albeit these formulas were less preferred by consumers in comparison to the controls, probably due to texture characteristics.

4.3 Effect of legume flours on baking characteristics of gluten-free bread

Legumes are important sources of food proteins. They contain high amounts of lysine, leucine, aspartic acid, glutamic acid and arginine, and provide well balanced essential amino acid profiles when consumed with cereals and other foods rich in sulphur-containing amino acids and tryptophan. In addition to their nutritional properties, legume proteins also possess functional properties that play an important role in food formulation and processing (Dakia et al., 2007; Boye et al., 2010; Roy et al., 2010).

Because of legume nutritional and techno-functional properties, many organizations such as the World Health Organisation are promoting their introduction in different staple food.

Although soya was considered a good source of protein to formulate gluten-free breads, the high allergenicity of soya and the associated digestive problems, are leading to more

research into alternative legume protein sources which may be able to provide gas-holding capacity and bake development.

Chickpea flour, pea isolate, and carob germ flour were introduced in corn-based gluten-free bread formulations at levels of 1.5% of protein in order to evaluate the potential functionality of these legume proteins.

Carob germ proteins showed higher visco-elastic properties than chickpea, pea and soya proteins, obtaining greater values of G'. Other authors had also found similar rheological properties (Rice and Ramstad, 1950; Plaut et al., 1953; Feillet and Roulland, 1998; Bengoechea et al., 2008; Wang et al., 2001; Smith et al., 2010), and stated similarities between gluten and carob germ proteins. Although the high number of disulphide bonds present on carob germ proteins, the different distribution of high molecular weight units and the low proline content make impossible to develop extensible batters similar to those of wheat.

Carob germ flour lacked extensibility and generated breads with lower volume and higher crumb hardness than soya, pea isolate and chickpea flours, probably due to remaining gums present in the flour after the extraction process. In terms of color, carob germ contributed to the yellowness of these breads, negatively affecting their general sensory acceptability.

Chickpea breads presented the higher specific volume values. The specific amino acid content of chickpea proteins, as previously reported by Boye et al. (2010), seemed to improve emulsion and foam capabilities. In gluten-free breads, the unstable structure created with starch, water and hydrocolloids is incapable of supporting foam, requiring additional surface active molecules to form a film around the bubbles, stabilizing them against coalescence and, hence, foam collapse. The additional help provided by chickpea amino acids, helped gluten-free batter development and improved the final quality of the product. Chickpea breads showed the lowest hardness values at day 1 (and overall the study) and the highest loaf specific volume, agreeing with the observation of Gómez et al. (2008) and Sabanis and Tzia (2011), who also observed a negative correlation between crumb hardness and loaf volume.

Sensory test revealed that chickpea and soya breads were, in general, the best evaluated in terms of acceptability. However, chickpea bread was only preferred by 16.6% of panelists while soya and pea were the most preferred and chosen by 54.5% and 21.2%

of the consumers, respectively, although these breads did not stand out in individual parameters. The dark crumb color, hard texture, compact structure and poor general appearance were the most important reasons to point carob germ bread as the less preferred.

In summary, carob germ flour generated batters with good rheological properties; however, its breads generally presented poor organoleptic attributes. Addition of chickpea flour improved foam stability and produced breads with good volume and texture characteristics. Pea isolate breads also yielded good results in all the evaluated parameters.

Our results demonstrate that chickpea and pea are promising ingredients as alternatives to soya that can provide gluten-free breads with good physico-chemical and organoleptic characteristics, in addition to a good balanced nutritional profile.

4.4 Liquid whey as an ingredient to formulate gluten-free bread

Dairy ingredients are usually included in bakery products due to its nutritional and functional properties, such as high protein and calcium content, and improvement of crust color, bread flavor and crumb structure (Erdogdu-Arnoczky et al., 1996; Yousif et al., 1998; Kenny et al., 2000; Secchi et al., 2011). Skim milk powder is the most common dairy ingredient used in bakery; however, other dairy ingredients like whey proteins or caseinates are also used.

Whey protein foams well by creating and stabilizing air bubbles in a liquid, they also stabilize emulsions by forming interfacial films between hydrophobic and hydrophilic food components (Renner and Abd El-Salam, 1991). Whey proteins are usually added to bakery products as a dry ingredient after a whey protein concentration process. However, many creameries are no capable of generate whey protein concentrate and have to manage the liquid whey. For this reason, direct utilization of whey could be an alternative to increase its value and reduce environmental management costs.

In this study, two types of liquid whey were used instead of water: sweet or fresh whey obtained after rennet coagulation, with pH values between 6.5 - 6.0; and acid or ripened whey, obtained after cheese making procedures that incorporate lactic acid bacteria, with pH values ranging from 4.5 to 5.5. Together with each type of whey soya flour levels of 0, 2.5 and 5% were also evaluated.

Irrespective of the type of whey used, its inclusion in the formulation affected significantly the gluten-free bread characteristics especially at 5% of soya flour. These batters showed the highest viscoelastic behavior in contrast to batters with 0% soya flour and water that exhibited the lowest values of G'. The water holding capabilities of both, whey and soya proteins extensively described in the literature, could partially explain this rheological behavior.

Regarding to baking characteristics, breads with maximum protein content showed the lowest bake loss and specific volume values. In contrast, breads with 0% soya flour and water had the highest bake loss and specific volume values.

Levels of soya flour clearly influenced specific volume so that this parameter decreased as soya concentration increased. Whey addition also caused a decrease in specific volume, which can be clearly observed comparing breads without soya. Other studies have shown contradictory results about the effect of whey incorporation in bread. Some authors reported a significant increase of gluten-free breads volume at whey protein levels of 10% (Nunes et al., 2009). In wheat bread, Divya and Rao (2010), also observed an increase in loaf volume when whey with 15% or 26% total solids was added. Similar results were also reported by Bishal et al. (1979) and Kadharmestan et al. (1998). On the contrary, others described a decrease in wheat bread loaf volume when whey protein was added (Gelinas and Lachance,1995; Yousif et al., 1998; and Kenny et al., 2000).

Bread with ripened whey showed the highest hardness values within the five days of storage, probably due to the higher protein and fat content. Soya incorporation produced harder breads but with a lower staling rate. The anti-staling effect of soya can be explained by the high water holding capacity of soya proteins and their strong interaction with starch, which hinders amylopectin recrystalization during storage so that bread staling is delayed (Zeleznak and Hoseney, 1987; Ryan et al., 2002)

Breads including fresh and ripened whey showed a darker crust color than breads formulated with water. This effect on color can be attributed to Maillard and caramelization reactions that occurred intensively in these breads due to whey's high lactose and protein content (Bilgin et al., 2006). Moreover, lactose content in fresh whey is expected to be higher than in ripened whey, which explains the darker colour of breads containing fresh whey.

Only three breads, that were selected as the most preferred in previous consumers test were compared in the final sensory test. Consumers found significant differences in hardness between the three samples. The formulation with 5% of soya and fresh whey was identified as the hardest, followed by 0% of soya and water and 0% soya and ripened whey, which was pointed as the softest. General appearance of was acceptable for all formulations, although the highest score was given to bread containing 0% soya and ripened whey.

Whey addition allows the production of gluten-free bread with good nutritional and technological properties. In general, breads containing fresh whey had better baking and physico-chemical characteristics and higher consumer acceptability than those made with ripened whey. The use of unprocessed cheese whey may help reducing the costs of gluten-free bread formulations and could also be a high quality soya protein substitute, while contributing to increase the value of this cheese by-product.

4.5 Detection of low-level gluten content in flour and batter by near infrared reflectance spectroscopy (NIRS)

Gluten is present in food products containing wheat, rye and barley, however, it may also be present in other food products where gluten is added as a texture agent or as a vegetable source of protein. As established by the European commission, gluten-free and "very low gluten" products must contain less than 20 ppm and less than 100 ppm of gluten, respectively. Thus, the control of gluten presence in food represents a big challenge.

Immunochemical analytical methods are currently used to determine gluten in food products. Polymerase chain reaction (PCR) has also been proposed as an effective alternative for the detection of wheat or other gluten-containing cereals (Dahinden et al., 2001). These methods are both laborious and expensive, and NIRS technique could be an alternative, as it has been already widely used as a valuable tool for quality control in food industry.

Two commercial gluten-free flour mixes and one corn starch were adulterated with two different commercial wheat flours and, in order to obtain more variation, each wheat flour were mixed with one gluten-free flour and with a combination of the gluten-free flours. We obtained seven different combinations of gluten-free flours, which were

mixed with different concentrations of two wheat flours. The manufacture of combinations resulted in 144 samples with final wet-gluten concentrations ranging from 0 to 4.5% (w/w). NIRS calibration was investigated to detect low-level gluten presence in flours and in initial and final batters. The batter samples were covered with a plastic layer and were scanned.

Reflectance data was used for calibration based on modified partial least squares (MPLS) regression. Independent prediction equations were developed for flour and for batter. Spectral models using mean spectra of two scans (average spectra), were compared with those using the two individual spectral data.

In batter samples, better results were obtained using individual spectral data (R^2=0.926; r^2=0.825) than average spectral data. Moreover, scanning samples with a plastic layer seem to be an easy and quick alternative to perform the analysis. In flour samples, the best results were obtained when working with average scan data (R^2=0.985; r^2=0.967), where the sample population size was smaller and the repeatability was higher.

When we analyzed flour and batter samples with a gluten range of 0-4.5% gluten presence, the 84% of the cases were correctly detected (with gluten vs. gluten-free) and the chi-square test confirmed that differences were significant (Xsquare = 40.085 with 1 degree of freedom; P = 0.0001). Checking the predicted values, it was observed that the effectiveness of calibrations decreased when the gluten presence was below 0.09% (we found false positives and false negatives). Although good calibrations were obtained, it was not possible to detect the absence of gluten at the concentrations currently demanded by the European food and safety laws, because when gluten concentration was between 0-100 ppm, the results obtained were not statistically significant (Xsquare= 3.506 with 1 degrees of freedom; P = 0.0611).

Good NIRS calibrations for wet gluten content in flour and batter have been developed. In batter samples, our results indicate that NIRS calibrations obtained directly with a plastic layer can be quick and accurate. Although the technique can predict the concentration of wet gluten it is not a reliable method for determining gluten content contamination in gluten-free products, and more research on this approach is needed.

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Chapter 5

Final Conclusions

The conclusions obtained in this thesis are:

1. The inclusion of unicellular protein in gluten-free breads causes a decrease in bake loss, an increase in hardness and a darkening of crumb and crust color. Moreover, unicellular protein decreases consumer acceptance of the breads.

2. Fiber rich ingredients, as buckwheat flour and carob germ flour, have a big impact on the quality of gluten-free breads, causing a decrease in volume and an increase in crumb hardness. Carob germ flour generates batters with good rheological properties but poor baking characteristics.

3. Chickpea flour dramatically improves bread volume and softens crumb texture, without increasing crumb porosity. Despite the good baking characteristics rendered by this ingredient, its taste seems to be the cause of the low preference demonstrated by consumers for this type of bread.

4. Pea protein isolate and soya flour produce breads with similar rheological and baking characteristics. However, consumers prefer soya breads to pea protein isolate breads probably due to their different taste attributes.

5. Combination of soya and whey proteins increases batter visco-elasticity due to the increase in water retention ability but it negatively affects specific volume of breads containing both ingredients. Whey addition allows the production of gluten-free bread with good nutritional and technological properties. In general, breads containing fresh whey show better baking and physico-chemical characteristics although breads containing ripened whey are the most preferred by consumers.

6. Accurate prediction of wet gluten content in flours and batters is achieved with NIRS. However, it is not possible to clearly detect the absence of gluten at the concentrations currently demanded by the European regulation.