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EFFECT OF WHEAT PROCESSING AND GENOTYPE ON THE GLUTEN PROTEINS

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“Plantar o trigo e refazer o pão de cada dia
Beber o vinho e renascer na luz de todo dia
Deixar a sua luz brilhar no pão de todo dia
Deixar o seu amor crescer na luz de cada dia
Vai ser, vai ser, vai ter de ser, vai ser muito tranquilo
O brilho cego de paixão e fé, faca amolada”

(Milton Nascimento, 1975)

EFFECT OF WHEAT PROCESSING AND GENOTYPE ON THE GLUTEN PROTEINS

Bruna Mattioni

ABSTRACT

Keywords: Wheat. Gluten. Ancient wheat. Heat treatment. Celiac Disease.

Wheat is worldwide utilized as a food grain since the late Stone Age and cultivated since 5000 b.c.. From wheat flour a diversity of baked products can be made because the unique ability of form a viscoelastic dough, characteristic controlled mainly by gluten proteins. Gluten proteins are among the most complex protein networks in nature due to numerous different components and distinct size, and due to variability caused by genotype, growing conditions and technological processes. On the other hand, Celiac disease (CD), the most common wheat intolerance worldwide, is a complex immune-mediated disease trigged by gluten ingestion. Assume a strictly gluten-free diet represents the only effective medical treatment for CD patients. On the other hand, treatments that involve time, temperature and pressure can change protein structure. The heat treatments can affect technological properties and reduce the allergenicity to varying extents in wheat flours and breads. Also, nowadays, there is growing interest in ancient wheats, such as einkorn, emmer, Khorasan (Kamut), faro, and spelt because might these varieties could be nontoxic to celiac and helthier than modern wheat. The aim of this project was to study the effect of wheat treatment and the different varieties of wheat on the gluten proteins and the implications in the immune response *in vitro.* This work was divided in 3 parts. I – Brazilian commercial wheat flour was subjected to to spray drying, oven heating, ultrasound and microwave radiation. Solubility, monomeric and polymeric proteins and glutenin and gliadin profile were analyzed. Also, digestibility and the amount of potential celiac disease immune stimulatory epitopes were measured with the R5 monoclonal antibody and G12 ELISA assays. Heat treatment leads to unfolding of peptide chains, changes in hydrophobicity and susceptibility to the action of proteolytic enzymes. The treatments affected solubility, and with exception of ultrasound, all treatment showed low solubility of

polymercis and monomerics proteins. Also, treatments affected glutenins and gliadins profile, the amount of the HMW-GS (High Molecular Weigh – glutenin) decrease after spray-dry, oven and extrusion and the amount of the LMW-GS (Low Molecular Weigh – glutenin) decrease too after spray-drying, microwave, oven and extrusion. Wheareas for gliadin, the amount of ω-gliadin increase after spra-drying and ultrasound treatments, and the amount of α/β- gliadin decrease after all treatments, while the treatment to do not affected γ-gliadins amount. Also, digestibility decrease after spray-dryier and ultrasound. This changes with decrease of solubility and changes in profile of proteins is results of rearrange of proteins during the treatments, resulting in a higher complex structure. And, finally, the potential celiac disease immune stimulatory epitopes were measured, and showed lower amount after spray-drying treatment by R5 monoclonal antibody and lower amounts after spray-drying and microwave treatment by G12 ELISA test in relation to control flour. At last, even with the alteration on the gluten structure and complexicity, these changes do not allow to produce a safe product to celiacs, the amount of the potential celiac disease immune stimulatory epitopes still were too high. II – Modern and ancients' wheat varieties are being tested against anti-Gliadin antibody. If aim to found a wheat nontoxic to celiacs, we tested pools of different wheats varieties, two of them with AA genome - *T. monococcum* ssp *monococcum* and *T. urartu.* Due agronomical caracteristic and comercial appeal, we used five *T. turgidum* wheat variety as follow: *T. turgidum* ssp *durum* (AABB), *T. turgidum* ssp *polonicum* (AABB), *T. turgidum* ssp *turgidum* (AABB) and *T. turgidum* ssp *turanicum* (AABB). All wheat flours were extracted, purified, separated using 2D gel eletrophoresis, stained with Comassie blue or were run a western blot with Gliadin antibody and Skeritt antibodies, and pictures of them were overlapping. All wheat varieties tested contained gluten proteins recognized by anti-gluten Skerritt and Gliadin antibodies related to T-cell stimulatory epitopes, at distinct levels. Einkorn and durum species tested here differ in toxicity depending on the sub-species. Eikorn tested showed higher immunogenicity that durum varaities. So, it is not safe for all celiacs to consume the wheat varieties tested, that include ancients' varieties. III - Additionally, this study verified if food products commercialized in Brazil were correctly labeled in relation to the presence or absence of gluten by using the ELISA R5 method. In relation to products sold in Brazil, the results show for the celiac population that companies are testing their food products to verify if labeling is correct to provide products that are safe and to accurately identify potential gluten levels. However, if 89% of gluten-free food

products are correctly labeled, this means that 11% represent a risk for the health of the celiac population. To improve this amount, routine auditing is necessary to verify the correct labeling about gluten in food products and adopt good manufacturing practices. Brazil is becoming a global economic player, so it is important to be aligned with global legislation concerning gluten presence and ensure that the label can be trusted. This results in greater confidence in both the global market and consumers and represents one more step toward health maintenance for celiacs.

EFEITO DO PROCESSAMENTO DO TRIGO E DIFERENTES VARIEDADES NAS PROTEINAS DO GLUTEN

Bruna Mattioni

RESUMO ESPANDIDO

Palavras-chave: Trigo. Glúten, Trigo crioulo, Tratamento térmico, Doença celíaca.

Introdução

O glúten é uma proteína encontrada nos grãos de trigo, centeio e cevada. Neste trabalho, considerar-se-á o trigo como a fonte de glúten, pelo fato desta matéria prima ser amplamente utilizada tecnologicamente no mundo, em pães, biscoitos, bolos e massas. Se, por um lado o consumo de pães é tido não somente como uma questão cultural, mas também religiosa através do mundo e do tempo, por outro lado, atualmente há discussões sobre o consumo de glúten. A adoção e indicação de dietas sem glúten, mesmo para quem não apresenta sintomas clínicos e fisiológicos de uma intolerância alimentar levou a Sociedade Brasileira de Alimentação e Nutrição a publicar um artigo sobre o posicionamento da mesma, esclarescendo que dietas sem glúten devem ser recomendadas apenas para indivíduos com alguma desordens relacionada ao glúten. Por outro lado, com esse debate, os casos de indivíduos que apresentam alguma disfunção ao ingerirem glúten, são mais facilmente identificados. Atualmente, o unico tratamento é a adoção de uma dieta sem gluten. Ainda hoje, o diagnóstico pode demorar, pois os sintomas podem ser diferentes de indivíduo para indivíduo, além disso, os sintomas se sobrepoem aos de outras doenças, o que leva médicos a investigarem outras doenças. Para facilitar o entendimento, diagnóstico e classificação, as desordens relacionadas ao glúten foram recentemente classificadas de acordo com a resposta em: autoimune, alérgica e de sensibilidade. Sendo que o presente trabalho é focado nas respostas autoumines provocadas pela ingestão de glúten, que são a doença celíaca, ataxia provocada pelo glúten e dermatite herpetiforme. A indentificação destas se dá por teste clínicos (sintomas), histológicos (danos no intestino), sorológicos (presença no soro dos anticorpos anti-gliadina, anti-endeomisio e anti-transglutaminase) e genéticos (presença dos genes DQ 2/8). Acredita-se que no Brasil dois milhões de indivíduos são celíacos ou possuem alguma desordem relacionada ao gluten. Como o único tratamento é a exclusão

do glúten da dieta, acaba movimentado um mercado de produtos alimentícios sem glúten. No Brasil, o número de empresas de alimentos que comercilizam produtos sem glúten e o volume de vendas tem aumentando exponencialmente. Nos Estados Unidos, o mercado de produtos sem glúten movimentou 8,8 bilhões de dólares até 2014. Devido à importância econômica do trigo em nível nacional e mundial, fazem-se necessários mais estudos, não apenas em relação à doença celíaca, mas também em relação ao glúten de trigo e suas variedades, e como o tratamento térmico deste cereal, pode afetar ou influenciar na resposta imune de indivíduos com pré disposição genética. Além disso, surgem especulações sobre o consumo de trigo de diferentes variedades, as quais poderiam ser não ser tóxicas, umas vez que não passaram por tantos processos de hibridização e manipulação gênica como o trigo hexaplóide moderno.

Objetivos

O principal objetivo deste trabalho foi estudar o efeito do tratamento térmico do trigo e as diferentes variedades de trigo sobre as proteínas do glúten e quais as implicações na resposta imune *in vitro*. Neste contexto, os seguintes objetivos específicos foram estabelecidos:

Submeter farinha brasileira comercial aos tratamentos de extrusão, *spray-dry*, ultrassom, microondas e forneamento.

Analisar as alterações em relação as características tecnológicas das proteínas que formam o glúten na farinha do trigo antes e após os tratamentos quanto à: solubilidade, proporção entre proteínas monoméricas e poliméricas, perfil de gliadinas e gluteninas.

Analisar se houve aumento ou diminuição da digetibilidade, e se os tratamentos térmicos afetam a quantidade de epitopos estimuladores de células T em celíacos pelo teste de ELISA R5 e G12.

Obter e analisar diferentes variedades de trigo sendo elas: *Triticum monococcum ssp monococcum, Triticum urartu, Triticum turgidum ssp durum, Triticum turgidum ssp polonicum, Triticum turgidum ssp turgidum, Triticum turgidum ssp turanicum, Triticum aestivium ssp spelta*.

Analisar se houve diferença entre as variedades na quantidade de epitopos estimuladores de células T em celíacos *in vitro*, usando western blott e anticorpos comerciais específicos.

Verificar se os produtos “sem glúten” comercializados no Brasil estão corretamente rotulados.

Contextualização

Para alcançar os objetivos propostos o trabalho foi dividido em três partes:

I – EFEITO DO PROCESSAMENTO DA FARINHA DE TRIGO NAS PROTEÍNAS DO GLÚTEN SOBRE AS CARACTERÍTICAS TECNOLÓGICAS E IMUNORREATIVIDADE

Os tratamentos que envolvem tempo, temperatura e pressão são capazes de alterar a estrutura das proteínas23. Em particular, com as altas temperaturas há degradação das proteínas do glúten, principalmente em reações envolvendo cisteína e lisina. Entretanto, faltam estudos para explicar como o tratamento térmico pode alterar as características tecnológicas e suas implicações na resposta autoimune em indivíduos susceptíveis. Para elucidar essas lacunas, um lote de farinha de trigo comercial foi submetido a diferentes tratamentos que envolveram tempo, temperatura e pressão: extrusão, *spray-dry*, forneamento, ultrassom e micro-ondas. As farinhas antes e após o tratamentos térmico foram analisadas em relação as características tecnológicas e de toxicidade *in vitro*. Todas as amostras foram analisadas quanto ao teor de proteína total (TP), proteínas poliméricas solúveis (SPP) e insolúveis (IPP). Para TP, somente houve diminuição após do tratamento de spray-dry. Entretanto, nesse tratamento foi evidenciado a perda de 43 % do peso inicial. Todos os tratamentos afetaram a solubilidade, sendo que o teor de IPP foi maior e o de SPP foi menor ($p < 0,05$), exceto para spray-dry. Por cromatografia líquida de alta eficiência por exclusão (HPLC-SEC), foram determinadas as proporções de proteinas poliméricas (majoriatariamente gluteninas) e proteínas monoméricas (majoritariamente gliadinas) totais nas diferentes frações proteícas, sendo elas, proteínas poliméricas totais (TPP), proteínas poliméricas não extractívies (UPP) e proteínas poliméricas extractíveis (EPP). Neste contexto, foi possível separar as gluteninas em poliméricas com maior massa molecular, e as gliadina em monoméricas com menor massa molecular. Por HLPC-SEC, gluteninas e gliadina apresentaram a mesma proporção, mas essa proporção foi alterada após os tratamentos em TPP. Em UPP e EPP o mesmo aconteceu, exceto para ultrassom e spray-dry. De modo geral, houve um aumento de proteínas monomericas e diminuição das poliméricas. Por HPLC em fase reversa (RP-HPLC) foi possivel separar as gluteninas em alta (HMW-GS) e baixa (LMW-GS) massa molecular e as gliadinas em γ-, α/β- e ω-gliadina. Foi observado que tanto HMW-GS quanto LMW-GS diminuem após o tratamento térmico, exceto para ultrassom e spray-dry. Em relação as gliadinas, apenas na fração das ω-gliadinas houve aumento significativo.

Esses dados levaram a concluir que o tratamento térmico alterou a estrutura/complexidade das proteína que compõe o glúten deixando mais solúveis. Os tratamentos que envolvem aquecimento utilizados levaram a alterações na conformação das proteínas que compoem o glúten, as quais foram vistas pelas alterações na solubilidade e perfis de proteínas, através de um rearanjo especial onde pode ter ocorrido uma maior exposição de grupos hidrofóbicos, tornando as proteínas do glúten mesnos solúveis ou ainda, pode ter alterado as ligações covalentes levando a uma estrutura mais complexa e menos solúvel. Uma vez que a os tratamentos resultaram em menor solubilidade, exceto para ultrassom. Adicionalmente, os perfis de gluteninas e gliadinas foram alterados, sendo a percentagem de HMW-GS diminuido após os tratamentos de spray-dry, forneamento e extrusão, enquanto que a percentagem de LMW-GS também dimunuiu após os tratamentos de spra-dry, microondas, forneamento e extrusão. Em contraste, a percentagem de ω-gliadina aumentou após o tratamento por ultrassom, já a percentagem de α/β-gliadinas diminuiu após os todos os tratamentos tratamentos, enquanto que as γ-gliadinas não foram afetadas pelos tratamentos. Os tratamentos de spray-dry e ultrassom também diminuiram a digestibildade das proteínas do glúten. E, finalmente, a concentração de epitopos potencialmente tóxicos para celíacos diminuiu após spray-dry quando testado com anticorpo R5, e após spray-dry e microondas com o anticorpo G12, em relação a farinha controle. Por fim, mesmo com alterações na solubilidade e provalmente na estrutura das proteínas que compõem o glúten, estas alterações não são suficientes para produzir um produto seguro para celíacos, uma vez que a quantidade de epitopos potencialmente tóxicos para celíacos ainda é muito alta.

II – DIFERENTES VARIEDADE DE TRIGO SÃO TÓXICAS PARA CELÍACOS – ESTUDO *IN VITRO*

O trigo moderno tem genoma AABBDD, o qual foi alcançado após poliploidismo, resultande da hidridização de espécies com genosma AA, BB e DD. Atualmente, as variedades com genoma AABB têm grande apelo comercial. Assim, variedades antigas e modernas foram testadas para verificar se podem ser consumidas de maneira segura por celíacos. Foram testadas diferentes variedades de trigo, sendo duas variedades com genoma AA - *T. monococcum* ssp *monococcum* e *T. urartu*, e devido as características de plantio, alto rendimento e apelo commercial, foram usadas 5 variedades com genoma AABB - *T. turgidum ssp durum*, *T. turgidum ssp polonicum*, *T. turgidum ssp turgidum* and *T. turgidum ssp turanicum*. Todas as amostras de trigo foram moídas e as proteínas foram extraídas, separadas em gel bidimensional, coradas com Coomassie blue ou transferidas para membrana de nitrocelulose para analises de Western

blot contra dois anticorpos: Anti-Gliadina a Anticorpo de Skerritt. Todas as membranas e géis foram digitalizados e as imagens sobrepostas. Ambos os anticorpos se ligaram às frações tanto de gliadina como de glutenina. Pode-se observar grandes diferenças entre as variedade com diferentes genomas. As variedade AA testadas apresaram maior imunogenicidade que as variedades AABB. Mas, embora algumas das variedades antigas apresentaram menor reatividade, ainda são tóxicas para celíacos e devem ser evitadas pelos mesmos. Desfazendo assim, o mito que as variedades antigas podem ser consumidas por celíacos.

III – CONCORDÂNCIA ENTRE PRODUTOS ROTULADOS COMO SEM GLÚTEN E LEGISLAÇÃO DE ALIMENTOS PARA PRODUTOS BRASILEIROS INDUTRIALIZADOS

A cada ano cresce o número de pessoas diagnosticadas como celíacos e, consequentemente, a adesão a uma dieta sem glúten bem como de seus familiares. Atualmente, a única terapia disponível para celíacos é adotar uma dieta "sem glúten" por toda a vida. Aliado a isso, há uma tendência mundial em seguir dietas sem glúten com o objetivo de perder peso, o que não é verdadeiro do ponto de vista nutricional. Todo esse cenário, levou a expansão do mercado de produtos sem glúten. A veracidade dos rótulo é um ponto importante para quem necessita seguir um dieta isenta de glúten. Assim, esse trabalho objetivou verificar se os produtos alimentícios comercializados no Brasil estão corretamente rolutados em relação a presença de glúten, usando o método ELISA R5. Em relação aos produtos vendidos no Brasil, os resultados mostraram que as empresas estão testando seus produtos a fim de verificar se o alimentos ofertado é seguro para celíacos. Entretanto, apenas 89 % dos produtos analisados, rotulados como sem glúten, estão corretos, ou seja, 11 % estão contaminados com glúten, representando um risco para a saúde dos consumidores celíacos. Para melhorar este resultado, há necessidade de maior pressão por parte da comunidade celíaca, aliado a uma verificação por parte dos governos. Pois, o Brasil tem assumido cada vez mais um papel de destaque na economia mundial, sendo necessário estar alinhado com as legislações vigentes, neste caso, em relação à presença ou ausência de glúten nos produtos comercializados.

Contribuições da Tese

De uma maneira geral esta Tese contribuiu para mostrar que o tratamento térmico afeta a estrutura e a imunorreatividade das proteínas do glúten, tornando as proteínas menos solúveis e menos tóxicas. Entretanto, essa redução ainda é capaz de causar danos em celíacos.

Também, que apesar de um apelo comercial as variedades antigas, pelos menos as amostras testadas neste trabalho, mesmo sendo menos reativas, ainda são tóxicas para celíacos e não devem ser consumidas pelos mesmos. Por fim, esta Tese demosntrou que 11 % dos produtos alimentícios comercializados no Brasil e analisados por este estudo, estão com níveis de contaminação com glúten, acima do considerado seguro para celíacos.

Conclusão

A farinha de trigo comercial foi submetida aos diferentes tratamentos, e foi possivel observar que os tratamentos alteraram as características da farinha. Houve um perda muito grande de proteína por spra-dry. E de maneira geral a solubilidade diminuiu. O perfil de gluteninas e gliadinas foi alterado. E houve redução na toxicidade. Entretanto, essa alterações não foram suficientes para produzir uma farinha de trigo segura para celíacos.

Em relação as diferentes variedade de trigo, *Triticum monococcum ssp monococcum* (genoma AA), *Triticum urartu* (AA), *Triticum turgidum ssp durum* (AABB), *Triticum turgidum ssp polonicum* (AABB), *Triticum turgidum ssp turgidum* (AABB), *Triticum turgidum ssp turanicum* (AABB), foi possível desfazer o mito que as variedades antigas não são tóxicas para celíacos e devem ser evitadas pelos mesmos.

Em relação aos produtos comercializados no Brasil como glúten-free, foi possível verificar que estamos no caminho de produzir produtos seguros, mas ainda temos 11 % de produtos que representam um risco para celíacos.

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LIST OF ABREVISTIONS AND ACRONYMS

CC - Instant Chocolate Powder and Candies
CD - Coeliac disease
CER - Corn flakes and granola
CG - Contain Gluten
CM - Cookies, and muffins
DH - Dermatitis Herpetiformis
EMA - Anti-endomysial antibodies
EPP - Extractable Polymeric Protein
FDA - Food and Drug Administration
FFA - Flours, *farofas* (toasted manioc flour mixtures) and starches
GA - Gluten Ataxia
GF - Gluten-Free
GR - Grains
GRD - gluten related disorders
HMW-GS - High Molecular Weight Glutenin
HPLC - High-Performance Liquid Chromatography
IPP - Insoluble Polymeric Protein
LMW-GS - Low Molecular Weight Glutenin
MHC - Major Histocompatibility Complex
MIX = Baking Mixture and Instant Products
MW - Molecular Weight
NCGS - Non-Celiac Gluten Sensitivity
NL - Not Label
OUT - Others
PAS - Pasta
PC - Potato Chip
QQPFP - glutamine-glutamine-proline-phenylalanine-proline
SBAN - Brazilian Society for Food and Nutrition
SDS - Sodium Dodecyl Sulfate
SG - Gluten sensitivity
SNK - Snacks
SPP – Soluble Polymeric Protein
SS - seasoning, herbs and spices
TP - Total Protein
TPP - Total Polymeric Protein
tTG - Anti-tissue transglutaminase
UPP - Unextractable Polymeric Protein
WA - Wheat allergy
WDEIA - wheat dependent, exercise-induced anaphylaxis

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1 INTRODUCTION

Gluten is the name given to a protein complex formed when flour and whater are mixed, this proteins are found in wheat, rye and barley. In this study, were consider wheat as gluten source, due its economical, technological and cultural importance. From wheat flour a large variety of baked products can be made.

When wheat flour is mixed with water to form dough, the proteins form a continuous gluten network which confers viscoelasticity and enables the dough to be processed into bread, and similar leavened food products (SHEWRY et al., 2001). The gluten network is composed of multiple subunits of prolamin and glutelin (WIESER, 2007). Prolamins are storage proteins found in wheat (gliadin), barley (hordein), rye (secalin) (SHEWRY; HALFORD, 2002) and some oats (avenins) varieties (SILANO et al., 2014).

During the baking process, the effects of heat on proteins are generally determined by changes in solubility, rheology (viscoelastic properties), conformation, interfacial behavior, distribution and reactivity of sulfhydryl and disulfide groups, which can be determined using gel electrophoresis and HPLC patterns and baking performance (HAYTA; ALPASLAN, 2001).

Due to nutritional characteristics and potential in sustainable agriculture there is growing interest in ancient wheats, such as einkorn (AA genome), emmer, Khorasan (Kamut), and spelt (ABDEL-AAL; HUCL, 2014).

Baking products, mainly bread, have been related to cultural and religious aspects worldwide and throughout the ages. However, there currently exists a concern about wheat consumption.

Since wheat is widely consumed all over the world, its potential to cause disease is a matter of concern (SCIBILIA et al., 2006).

Digestion-resistance of gluten peptides are the primary trigger of the immune response in the celiac disease, one of the most common immune based diseases in present-day society (RIBEIRO et al., 2016). Immune-mediated disease triggered by gluten ingestion include celiac disease, gluten ataxia and dermatitis herpetiformis (SAPONE et al., 2012). Besides, immune-mediated, there are others responses to gluten and wheat proteins, as well as allergic and sensitivity responses (BATTAIS et al., 2005; CATASSI et al., 2013). These were classified as gluten related disorders (SAPONE et al., 2012).

The only effective therapy is to assume a gluten-free (GF) diet (CATASSI et al., 2013; COSNES et al., 2008; LEWIS et al., 2011),

except in the case of wheat allergy when it is necessary to avoid all wheat proteins (CATASSI et al., 2013; SCIBILIA et al., 2006).

With an increase in diagnostics and in the adoption of GF diet the demand for product variety has led to an exponential increase in the number of food companies that manufacture GF products in Brazil and around the world (LEITE, 2015; SEBRAE 2014; ACELBRA 2015; THE ECONOMIST 2016; MARKETS AND MARKETS 2015). Gluten free market reach US$ 2.84 billions in 2014 (TRANSPARENCY MARKET RESEARCH, 2015).

As a result, the high demand provokes new regulations to give more security to GF consumers. Some countries have their own regulations, but in general the *Codex Alimentarius* is followed.

Due to the importance of wheat in Brazil, it is necessary to have more studies about labelling of gluten-free products sold in Brazil, as well as how different wheat varieties and heat treatment affect the immune response in celiacs.

This study was divided in 3 phases:

Phase 1 - Effect of thermal and others treatments treatment on wheat gluten proteins and their effect on digestibility and in vitro response.

Phase 2 - *In vitro* response of commercial antibodiesto modern and ancient wheat varieties.

Phase 3 - Compliance with gluten-free labelling regulation in the Brazilian food industry.

1.1 OBJECTIVES

The aim of this research project is to study the effect of wheat treatment and the different varieties of wheat on the gluten proteins and the implications in the immune response *in vitro.*

1.1.1 Specific objectives

Phase 1

Submit Brazilian commercial wheat flour to spray-dry, oven, ultrasound and microwave processing.

Analyze changes on wheat flour proteins before and after treatments: in relation to solubility, gliadin and glutenin profile, *in vitro* digestibility and immunotoxicity by ELISA and Western blot methods.

Phase 2

Acquire and mill different wheat varieties: *T. monococcum ssp monococcum*, *T. urartu*, *T. turgidum ssp durum*, *T. turgidum ssp polonicum*, *T. turgidum ssp turgidum*, *T. turgidum ssp turanicum.*

Analyze if different wheat varieties can cause *in vitro* changes of the immune response, using commercial antibody techniques.

Phase 3

Verify if food products sold in Brazil are correctly labeled in relation to the presence or absence of gluten.

1.2 STRUCTURE OF THE THESIS

This document is organized as follows:

In Chapter 2 - Importance of wheat gluten, some fundamental concepts on the origin, economic, and cultural importance of wheat are presented, as well as discussions concerning the nutritional impact of wheat proteins on immuno-mediated response in susceptible people, as well as a global view about GF product, diet and labeling.

Chapters 3, 4 and 5 are composed of the main contributions of this thesis, the results of which have been published or submitted in international conferences and journals. Where, Chapter 3 – Effect of heat treatment on gluten proteins and *in vitro* toxicity, Chapter 4 – Wheat varieties are toxic to celiac – *in vitro* study, and, Chapter 5 – Compliance with gluten-free labeling regulation in the Brazilian food industry

In Chapter 6 some conclusions and recommendations for future research are discussed.

2 BIBLIOGRAPHIC REVIEW

The term gluten refers to the proteins able to form a network composed of multiple subunits of prolamin (gliadin, in wheat) and glutelin (glutenin, in wheat) (WIESER, 2007). When wheat flour is mixed with water to form dough, the proteins form a continuous gluten network (SHEWRY et al., 2001). This gluten network determines the unique baking quality of wheat by conferring water absorption capacity, cohesivity, viscosity and elasticity to the dough (WIESER, 2007), enabling it to be processed into bread, and similar leavened foods (SHEWRY et al., 2001).

2.1 WHEAT

Bread wheat (*Triticum aestivium*, genome AABBDD) is one of the most important crops in the world and is a staple food throughout the temperate world and an important source of nutrients for many millions of people (LING et al., 2013). From wheat flour a large variety of bakeries products can be made (JIANG et al., 2008).

Ancient wheat species were replaced at various times with domesticated species that fit better with evolving farming and processing technologies (ABDEL-AAL; HUCL, 2014). The hexaploid wheat genome was formed from multiples hybridization events between different progenitor species, resulting in three subgenomes: A, B and D in modern wheat (*Triticum aestivium*) (EVERSOLE et al., 2014).

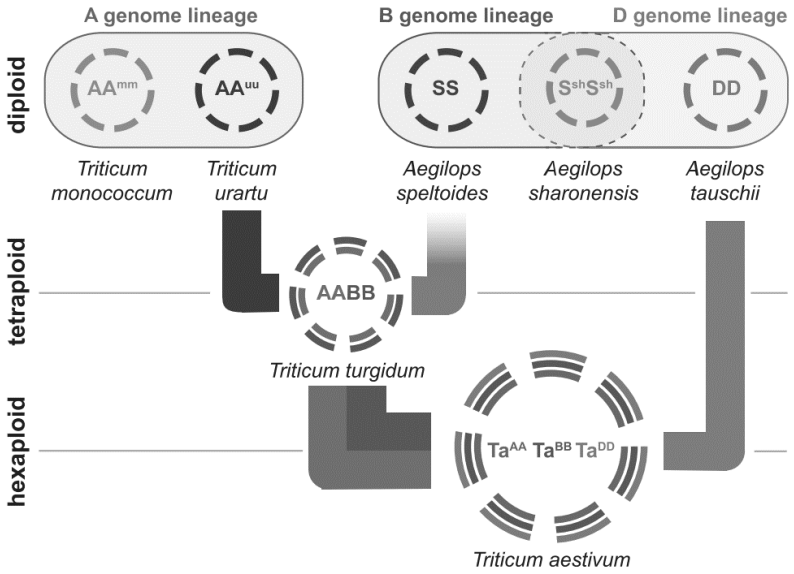
The first cultivation of wheat occurred around 10 000 years ago, as part of the 'Neolithic Revolution', when a transition from hunting and gathering of food to settled agriculture happened (SHEWRY, 2009). It has been utilized as a food grain since the Stone Age (6700 b.c.) and cultivated since 5,000 B.C. (ABDEL-AAL; HUCL, 2014).

These earliest cultivated varieties were diploid (genome AA - *einkorn*) and tetraploid (genome AABB - *emmer*) wheats and their genetic relationships indicate that they originated in Turkey. The earliest cultivated forms of wheat were selected by farmers from wild crops presumably because of their superior yield and reduced loss of the spike at maturity, which results in seed loss at harvesting (SHEWRY, 2009).

The A genome, as a basic genome of bread wheat and other polyploid wheats, for example, *T. turgidum* (AABB) and *T. timopheevii* (AAGG), is a key to wheat evolution, domestication and genetic improvement (LING et al., 2013).

The progenitor species of the A genome is the diploid wild einkorn wheat *T. urartu*, which has a closer resemblance to cultivated wheat than *Aegilops speltoides* (BB, related with SS genome) and *Ae. tauchii* (DD), especially in the morphology and development of spike and seed (LING et al., 2013; SHEWRY, 2009; VENSEL; ADALSTEINS; KASARDA, 1997). The hybridization process started before domestication, the firt hybridization is estimated to have occurred several hundred thousand years ago between two diploid *Triticum urartu* (AA) and *Aegilops speltoides* (donator of the B genome – resemble S genome) resulting in the allotetraploid *Triticum turgidum* (AABB), a wild ancestor of modern *T. turgidum* sp. *Durum*. Next hybridization was between *Triticum turgidum* (AABB) and *Aegilops tauschii* (DD) resulting the ancestral allohexaploid *T. aestivum* (AABBDD) (Figure 2.1) (MAYER et al., 2014).

Figure 2. 1. Schematic diagram of the relationships between wheat genomes with polyploidization history and genealogy.



Source: Mayer et al. (2014).

Each of the three different genomes that have become additively combined in hexaploid wheat is likely to have contributed different properties to it. For example, breadmaking quality could be one of the most important contributions (VENSEL; ADALSTEINS; KASARDA, 1997).

About 95 % of the wheat grown worldwide is hexaploid wheat (*T. aestivum*), with most of the remaining 5 % being tetraploid durum wheat (*T. turgidum*). However, other wheat species (*einkorn*, *emmer*, *spelt*) are still grown in some regions including Spain, Turkey, the Balkans, Indian subcontinent and some other regions of Europe (SHEWRY, 2009).

The wheat provides approximately 20 % of calories consumed by humans (PFEIFER et al., 2014).

In Brazil, wheat is cultivated in the Southern (Rio Grande do Sul, Paraná, Santa Catarina) and Center-West (Mato Grosso, Goiás, Distrito Federal) areas, but 90 % of total production is located in Parana State (CONAB, 2014).

The national estimate is that the area planted in 2016 was roughly 2.1 million hectares and production around 6.2 million tons (CONAB, 2014).

Brazilian wheat consumption for 2017 is projected to be 10.5 million tons, 2 % higher than the previous crop 2015/2016. To overcome this demand, good quality wheat grain might is imported from Argentina (64 % of total imported), Paraguay (17.8 %), Uruguay (9.1 %), USA (8 %) and Canada (0.6 %) (CONAB, 2016).

Beyond adaptation and agronomical characteristics of wheat, the main property of wheat is gluten protein and it´s unique ability of dough formation (WIESER, 2007).

Wheat is also used in a range of other products such as unleavened breads, cakes, and biscuits, pasta (from durum wheat), and noodles (JIANG et al., 2008), and thus it present as the main source of nutrients for most of the world´s population (ŠRAMKOVÁ; GREGOVÁ; ŠTURDÍK, 2009).

Nowadays, there is growing interest in ancient wheats, such as einkorn, emmer, Khorasan (Kamut), and spelt, because their potential in sustainable agriculture and special nutritional aspects (ABDEL-AAL; HUCL, 2014). The use ancient wheat varieties as nontoxic varieties for celiacs has been suggested (MARIETTA; MURRAY, 2012).

Some studies with ancient varieties showed different results on this topic. One study showed that *T. monococcum* has high immunogenicity property on polyclonal CD intestinal T-cell cultures comparable to *T.*

aestivium (GIANFRANI et al., 2015). Another, with celiac patients, *T. monococcum* was better tolerated than *T. aestivium* (ZANINI et al., 2015).

There are a relationship between the *T. aestivum* and ω-gliadins and the *T. urartu* ω-gliadins (DUPONT et al., 2004). Gliadins present high immunotocixty to celiac (TYE-DIN et al., 2010).

Tetraploid wheat varieties, e.g. *T. turgidum*, showed lower toxicty to celiacs (RIBEIRO et al., 2016).

Nonetheless, positive significant correlations were observed for ω- and γ-gliadins, and R5 reactivity, in spelt and tetraploid wheat varieties, respectively, explaining approximately 40 % of the variation in the case of tetraploid varieties. This is of particular interest because it was described that a α-gliadin/C-hordein – derived peptide may be considered the canonical dominant T cell – stimulatory peptide in HLA-DQ2–associated celiac disease (RIBEIRO et al., 2016; TYE-DIN et al., 2010).

2.2 WHEAT GLUTEN PROTEINS

Protein is considered the most important nutrient for humans and animals, as manifested by the origin of its name, from the Greek *proteios* for primary (ŠRAMKOVÁ; GREGOVÁ; ŠTURDÍK, 2009). A protein is characterized by three properties: amino acid sequence, three-dimensional structure and function (KOLODNY et al., 2013).

A protein consists of several domains and there are many examples of different ways of combining the same domains in different protein chains (KOLODNY et al., 2013).

Cereal proteins were classified by Osbourne according to extractability and solubility in: albumins - water soluble; globulins - salt solubles; prolamins - alcohol soluble and; glutelin acid- and alkali-soluble proteins (DELCOUR; HOSENEY, 2010). Prolamins are the major endosperm storage proteins of all cereal grains, they represent around 50 % of total protein (SHEWRY; HALFORD, 2002).

Gluten is the name given to a particulate prolamins (50 %) and particulate glutelins (50 %) that when mixed, form a continuous protein network, which confer the unique baking quality cohesivity, viscosity and elasticity on dough (WIESER, 2007). In the wheat, prolamins are refered as gliadina and glutelin as glutenins (WIESER, 2007).

The protein composition of wheat flour is complex (DUPONT et al., 2004). End-use quality of bread wheat (*Triticum aestivum*) is influenced by the composition of gliadin and glutenin (LAGRAIN et al, 2014). Due to its functionality, in nutritional and technological aspects,

these proteins are largely explored in wheat (SHEWRY, 2009; SHEWRY; HALFORD, 2002; WIESER, 2007).

They are unique in terms of their amino acid composition, which is characterized by high contents of glutamine and proline and by low contents of amino acids with charged side groups (WIESER, 2007). Besides, a small amount of cysteine residues that are extremely important to structure and funcionatily of gluten, are present in an oxidized state and form either intrachain disulfide bonds within a protein or interchain disulfide bonds between proteins (KECK; KÖHLER; WIESER, 1995), as well as, tyrosine–tyrosine crosslinks covalent bonds that give raise to the gluten network formed during breakmaking (TILLEY et al., 2001).

The exact number of gluten protein has not been determinated, but it is belivied to be a complex mixture of several hundred proteins (HURKMAN et al., 2013).

2.2.1 Gliadins

Glidins represent 50 - 60 % of gluten proteins. The classification depends on the methodology used, but they are usually divided according to their mobility in electrophoresis, terminal amino acid sequence and sulphurcontent (rich or poor). Characterization by electrophoretic divides as α-, β-, γ-, ω-gliadin. The molecular weight varies from 30 to 74 kDa (JOHANSSON et al., 2013) (Table 2.1). ω-gliadins are classified as S-poor, because they have fews cysteine residues in their aminoacid sequence, whereas, α/β- and γ-gliadins are S-rich, due higher content of cysteines located in the C-terminal domain (WIESER, 2007).

Gliadins are encoded on the A, B and D genome (PFEIFER et al., 2014), and amino acid sequence analysis of gliadin show that the different classes have a high degree of homology particulary within the central domain (JOHANSSON et al., 2013).

Gliadins are monomeric proteins (MACRITCHIE et al 2014), stabilized by intramolecular disulphide bonds between cysteine residues (SHEWRY and TATHAM, 1997). These cysteine residues are present in α/β-type and γ-type gliadins and absent in ω-gliadin (SHEWRY and TATHAM, 1997). The ω-gliadins present high contents of glutamine, proline and phenylalanine which together account for around 80% of the total composition, with repetitive sequences (WIESER, 2007).

Table 2. 1. Characteristics of gliadins.

Gliadins - monomeric					
α/β-gliadin		γ-	ω-		
α-	β-		ω_1-	ω_2-	ω_5-
S-rich			S-poor		
N- and C-terminal domain non-repetivive. repetitive central domain		N- and C- terminal domain non-repetivive. heptapeptide repetitive central domain	N- and C- terminal domain non-repetivive. repetitive central domain		
MW 30 – 45 kDa			MW 46 – 74 kDa		

Source: JOHANSSON et al. (2013).

The gliadins when hydrated contribute to dough viscosity and extensibility (SHEWRY, 2009; WIESER, 2007). Addictionaly, hydrogen bond binds α/β- and γ-gliadins with ω-gliadins (KASARDA; BERNARDIN; QUALSET, 1976). Hydrogen bonds are also responsible for binding gliadins and glutenins together (SCARDONE; FORLANI; CERLETTI, 2000; SHEWRY, 2009).

2.2.2 Glutenins

Glutenins are polymeric proteins, with a classification somewhat more complicated than gliadins. Glutenins are classified according to molecular weight into High Molecular Weight (HMW-GS) (80 – 160 kDa) and Low Molecular Weight (LMW-GS) (30 – 51 kDa) (JOHANSSON et al., 2013) that when linked form polymers of about 150 kDa to over 1,500 kDa (VENSEL et al., 2014). HMW-GS are also, classified according to genotype as x-type or y-type (each A, B and D genome encode one x- and one y-type) (LI et al., 2016). While, LMW-GS can be classified according to their N-terminal amino acid as *i*-, *s*- and *m*-LMW-GS (isoleucine, serine and methionine, respectively) (JOHANSSON et al., 2013) (Table 2.2).

The major structural characteristics of gluten proteins that have been shown to influence rheological characteristic are the number and position of cysteine residues and length and regularity of the repetitive domain. The polymeric structure is stabilized by intra- and intermolecular disulphide bonds between cysteine residue (SHEWRY and TATHAM, 1997), hydrogen interaction (JOHANSSON et al., 2013).

Table 2. 2. Characteristics of glutenins.

Glutenins - polymeric		
LMW-GS	HMW-GS	LMW-GS
i-, *s*-, *m*-, B-, C- and D-types	x-type larger	y-type smaller
S-rich		
30 – 51 kDa	80 – 160 kDa	
Non-repetitive N- and C termini, repetitive central domain	Non-repetitive N- and C termini, repetitive hexa, octa, nona-peptide central domain	

Source: Johansson et al. (2013).

Glutenin are related to the rheological properties of the dough (CHAUDHARY; DANGI; KHATKAR, 2016; LI et al., 2016; ZHANG et al., 2014).

The quantity of HMW-GS and the size distribution of glutenin polymeric protein are strongly correlated with dough properties and bread-making quality (DON et al., 2006; ZHANG et al., 2014). Hydrated glutenins contribute to dough cohesiviteness and elasticity adding stretching and volume properties to the dough (SHEWRY, 2009).

The tridimensional structure of gluten protein in not clear, because gluten protein does not from a crystalline structure, but an amorphous structure (JOHANSSON et al., 2013). Some gliadins however have been found in the glutenin polymer (VENSEL et al., 2014).

2.3 TREATMENT OF WHEAT FLOUR

Because of global, economic, and social trends, wheat-processing industries are always looking at different treatments to modify grain-quality attributes (PFEIFER et al., 2014).

Treatments that involve time, temperature and pressure can change protein structure. Specifically, high temperatures cause gluten protein degradation, mainly with lysine, but with cysteine (mainly gluten amino acid) too, and it may affect gluten protein types and somehow this can affect autoimmune response to gluten.

The effects of heat treatment on protein generally cause changes in solubility, rheology (viscoelastic properties), conformation, interfacial behavior, distribution and reactivity of sulfhydryl and disulfide groups,

gel electrophoresis, HPLC patterns and baking performance (HAYTA; ALPASLAN, 2001).

To better understand these possible alterations, different treatments with varying temperatures, times and pressures were tested: extrusion, spray-dry, ultrasound, microwave radiation and oven treatments.

2.3.1 Extrusion

Extrusion is a thermal process that combines different unitary steps: homogenization, cooking, mixing and molding applied to food preparation (SINGH; GAMLATH; WAKELING, 2007). This process involves the application of high heat, high pressure, and shear forces (SINGH; GAMLATH; WAKELING, 2007; ZHANG; BAI; ZHANG, 2011) resulting in improving digestibility through the destruction of antinutritional factors, such as, trypsin inhibitors, tannins and phytates, all of which inhibit protein digestibility (FELLOWS, 2006; FERNANDES et al., 2003; SINGH; GAMLATH; WAKELING, 2007).

During extrusion some amino acid residues are affected, in part due to the Maillard reaction (chemical reaction involving amino groups and carbonyl groups, which are common in foodstuffs, and leads to browning and flavor production), and in part due other factors such as, acrylamide formation and hydrolysis (SINGH; GAMLATH; WAKELING, 2007), and cross-linking of denatured proteins (ZHANG; BAI; ZHANG, 2011).

2.3.2 Spray-dry

Spray-dry is a unitary process well established for processing liquids into powders (MUMENTHALER; HSU; PEARLMAN, 1994). Spray-dry utilizes heat from a hot gas stream to evaporate microdispersed droplets created by atomization of a continuous liquid feed and is therefore a very fast and cost-effective dehydration method, but there is degradation and oxidation of volatile compounds (KESHANI et al., 2015; MUMENTHALER; HSU; PEARLMAN, 1994).

Keshani et al. (2015) described a spray-dry process more efficient without heat degradation, where the spray-air contact is determined by the position of the atomizer in relation to the drying air inlet; in a co-current flow design, spray evaporation is rapid and as the drying air cools, accordingly the evaporation time is shortened; when the spray comes into contact with the drying air, evaporation takes place in the droplets until the moisture content becomes too low to diffuse through the dried droplet

surface; finally, the recovery of dried powder is carried out either in the cyclone, filter bag or electrostatic precipitator.

2.3.3 Microwave

In the microwave treatment, heat is generated by the interaction of microwaves with charged and polar molecules of food components (LESZCZYNSKA et al., 2003). The electromagnetic energy is converted to heat by ionic conduction (movement of ions in a solution under an electromagnetic field) and dipole rotation (reorientation of dipoles under microwave radiation) (WINEFORDNER, 2011).

Heating by microwaves offers some advantages such as shorter time of treatment, energy saving, high nutritional quality of treated food and lack of Maillard browning reactions (LESZCZYNSKA et al., 2003).

Furthermore, immunotoxicity of gliadin seems to decrease with an increase in energy when to wheat flour (LESZCZYNSKA et al., 2003). Other researchers, found reduced antigenicity (by 99% in comparison with the control samples), applying microwave to wheat kernels (LAMACCHIA; LANDRISCINA; D'AGNELLO, 2016).

2.3.4 Ultrasound

Ultrasound makes use of physical and chemical phenomena that are fundamentally different compared with those applied in conventional extraction, processing or preservation techniques (CHEMAT; ZILL-E-HUMA; KHAN, 2011). Ultrasound is used to degrade polymers through the effects of cavitation (CHEMAT; ZILL-E-HUMA; KHAN, 2011) which generates numerous tiny bubbles in liquid media and mechanical erosion of solids, including particle rupture (TADEO et al., 2010).

This treatment affects the secondary structure of proteins and might be disrupt intermolecular disulfide bonds (HU et al., 2013).

Ultrasound treatment leads to depolymerization of very large glutenin polymers by breaking of disulfide bonds, resulting in reduction in size of protein molecules and increased gluten solubility (HU et al., 2013; MARCUZZO et al., 2010).

2.3.5 Oven

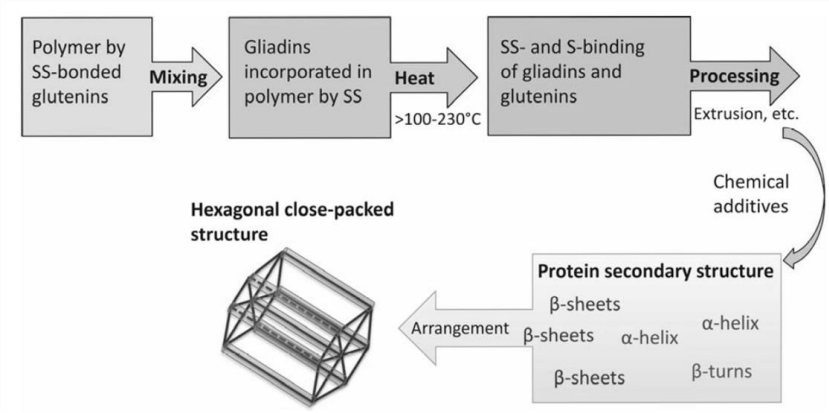
Wheat, consumed as bread, is a staple food for 30 % of worldwide population (CHOULET et al., 2014). For this study it was used traditional

chapatti method which one use just wheat and water to formulation of dough (RAO; LEELAVATHI; SHURPALEKAR, 1986).

During the dough preparation and baking the gluten proteins are kneading and a series of inter- and intra-chain bind between gluten proteins give a gluten protein network (KECK; KÖHLER; WIESER, 1995; WIESER, 2007). Additional covalent bonds formed during breadmaking are di- and iso-tyrosine crosslinks between gluten proteins, especially in gliadins (TILLEY et al., 2001).

The covalent structure of the gluten network is superimposed by non-covalent bonds (hydrogen bonds, ionic bonds, hydrophobic bonds) (Figure 2.2) (WIESER, 2007).

Figure 2. 2. Changes in the gluten proteins during the mixing, heating and extrusion.



Legend: SS = disulfide.
Source: Johansson et al. (2013).

2.4 GLUTEN RELATED DISEASE (GRD)

Most of the people, at some time, experienced an adverse reaction caused by ingestion of any kind of food.

An important case of food intolerance are those triggered by gluten, now know as gluten related disorders (SAPONE et al., 2012). These manifestations can be noticed by respiratory symptoms as well as by skin, gastrointestinal or neurological problems. These reactions are

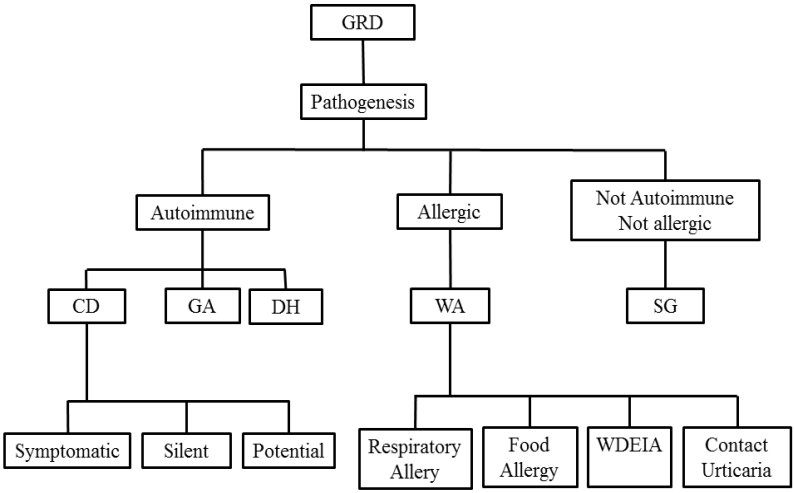
classified as immunological, non-immunological or of sensitivity (MOREIRA; LINS, 2001).

The consumption of gluten free products is beyond what the celiac population alone would consume (< 1%) (WOROSZ and WILSON, 2012).

In 2009, Shewry´s review article cited the different adverse reactions to gluten ingestion and/or contact. The same author mentioned that ancient wheats and durum wheat might be healthier.

Sapone et al (2012) reported that in addition to the celiac disease, there are other gluten related disorders (GRD) (Figure 2.3).

Figure 2 3. Nomenclature and classification of gluten-related disorders.



Legend: GFD = Gluten-free diet. CD = Celiac disease. GA = Gluten Ataxia. DH = Dermatitis herpetiformis. WA = Wheat allergy. SG = Gluten sensitivity. WDEIA = wheat-dependent exercise-induced anaphylaxis.
Source: Sapone et al. (2012).

In some way, this explains the increase of gluten-free food product consumption, aside from trends. GRD can be classified according the three main forms of reactions: autoimmune (celiac disease, dermatitis herpetiformis and gluten ataxia), allergic (wheat allergies) and possibly immune-mediated (gluten sensitivity) (KOEHLER et al., 2013; SCARDONE; FORLANI; CERLETTI, 2000; SHEWRY; HALFORD, 2002).

2.4.1 Immune-mediated response

Around 1 % of the world´s population is genetically predisposed to manifest an immune-mediated response to gluten ingestion (LINDFORS; MÄKI; KAUKINEN, 2010; MURRAY, 1999; TRONCONE; FERGUSON, 1991). Most CD patients (approximately 95 %) express genes encoding the major histocompatibility complex (MHC) class II protein HLA-DQ2 and/or DQ 8 (SCHUPPAN, 2000), located on chromosome 6p21 (SAPONE et al., 2012). However, the presence of HLA-DQ2 and/or HLA-DQ8 together represents 36 to 53 % of risk, others genes collectively contribute more than HLA to the immune-mediated response genetic background (MURRAY, 1999). Recent advances in the genetic and environmental contributions to autoimmunity suggest that interactions between genetic elements and epigenetic changes caused by environmental agents may be responsible for inducing autoimmune disease (HEWAGAMA; RICHARDSON, 2009).

Immuno-mediated response include Gluten Ataxia (GA), Dermatitis Herpetiformis (DH), and, the most know, Celiac Disease (CD) (SAPONE et al., 2012).

Individuals with dermatitis herpetiforms present skin manifestations with blistering rash and pathognomonic cutaneous IgA deposits in the lesions, inflammatory cells and cytokines are also found (SAPONE et al., 2012; SHEWRY, 2009). Dermatitis herpetiformis is characterized by intensely pruritic papulovesicular lesions that occur symmetrically over the extensor surfaces of the arms and legs as well as the buttocks, trunk, neck, and scalp (SAPONE et al., 2012). The diagnosis requires the confirmation of granular deposits of IgA in an area of normal-appearing skin, by immunofluorescence studies. The skin lesions respond to the withdrawal of gluten from the diet or to treatment with dapsone (FARREL; KELLY, 2002). A small-bowel biopsy in patients with dermatitis herpetiformis shows a mild and patchy gluten-sensitive enteropathy (FARREL; KELLY, 2002).

Gluten ataxia (GA) is a sporadic cerebellar ataxia associated with the presence of anti-gliadin antibodies (more specific anti-TG6 antibodies) without other apparent etiology for the ataxia (LINDFORS; MÄKI; KAUKINEN, 2010; MITOMA et al., 2016; SHEWRY; PELLNY; LOVEGROVE, 2016).

Widespread deposition of transglutaminase antibodies has been found around brain vessels and small intestine (SAPONE et al., 2012). Deposits have been reported also to be present in other organs such as lymph nodes, skeletal muscle and liver in untreated celiac disease patients

(LINDFORS; MÄKI; KAUKINEN, 2010). The deposition is most pronounced in the cerebellum, pons and medulla (SAPONE et al., 2012).There is evidence to suggest that there is antibody cross-reactivity between antigenic epitopes on Purkinje cells and gluten proteins (SAPONE et al., 2012).

Mitoma et al. (2016) reviewed ataxias, and described some common symptoms usually present, such as pure cerebellar ataxia or rarely ataxia in combination with myoclonus, palatal tremor or opsoclonus myoclonus. Gaze-evoked nystagmus and other ocular signs of cerebellar dysfunction are seen in up to 80 % of the cases. All patients had gait ataxia, and the majority limb ataxia (MITOMA et al., 2016).

The most typically immuno-mediated response to gluten known by people in general, is celiac disease (CD) (LEWIS et al., 2011). CD includes symptomatic cases with either classical intestinal symptoms (chronic diarrhea, weight loss and related) or non-classical extraintestinal symptoms, as well as, damage to the intestinal villi and crypt hyperplasia (SPATOLA et al., 2014). In addition, a specific serologic autoantibodies test should be performed to confirm the presence of anti-tissue transglutaminase (tTG) and anti-endomysial antibodies (EMA). All of these are characteristics of CD (DIETERICH et al, 1997; COSNES et al, 2008; MOREIRA et al, 2009). Despite small-bowel inflammation, atypical symptoms or physiological derangements such as iron deficiency anemia or osteoporosis, might be present, resulting in a low quality life of the celiacs (LEWIS et al., 2011; LINDFORS; MÄKI; KAUKINEN, 2010). Deficiencies of the fat-soluble vitamins D, E, A, and K; iron; folic acid; and calcium are also common (MURRAY, 1999).

Consumption of gluten causes the production of antibodies targeted against the enzyme transglutaminase 2 (TG2) and gluten-derived gliadin peptides, and, by deamidation, TG2 transform glutamate in glutamic acid (LINDFORS; MÄKI; KAUKINEN, 2010).

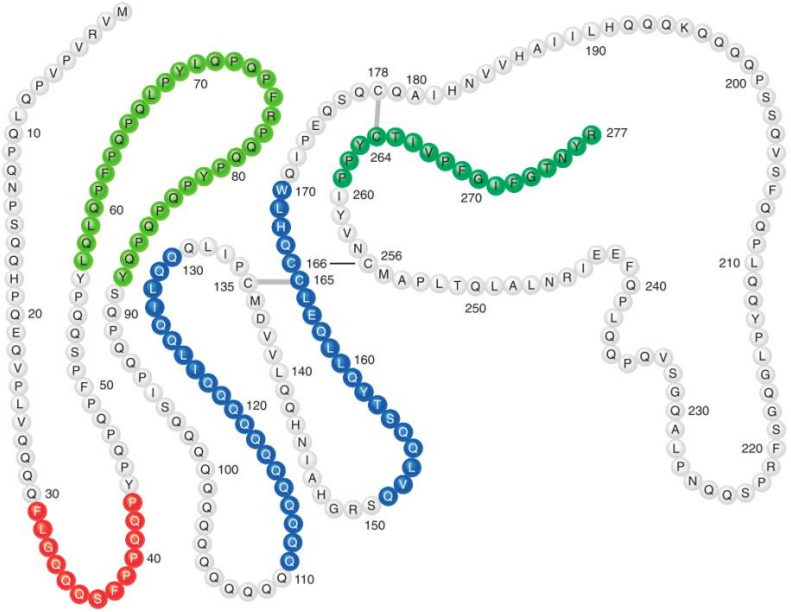
Different gliadin peptides bind with CXCR3 as the receptor that releases zonulin in a MyD88-dependent manner a, other receptors (FASANO, 2011).

When accumulated in the tissue, the deamidated immunogenic peptides bind to the DQ2 or DQ8 molecule on the antigen-presenting cells transporting them to gliadin-specific CD4+ cells in the lamina propria (LINDFORS; MÄKI; KAUKINEN, 2010). These cells become activated and begin to secrete proinflammatory cytokines that also contribute to the small-intestinal mucosal deterioration (SHEWRY, 2009). This cytokine overexpress MHC class I molecules and natural killer cells (LINDFORS; MÄKI; KAUKINEN, 2010).

The so-called toxic peptides, of which p31-43 is probably the most fully studied, modulate the small-intestinal mucosal biology via an innate inmune mechanism (LINDFORS; MÄKI; KAUKINEN, 2010)

There are more than 50 T cell-stimulatory epitopes for only one gliadin fragment, the 33-mer peptide from wheat α-gliadin is the immunodominant antigen (SHEWRY, 2009; TYE-DIN et al., 2010). There are other important epitopes from wheat a-gliadin related with celiac response (Figure 2.4) (FASANO, 2011).

Figure 2.4. Mapping of α-gliadin that modulate celiac response.



Legend: Exerting cytotoxic activity (red), immunomodulatory activity (light green), zonulin release and gut-permeating activity (blue), and CXCR3 release in CD patients (dark green). Source: Fasano (2011).

CD occurrence is increased in at risk conditions, such as a family history of CD, autoimmune diseases, IgA deficiency, some genetic syndromes (Down, Turner and William syndromes) and especially type 1 diabetes and thyroiditis (COSNES et al., 2008; FARREL; KELLY, 2002; SAPONE et al., 2012).

2.4.2 Allergic response

Respiratory allergies triggered by wheat are documented since the times of the Ancient Romans (SHEWRY, 2009), known as “baker’s asthma” (SCIBILIA et al., 2006).

Wheat allergy (WA) is defined as an adverse immunological reaction to gluten proteins, IgE- mediated reactions to wheat (SAPONE et al., 2012; SCIBILIA et al., 2006).

Food allergic reactions to wheat can give way to an array of clinical manifestations that can be immediate and/or delayed (SCIBILIA et al., 2006). WA can be classified according with the route of the allergen exposure into: classic food allergy affecting the skin, gastrointestinal tract or respiratory tract; wheat dependent, exercise-induced anaphylaxis (WDEIA); occupational asthma (baker’s asthma)/ rhinitis; and contact urticaria (BATTAIS et al., 2005; SAPONE et al., 2012)

In WA not just gluten protein cause an allergic response, but a number of other proteins, including enzymes, such as α- and β-amylase inhibitors, germ agglutinin, peroxidase and non-specific lipid transfer proteins and serpins (BATTAIS et al., 2005; LARRÉ et al., 2011; SAPONE et al., 2012; SHEWRY, 2009).

In WDEIA, allergic response leads to severe allergic reactions including anaphylaxis (SHEWRY, 2009).

2.4.3 Non-celiac gluten sensitivity

In recent years the gluten-free diet has become a trend, with some individuals self-diagnosed as non-celiac gluten sensitivity (NCGS) (BARDELLA; ELLI; FERRETTI, 2016).

The mechanism of non-celiac gluten sensitivity is not clear, but neither allergic nor autoimmune mechanisms could be identified (CATASSI et al., 2013; EL-SALHY et al., 2015). The remission of symptoms occurs naturally or with concomitant use of supplement and allopathic drugs (DICKEY, 2007). Intestinal clinical symptoms include bloating, abdominal pain, diarrhea, constipation, epigastric pain, nausea, acid reflux, aerophagia, and aphthous stomatitis are less frequent (BARDELLA; ELLI; FERRETTI, 2016). Also extra-intestinal symptoms Such as tiredness, foggy mind, headache, muscle or joint pain, arm/leg numbness, anxiety and/or depression, have been related (CATASSI et al., 2013).

It is believed that possibly NCGS could be a irritable bowel syndrome underdiagnosed (BARDELLA; ELLI; FERRETTI, 2016; EL-SALHY et al., 2015).

2.5 LABEL AND LEGISLATION

Currently available digestive enzyme supplements are ineffective in degrading immunogenic gluten epitopes (JANSSEN et al., 2015). In consequence, for all cases, the only effective therapy is to assume a gluten-free diet (COSNES et al., 2008; DON; KOEHLER, 2014).

However, some gluten-free products labeled as such may contain gluten owing to contamination with gluten-containing items during harvest, storage, processing, or transport (MARCÍLIO et al., 2005; MURRAY, 1999). For people who have a gluten-restricted diet, consumption of food products with gluten worsens their pathologic and nutritional state (MURRAY, 1999).

To garantee safe products to celiac, some contries are making efforts to news regulations in relation to presence of gluten in food products.

Considerable controversy exists regarding the definition of gluten-free food products. The term gluten-free varies from country to country. According to the U.S. *Food and Drug Administration* (FDA), any product labeled as gluten-free should contain less than 20 mg/kg of gluten; however, it is not mandatory to label any food item regarding the presence or absence of gluten in the United States (FDA, 2013).

According to the Codex Alimentarius Commission (2008), food that contains less than 20 mg/kg of gluten should be labeled as gluten-free, above 20 and up to 100 mg/kg of gluten as reduced gluten content, and above 100 mg/kg of gluten as containing gluten.

For countries in the European Community it is obligatory to label all food products regarding the presence of gluten, and they follow Codex Alimentarius (Commission of the European Communities 2009).

In 2002, Brazilian health authorities defined the resolution that all food and beverage products must be labeled regarding the presence or absence of gluten from wheat, barley, rye, oats, and their derivatives (Brasil 2002). In 2003 laws for foods and also drugs were enacted (Brasil 2003a, 2003b).

Recently, the Brazilian government launched a new clinical trial protocol and therapeutic guidelines regarding celiac disease (Brazil 2015) to help in the correct diagnosis of celiac disease and to monitor trends in nutrition.

The Brazilian Society for Food and Nutrition (SBAN) released a paper about indications of a gluten-free diet on healthy population (PANTALEÃO; ROGERO; AMANCIO, 2016).

According with Codex Alimentarius and the Working Group on Prolamin Analysis and Toxicity the enzyme-linked immunoassay (ELISA) that uses the R5 monoclonal antibody (Mendez method) is the best method for gluten determination. This method is specific for the glutamine-glutamine-proline-phenylalanine-proline (QQPFP) amino acid sequence and represents a valuable tool for the quality control of gluten-free foods (OSMAN et al., 2001).

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3 EFFECT OF HEAT TREATMENT ON GLUTEN PROTEINS AND *IN VITRO* TOXICITY

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ABSTRACT

Brazilian commercial wheat flour was submitted to spray-dry, extrusion, oven, ultrasound and microwave treatments. Solubility, monomeric and polymeric proteins and glutenin and gliadin profiles were analyzed. Also, *in vitro* digestibility and the response against potential celiac disease immune stimulatory epitopes was measured with the R5 monoclonal antibody and G12 ELISA assays. The treatments affected solubility with exception of ultrasound, all treatment showed low solubility of polymercis and monomerics proteins. Also, treatments affected glutenins and gliadins profile, the amount of the HMW-GS decrease after spray-dry, oven and extrusion and the amount of the LMW-GS decrease too after spray-dry, microwave, oven and extrusion. Wheareas for gliadin, the amount of ω-gliadin increase after spra-dry and ultrasound treatments, and the amount of α/β- gliadin decrease after all treatments, while the treatment to do not affected γ-gliadins amount. Also, digestibility decrease after spray-dry and ultrasound. This changes with decrease of solubility and changes in profile of proteins is results of rearrange of proteins during the treatments, resulting in a higher complex structure. And, finally, the potential celiac disease immune stimulatory epitopes were measured, and showed lower amount after spray-dry treatment by R5 monoclonal antibody and lower amounts after spray-dry and microwave treatment by G12 ELISA test in relation to control flour. At last, even with the alteration on the gluten structure and complexicity, these changes do not allow to produce a safe product to celiacs, the

amount of the potential celiac disease immune stimulatory epitopes still were too high.

Key-Words: Wheat, Processing, ELISA, Celiac Disease

3.1 INTRODUCTION

Wheat is one of the most important crops in the world and is a staple food throughout the temperate areas and an important source of nutrients for many millions of people (SCHEUER et al., 2011; SHEWRY; HEY, 2016).

From wheat flour a large variety of baked products can be made (WIESER, 2007), due to the ability to form a viscoelastic dough. Wheat processing quality is mainly controlled by gluten proteins (HAMER, 2003). Gluten proteins are among the most complex protein networks in nature due to numerous different components and different sizes, from dimers to polymers, with molecular weight up to millions and, due to variability caused by genotype, growing conditions and technological processes (JOHANSSON et al., 2013). They play a key role in determining the unique rheological dough properties and baking quality of wheat (HAMER, 2003; JIANG et al., 2008; LÁSZTITY, 1986; SHEWRY; HALFORD, 2002). The two main protein components that determine the technological characteristics of wheat flour are the gliadins and glutenins (gluten proteins) (HAMER, 2003).

Gliadins are monomeric proteins soluble in alcohols, subdivided according with the methodology used but in general, gliadins are divided into α, β, γ, ω-gliadin (according to their mobility in electrophoresis), sulfur rich or poor (S-rich or S-poor), with molecular weight from 30 – 74 kDa (JOHANSSON et al., 2013). Glutenins are polymeric proteins, with a classification somewhat more complicated than gliadins. Glutenins are classified according to molecular weight into High Molecular Weight (HMW-GS) (80 – 160 kDa) and Low Molecular Weight (LMW-GS) (30 – 51 kDa) and according to genotype as x-type or y-type. There are 7-16 LMW-GS in each genotype and they can be classified according to their N-terminal amino acid as *i*-, *s*- and *m*-LMW-GS (isoleucine, serine and methionine, respectively) (JOHANSSON et al., 2013). The quantity of HMW-GS and the size distribution of glutenin polymeric protein are strongly correlated with dough properties and bread-making quality (DON et al., 2006; ZHANG et al., 2014).

Processes that involve time, temperature and pressure can change protein structure. The heat process can affect technological properties and reduce the allergenicity to varying extents in wheat flours and breads

(SUDHA; SOUMYA; PRABHASANKAR, 2016). With high temperature protein degradation occurs, mainly involving cysteine and lysine amino acids (ROMBOUTS; LAGRAIN; DELCOUR, 2012). Immuno-reactivity of gliadin decreases after microwave irradiation (LESZCZYNSKA et al., 2003). Ultrasound treatment leads to depolymerization of very large glutenin polymers by breaking disulfide bonds, resulting in a reduction in size of the proteins and increased gluten solubility (HU et al., 2013; MARCUZZO et al., 2010). During dough preparation and baking the large glutenin polymer is characterized by three competitive redox reactions: (1) the oxidation of free SH groups which support polymerization; (2) the presence of chain 'terminators' that stop polymerization and (3) SH/SS interchange reactions between glutenins and thiol compounds such as glutathione that depolymerize polymers (KECK; KÖHLER; WIESER, 1995; WIESER, 2007). Additional covalent bonds formed during breadmaking are di- and iso-tyrosine crosslinks between gluten proteins, especially in gliadins (TILLEY et al., 2001), and lanthionine which is formed during production of hard pretzels. The covalent structure of the gluten network is superimposed by non-covalent bonds (hydrogen bonds, ionic bonds, hydrophobic bonds) (ROMBOUTS et al., 2012).

Yet, digestion-resistance of gluten peptides is the primary trigger of the immune response in celiac disease, one of the most common immune based diseases in present-day society (RIBEIRO et al., 2016). Immune-mediated diseases triggered by gluten ingestion include celiac disease (CD), gluten ataxia, and dermatitis herpetiformis (SAPONE et al., 2012). Genetic studies have implicated that the presence of HLA-DQ2/8 loci is responsible for 20 - 40 % of CD cases, non-HLA locus contributes with 15 % of risk in CD development, currently 43 loci that collectively explain some 50% of the genetic predisposition for CD have been identified (WITHOFF et al., 2016). The most common symptoms include malnutrition, diarrhea, growth retardation, anemia, and fatigue (COSNES et al., 2008) resulting from inflammatory injury to the mucosa of the small intestine after the ingestion of gluten (FARREL; KELLY, 2002) and is characterized by a dysregulation of gluten-specific T-cell responses (FREITAG et al., 2009). Besides, CD, gluten related disorders (GRD) include Wheat Allergy, which is defined as an adverse immunologic reaction to gluten proteins, and Gluten Sensitivity a kind of reaction to gluten ingestion in which neither allergic nor autoimmune mechanisms (CATASSI et al., 2013; EL-SALHY et al., 2015; SAPONE et al., 2012).

Different gliadin types (α/β-type, ω-type and γ-type gliadins) (RIBEIRO et al., 2016; TYE-DIN et al., 2010) as well as glutenins

(KONAREV et al., 2011) have been shown to have an important and variable role in the pathogenesis of the disease and elaboration of a hierarchy of T-cell–stimulating peptides (CATASSI et al., 2007; FARREL; KELLY, 2002; FREITAG et al., 2009).

The aim of this study was to provide a better understanding of changes in gluten promoted by processes. Changes in solubility, monomeric and polymeric protein distribution and glutenins and gliadins were analyzed. Also, digestibility and the response against to the potential celiac disease immune stimulatory epitopes were measured with the R5 monoclonal antibody and G12 ELISA assays.

3.2 MATERIAL AND METHODS

Brazilian commercial wheat flour (*Triticum aestivum*), used as control, from Cooperativa Agrária Agroindustrial, de Guarapuava, Paraná – Brasil, 2012 harvest was tested before and after the following treatments:

Extrusion: Pilot scale extrusion processes were carried out under optimal operating conditions for commercial wheat using a single screw MX40 pilot extruder (INBRAMAQ, Ribeirão Preto, Brazil). The extrusion conditions were adjusted and based on upon previous experience. The processing conditions of extrusion were as follows: the temperature of extrusion in the barrel head was 120 °C, there was a water addition of 30 % in relation to flour, and the screw speed was 220 rpm. The flow rate was about 20% of the nominal capacity and amounted to 50 kg/h. The L/D ratio was 2.3:1; the screw diameter was 92.5 mm and the processing barrel length was 210 mm. The diameter of 10 circular nozzles was 3 mm. The dough dosage to the screw was 40% and to the barrel 50 %. Extrusion were made in aquaculture nutrition laboratory (LABNUTRI) of department of aquaculture at UFSC.

Spray-dry: Flour was suspended in 90 % of water (w/v) and dried in spray-dryer. The suspensions were atomized using a Mini Spray-Dryer B-290 (BÜCHI Labortechnik AG, Postfach, Switzerland). The drying parameters were set as follows: inlet air temperature at 130 °C; air flow of 30 mL/min; aspiration capacity at 100 %; and pump capacity at 25 %. Spray-dry were made in Laboratory of Rheology (REOLAB), Department of Food Science at UFSC.

Oven: Chapatti dough was made with 200 g of flour and enough water previously determined (SCHEUER et al., 2014). The dough was hand mixed and approximately 40 g of dough was rounded to uniform thickness (3.0 mm) and placed in an oven at 250 °C for 5 min (HARIDAS

HAO; LEELAVATHI; SHURPALEKAR, 1986). Chapatti were made at Cereal science laboratory (LABCERES), Department of Food Science at UFSC

Ultrasound: Wheat flour was suspended in water 90 % (w/v) and sonicated at a frequency of 20 kHz, 500 W of power, at room temperature for 1 h, using an ultrasound Quimis 03360 (QUIMIS APARELHOS CIENTÍFICOS, Diadema, Brasil) (MARCUZZO et al., 2010). Ultrasound were made in the laboratory of food analysis (LABCAL), Department of Food Science at UFSC.

Microwave: Wheat flour was suspended in water 90 % (w/v) and exposed to microwave radiation in a laboratory microwave for 5 min at 500 W (LESZCZYNSKA et al., 2003). Ultrasound were made in the laboratory of food analysis (LABCAL), Department of Food Science at UFSC

After all treatments, the samples were lyophilized before proceeding with analysis.

Lyophilization and analysis describe below were performed in Grain Quality & Structure Research Unit (CGAHR) at USDA-ARS Center for Grain and Animal Health Research.

3.2.1 Determination of Total Protein (%TP) – LECO

Control Flour and treated flours were analyzed by nitrogen combustion (Leco FP-428 nitrogen determinator (St. Joseph, MI USA) according to AACC method 46-30.01 (AACC, 2000).

3.2.2 Determination of percentage of insoluble polymeric protein (%IPP) and monomeric and soluble polymeric protein (%SPP)

Proteins were extracted according to Bean et al., (1998) briefly, 100 mg of flour was weighed and suspended in 1 mL of 50 % 1-propanol, with shaking by vortex for 5 min. The sample was centrifuged for 5 min at 12,000 x g, this step was repeated two more times. The supernatant contains SPP and the pellet contains IPP. The pellets were lyophilized and protein content was determined by LECO.

% SPP was determined by the Equation 1:

$$\%SPP = \%TP - \%IPP \qquad \text{eq. 1}$$

3.2.3 Size exclusion HPLC

Size exclusion high-performance liquid chromatography (SE-HPLC) was used to determined monomeric and polymeric distribution of wheat protein as previously described (LARROQUE et al., 2000). Previous studies using this methodology demonstrate that the chromatogram can be divided in 3 peaks, peak 1 are polymeric glutenins (mainly HMW-GS and LMW-GS); peak 2 are monomeric gliadins (α-, β-, γ- and ω-gliadins); and peak 3 (GUPTA; KHAN; MACRITCHIE, 1993; VENSEL; TANAKA; ALTENBACH, 2014). Also, α-, γ- and ω-gliadins with odd numbers of cysteine residues are present in all fractions (VENSEL; TANAKA; ALTENBACH, 2014).

Furthermore, using this method, wheat proteins can be separated into distinct fractions according to their solubility into SDS extractable (EPP) and SDS unextractable (UPP) protein fractions (GUPTA; KHAN; MACRITCHIE, 1993).

Total Polymeric Protein (TPP), Extractable Polymeric Protein (EPP) and Unextractable Polymeric Protein (UPP) were extracted as described by Larroque et al. (2000) and Gupta; Khan; MacRitchie, (1993). Extraction procedures are described below:

TPP; 10 mg of sample was extracted with 1 mL of 50 mM sodium phosphate, 0.5 % SDS buffer pH 6.9, vortexed for 5 min followed by sonication for 15 sec at 6 W. Samples were centrifuged at 12,000 x g for 20 min, the supernatant was filtered through 0.45 µm filter and immediately heated for 2 min at 80 °C.

EPP; 10 mg of sample was extracted with 1 mL of 50 mM sodium phosphate, 0.5 % SDS buffer pH 6.9, using vortex for 5 min and centrifuged at 12,000 x g for 20 min. The supernatant was filtered through 0.45 µm filter and immediately heated for 2 min at 80 °C.

UPP; 1 mL of 50 mM sodium phosphate, 0.5 % SDS buffer pH 6.9 was added to the EPP pellet to extract remaining protein using vortex by 10 min followed by sonication for 25 sec at 6 W, and centrifuged at 12,000 x g for 20 min. The supernatant was filtered through 0.45 µm filter and immediately heated for 2 min at 80 °C.

After extraction, analysis was carried out using an Agilent 1100 HPLC (Agilent, Palo Alto, CA, USA) instrument. The protein extract (20 µL) were injected into a BioSep-SEC s4000 analytical column (300 mm length x 7.8 mm ID) (Phenomenex, Torrance, CA, USA) and run for 30 min on an isocratic gradient of 50 % water containing 0.1 % trifluoroacetic acid (TFA) and 50 % acetonitrile (ACN) containing 0.1 % of TFA at constant flow rate of 0.5 mL/min. The column temperature was

30 °C, pressure was 60 bar. Post run was 10 min. Absorbance was mesured using a UV detector at 210 nm. The relative molecular weight distributions of polymeric proteins were obtained according (LARROQUE et al., 2000).

3.2.4 Gliadin and glutenin profile by reverse phase HPLC (RP-HPLC)

Gliadin and glutenin were extracted according to the method of Fu and Kovacs (1999).

Gliadins were extracted from 100 mg flour with 1 mL of 0.3 M NaI, 7.5 % propanol by vortex for 15 min at room temperature. Following centrifugation at 12,000 x g for 5 min at room temperature, the supernatant was transferred to 0.45 um spin filter and centrifuged for 5 min at 12,000 x g at room temperature. The filtrate was collected in an HLPC vial for analysis of gliadins. Pellets were used to extract glutenins as described below:

Glutenins were extracted from the pellets of the above extraction. The pellet was washed with 1 mL water by vortexing for 15 min and centrifuged for 5 min at 12,000 x g at room temperature. The pellet was resuspended in 1 mL of 50 % isopropanol + 2 % BME, vortexed for 30 min and centrifuged for 5 min at 12,000 x g at room temperature. The supernatant was collected and proteins were alkylated by the addition of 20 uL 4-vinylpyridine (4VP) to 300 uL of extract. The sample was vortexed for 15 min, the contents were transferred to 0.45 um spin filter and centrifuged for 5 min at 12,000 x g at room temperature. The filtrate was collected in an HPLC vial for analysis of glutenins.

After extraction glutenins and gliadins were analyzed by reversed phase HPLC using an Agilent Technologies 1260 Infinity HPLC (Agilent, Palo Alto, CA, USA). Extracts (20 µL injection) were analyzed using a Jupiter C18 analytical column 5µ particle size, 300 Å pore size (250 mm length x 4.6 mm ID) (Phenomenex, Torrance, CA, USA) eluant absorbance was measured using a UV detector at 210 nm.

For gliadins, proteins were eluted using solvents (A) water containing 0.1 % TFA and (B) ACN containing 0.05 % TFA in a 25 % to 50 % linear gradient of B over 80 min at constant flow rate of 1 mL/min. The column temperature column was 70 °C. Post run was 10 min.

For glutenins, proteins were eluted using solvents (A) and (B) in a 23 to 60 % linear gradient of B over 40 min at constant flow rate of 1 mL/min. The column temperature was 70 °C. Post run was 10 min.

3.2.5 Standard *in vitro* pepsin digestibility

The protein digestibility of all samples was performed according to Mertz et al., (1984) modified by Aboubacar et al., (2001). The flour samples (200 mg) were incubated with 35 mL pepsin (porcine pepsin Sigma P-7000, activity: 890 U/mg) solution (1.5 mg/mL, 0.1 $molL^{-1}$ KH_2PO_4 pH 2.0) for 2 h at 37 °C in dry shaking. Digestion was stopped by addition of 2 mL of 2 $molL^{-1}$ NaOH. Samples were centrifuged (3220 x g for 15 min at 4 °C), the supernatants were discarded and residue washed and centrifuged twice with 10 mL of buffer (0.1 $molL^{-1}$ KH_2PO_4 pH 2.0). Undigested protein was determined by LECO. Digestibility was calculated according Equation 2:

$$\%\ digestibility = \frac{P_{total} - P_{undigested}}{P_{total} x 100} \qquad \text{Eq. 2}$$

Where:

N_{total} = Total protein

$N_{undigest}$ = Undigested protein

3.2.6 ELISA R5 and G12

The flours were analyses by ELISA R5 and G12, after gluten extraction. Flours were extracted with the Mendez Cocktail (LÓPEZ et al., 2002) followed by the addition of 80 % ethanol to a final concentration of 60 % ethanol.

ELISA R5: The extracted samples were analyzed by Méndez ELISA R5 Method as previously described (VALDÉS et al., 2003) using Prolamins Kit (TRANSIA® Plate Prolamins) (BioControl Systems, Bellevue, WA USA) according to the procedure of manufacture. A standard curve was plotted using reference samples (1.56, 3.12, 6.25, 12.5 and 25 ng/mL of gliadin) and gliadin values were multiplied by 2 to calculate gluten concentrations.

ELISA G12: The extracted samples were analyzed by G12 antibody-based sandwich ELISA test kit (AgraQuant® Gluten G12 ELISA) from Romer Labs (Romer Labs, Union, MO USA) according to the procedure of manufacture.

3.2.7 Statistical analysis

The results are expressed as mean ± standard deviation (SD). Differences among the different treatments groups concerning toxic epitopes amount were determined by one-way analysis of variance

(ANOVA). Multiple comparisons were made using the Tukey's post hoc test, and the criterion for significance was $p < 0.05$. Bartlett's test was used to verify the homogeneity of variances.

3.3 RESULTS AND DISCUSSION

3.3.1 Total protein, soluble and insoluble polymeric protein

Heating treatment conditions had no effect on the total protein (TP) ($p < 0.05$) with the exception of the spray-dry treatment that caused 43 % loss of initial weigh and 36.4 % loss of protein. This occurred because during the drying process, the flour stuck onto the glass dryer wall, and it was impossible to recover. However, for the other treatment there were no losses. This TP value was reported to be common in Brazilian wheat cultivars (SCHEUER et al., 2011).

All treatments affected solubility as determined by the amounts of polymeric and monomeric proteins. Except in spray-dry, in all process the insoluble polymeric proteins (IPP) were higher than in control flour (Table 3.1). Thus, the opposite was observed in relation to soluble polymeric proteins (SPP).

Table 3. 1. Results of the total protein, soluble and insoluble polymeric protein (%) befor and after treatment.

Treatment	TP (%)	IPP (%)	SPP (%)
Control Flour	12.1 ± 0.3^{a}	5.6 ± 0.2^{a}	6.5 ± 0.2^{a}
Spray dry	7.7 ± 0.4^{b}	3.0 ± 0.3^{b}	4.7 ± 0.2^{b}
Microwave	12.4 ± 0.2^{a}	9.1 ± 0.6^{c}	3.3 ± 0.5^{c}
Oven	12.2 ± 0.2^{a}	10.7 ± 0.2^{d}	1.5 ± 0.2^{d}
Ultrasound	12.5 ± 0.1^{a}	6.7 ± 0.1^{e}	5.8 ± 0.1^{e}
Extrusion	12.0 ± 0.2^{a}	9.8 ± 0.3^{c}	2.3 ± 0.1^{f}

Legend: TP = Total protein, IPP = Insoluble polymeric proteins, SPP = Soluble polymeric proteins. Means ± standard deviations in the same column followed by a different letter are significantly different ($p \leq 0.05$).

Higher IPP was observed after treatment that involve high mechanical work and temperature. These conditions increase the intra- and interchain bonds, increasing the disulfide, tyrosine cross-link and hydrophobic bonds, which result in lower solubility.

The IPP values are in agreement with previous studies for wheat flour (SILVAS-GARCÍA et al., 2014). IPP is a protein quality indicator

that correlates better than protein content to bread loaf volume, bake mix time and mixing tolerance (BEAN et al., 1998).

In terms of dough quality, higher content of IPP increases the retention of CO_2, and bread dough was harder and less springy. On the other hand, low IPP is related to lower elasticity and the weakening of the gluten network, and high SPP is related to low extensibility strength of the dough (SILVAS-GARCÍA et al., 2014).

Covalent and non-covalent bonds are essential to form and stabilize the polymeric and monomeric structure of gluten network. The most important bonds are disulfide, tyrosine and hydrophobic bonds. Disulfide bonds play an important role in determining the structure and properties of gluten proteins. Monomeric α/β- γ- and ω-gliadins present three and four intrachain disulfide bonds respectively, whereas polymeric LMW- and HMW-GS include both intra- and interchain bonds (WIESER, 2007).

Hydrophobic interactions continuously increased with increasing temperatures, because ionic and hydrogen bonds were disrupted during heat treatment of gluten. These hydrogen bonds primarily contribute to holding the gluten dough together, and after heat treatment the number of ionic bonds and hydrogen bonds decrease as the temperature increase. Temperatures above 60 ^{o}C lead wheat gluten to unfold, resulting in free SH groups that were susceptible to oxidation and intra- or intermolecular disulfide bond formation (WANG et al., 2017).

During dough formation and bread-making processes, tyrosine cross-links occur via tyrosine bond formation at specific tyrosine-containing amino acid sequences commonly found in the repeated sections of glutenin proteins (TILLEY et al., 2001). Also, the hydrophobic interactions that occur during heating process due exposition of hydrophobic groups, promote formation of aggregates (WANG et al., 2017). Hydrophobic bonds contribute significantly to the stabilization of gluten structure. They are different from other bonds because their energy increases with increasing temperature; this can provide additional stability during the baking process (WIESER, 2007).

As reported by Silvas-García et al. (2014), changes in IPP and SPP values may indicate gluten polymer chains has suffered modification. So, the heat treatments seems to increase the molecular size of the gluten proteins.

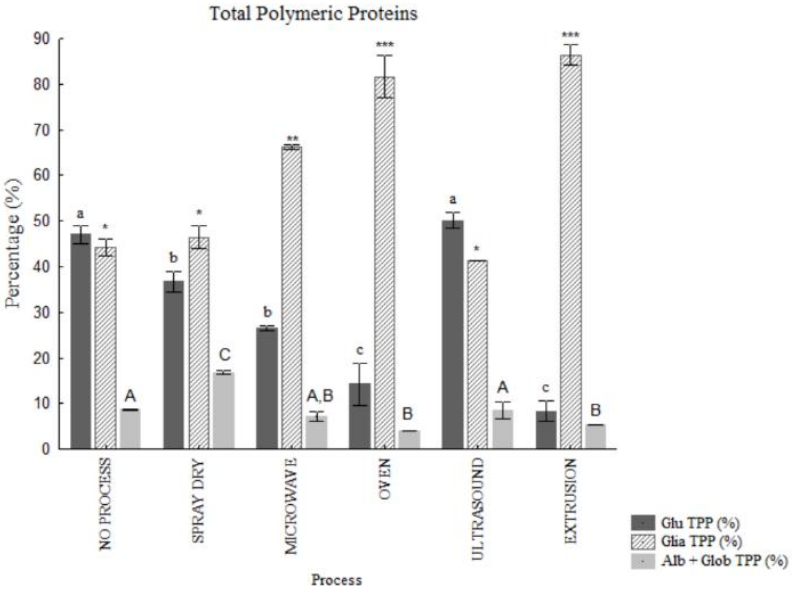
After ultrasound treatment the solubility of gluten proteins increases particularly of the glutenin fractions, whose solubility increases, according to Marcuzzo et al. (2010) by a reduction in size due the disruption of the major non-covalent and covalent interactions.

3.3.2 Total, extractable and unextractable polymeric proteins by HLPC – SEC

This procedure is useful to obtain information on changes in the solubility of the protein fractions induced by the heat treatment.

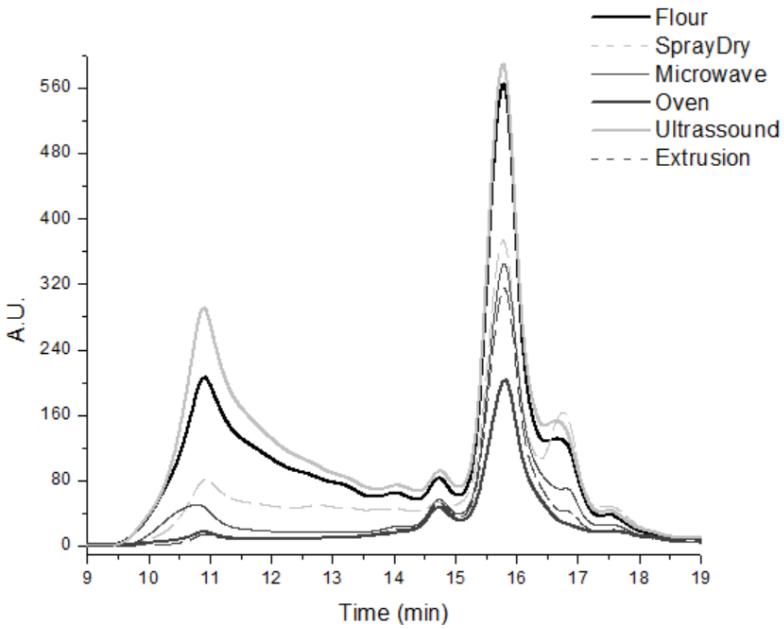
TPP, UPP, EPP and Glu/Glia ratios differ among treatments. When Total Polymeric Proteins (TPP) of the control flour were extracted, the polymeric and monomeric proportion were similar, but after treatment it changed (Figure 3.1, Figure 3.2). With exception of ultrasound and spray-dry treatments, the amount of polymeric proteins decreased while monomeric increase ($p < 0.05$), most notably with oven and extrusion treatments. In relation of Glu/Glia ratios (Figure 3.7), control flour and ultrasound treatment were similar ($p > 0.05$), while in all the other treatment Glu/Glia decreased, as expected.

Figure 3. 1. Total polymeric protein (TPP, %) fraction distribution in flour before and after treatments.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour.

Figure 3. 2. Size-Exclusion HPLC separation of total polymeric protein in control flour before and after treatments.

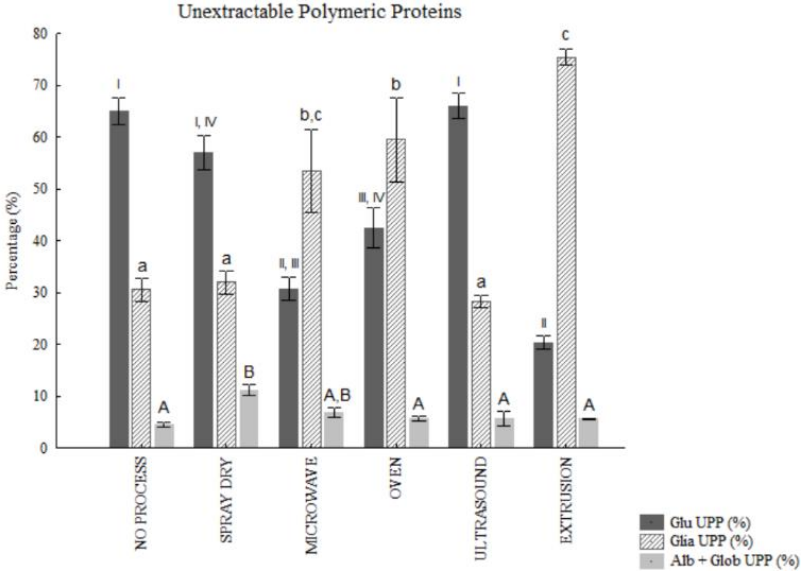


Legend: Flour = Control Flour.

Results are according with Vensel, Tanaka and Altenbach (2014) and Gupta, Khan and Macritchie (1993).

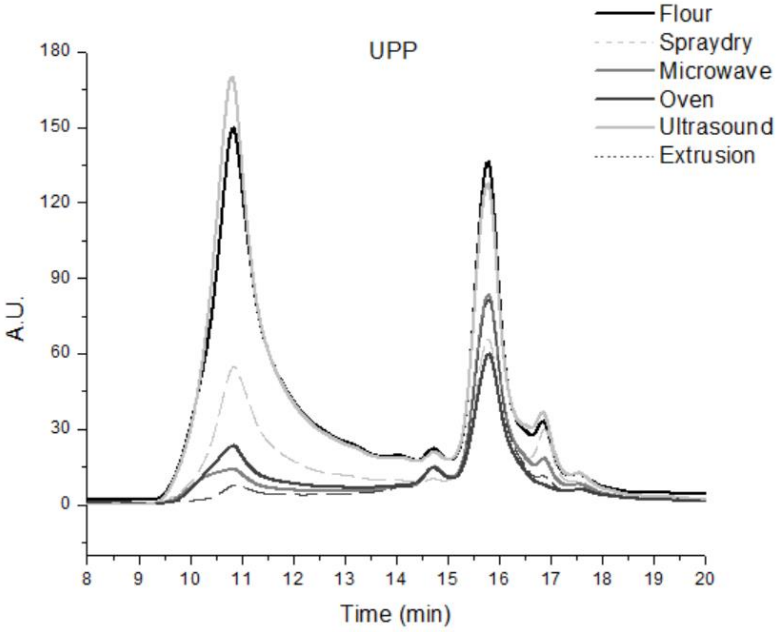
In the UPP fraction, polymeric proteins are in higher amounts in the control flour than monomeric, due to higher complexity and consequently, lower solubility. After ultrasound and spray-dry treatments remained the same, but, other treatments showed a decrease in the polymeric proteins (Figure 3.3, Figure 3.4) and consequently the Glu/Glia ratios (Figure 3.7), as the monomeric proteins increased.

Figure 3. 3. Unextractable Polymeric Protein (UPP, %) fraction distribution in control flour before and after treatments.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour

Figure 3. 4. Size-Exclusion HPLC separation of unextractable polymeric protein in control flour before and after treatments.

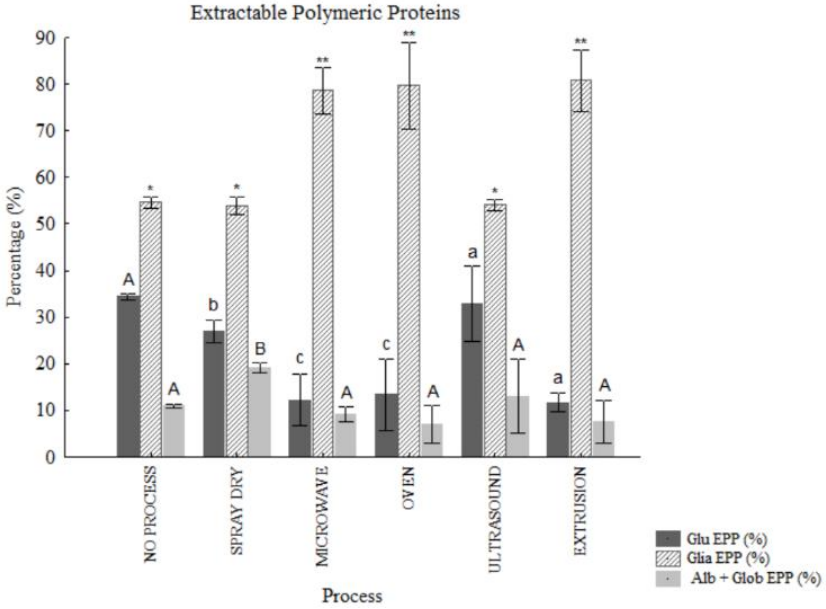


Legend: Flour = Control Flour.

In the EPP fraction, there were more monomeric proteins. Nevertheless, after treatments the monomeric proportion increased in all conditions, except ultrasound and spra-dry treatments (Figure 3.5, Figure 3.6). This indicates that polymeric proteins have been broken proteins of lower size or less complexity similar in size to monomeric gliadins. Additionally, a decrease in glutenins was observed.

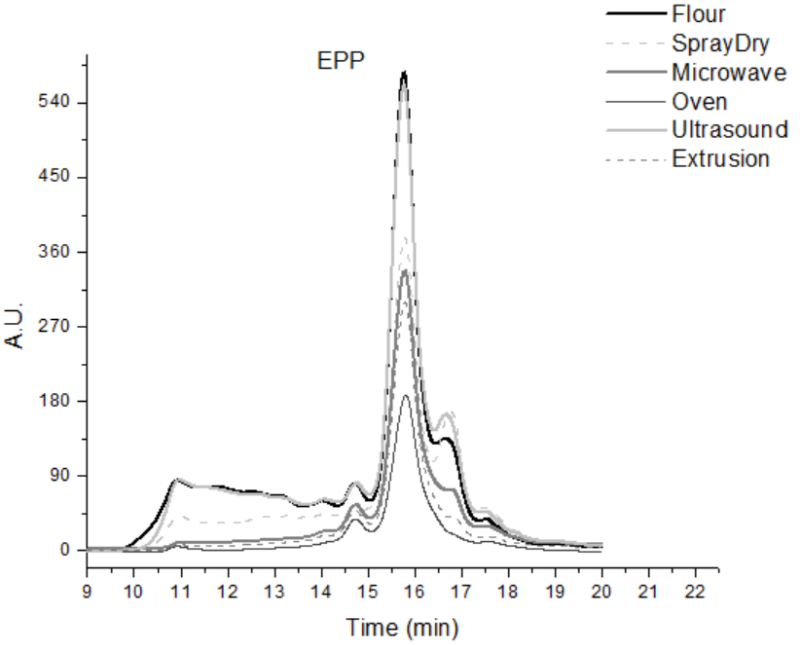
In all fractions, except ultrasound, polymeric proteins and the Glu/Glia ratio decreased and in relation to the control flour which suggest poor rheological properties (LI et al., 2016), affecting dough development and dough stability (CHAUDHARY; DANGI; KHATKAR, 2016).

Figure 3. 5. Extractable polymeric protein (EPP, %) fraction distribution in control flour before and after treatments.



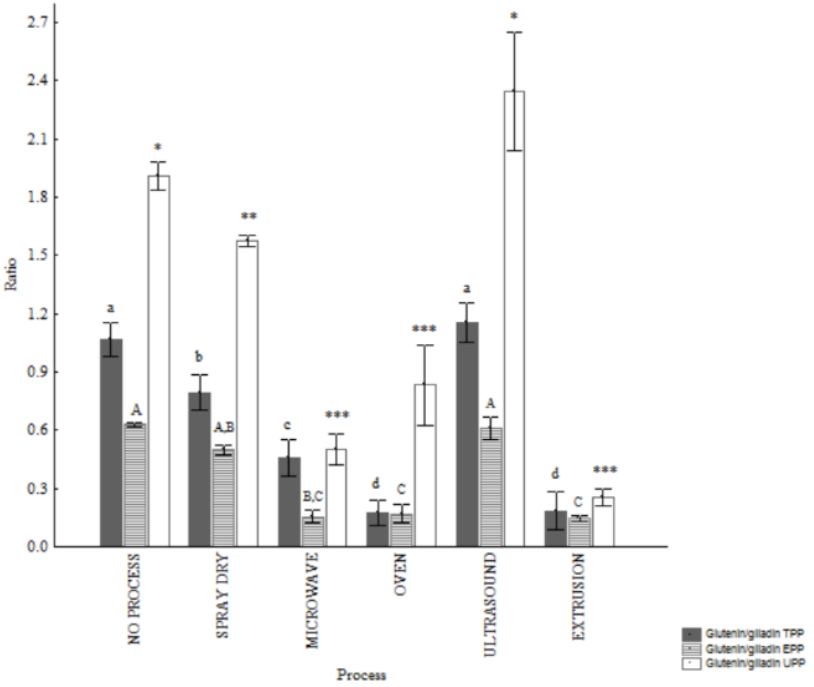
Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour

Figure 3. 6. Size-Exclusion HPLC separation of extractable polymeric protein in control flour before and after treatments.



Legend: Flour = Control Flour.

Figure 3. 7. Ratios of Glutenin/Gliadin extract from all fraction (TTP, UPP and EPP) in control flour before and after treatments.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour.

The data shows that treatments modifies size and/or complexity of gluten proteins, resulting in changes in their solubility. It was expected that in the UPP fraction resulting from all the treatments, polymeric glutenins were the predominant protein group, however, this was not observed. This result may indicate that treatments can modify structure and chemical bonds, making the molecules smaller due to hydrolysis, temperature, pressure and vibration. In turn, these changes could result in increased solubility by exposing of the hydrophobic amino acids.

SEC-HPLC is commonly used for analysis of cereal endosperm proteins. Analyzing molecular size distribution, it is possible separate cereal proteins into polymerics proteins (glutenins), monomeric protein (gliadin), albumins, and globulins, as well as calculating glutenin/gliadin ratio. These measurements are correlated with breadmaking quality

parameters (GUPTA; KHAN; MACRITCHIE, 1993; LARROQUE; BEKES, 2000).

The UPP is the polymeric glutenin protein (> 158 kDa) fraction, with lowest solubility and therefore highest molecular weight (GUPTA; KHAN; MACRITCHIE, 1993; MACRITCHIE, 2014). It is also related to the size and/or to the complexity of the gluten polymer (JOHANSSON et al., 2013) and, to the total amount of HMW subunits (DON et al., 2006). In this work, it was possible to observe that microwave, oven and extrusion processes had a strong effect on the solubility of this fraction.

On the other hand, monomeric gliadins, in EPP and TPP showed higher values. It is well known that mechanical work and temperature increase S-S bonds and di- and iso-tyrosine bonds (TILLEY et al., 2001).

According to Chaudhary, Dangi and Khatkar (2016) polymeric proteins affect the specific loaf volume favorably and bread crumb firmness adversely, while monomeric have a reverse impact on bread quality parameters. We noted that the amount of monomeric proteins increase, which means, that the breadmaking qualities are poor after microwave, oven and extrusion processing

We did not observe significant changes with the ultrasound treatment. However, Marcuzzo et al. (2010) noticed that after ultrasound treatment, breaking intrachain disulfide bonds are the most likely mechanism involved on reduction of size and increase on solubility (MARCUZZO et al., 2010).

Our results showed significant changes just in an increase of extractability of monomeric proteins. It is well know that overmixing decrease the HMW-GS and gliadins extractability, and it is possible to extract more proteins than from flour (PATEY; SHEARER; MCWEENY, 1977). Ionic, S-S and hydrogen bond are affected by heating treatment, leading to unfolding of wheat gluten (WANG et al., 2017). These changes affect the secondary structure of gluten, and influences the dough's rheological properties (LI et al., 2016).

3.3.3 Gliadins and glutenins protein characterization by RP-HPLC

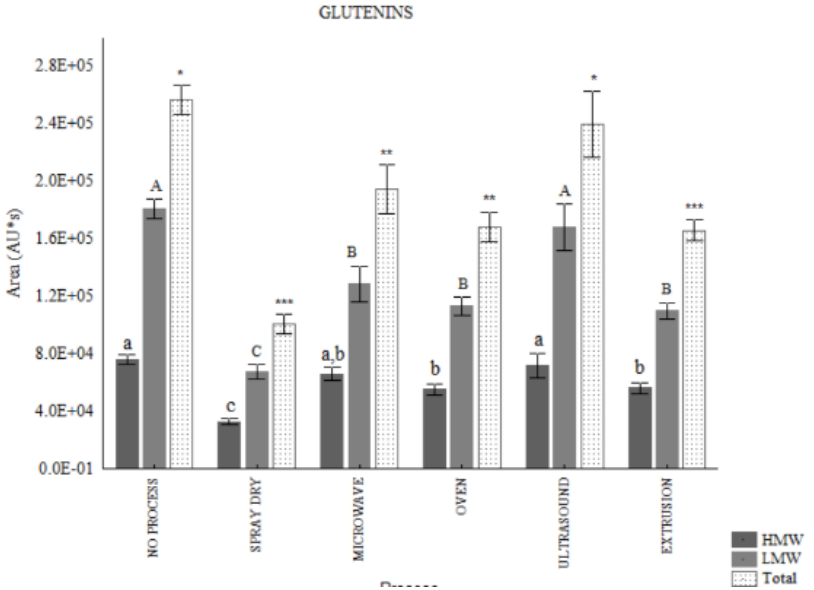
3.3.3.1 Glutenins

Using RP-HPLC glutenins can be divided into HMW-GS, LWM-GS, HMW-GS/LMW-GS ratio and changes in fractions (increase or decrease after treatment) can be measured.

The amount of HMW-GS decreased after spray-dry, oven and extrusion in relation to the control flour. For LMW-GS a decreased

($p<0.05$) was observed in all conditions, except for ultrasound treatment and similar results were observed Total glutenins (Figure 3.8).

Figure 3. 8. Area of Total glutenins, HMW-GS and LMW-GS in control flour before and after treatments.



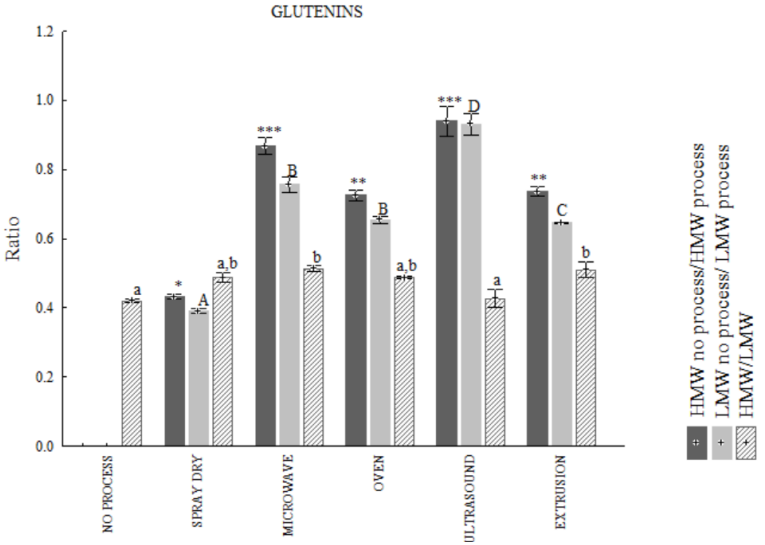
Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour

HMW-GS are directly related with technological applications of wheat as they are major determinants of gluten and dough elasticity (KONAREV et al., 2011).

HMW-GS are required for glutenin particle formation and affect the internal structure of glutenin (DON et al., 2006).

In relation to the HMW-GS/LMW-GS ratio it changed after all treatments, except ultrasound where the HMW-GS increased and LMW-GS decreased, indicating an increase in size or complexity of glutenin. These results are in accordance with (DON et al., 2006) that observed that alteration in HMW/LMW-GS ratio is an indicative of glutenin particles size alteration too.

Figure 3. 9. Ratios between HMW-GS control flour/ HMW-GS treatment, LMW-GS flour/LMW-GS treatment and HMW/LMW-GS.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour

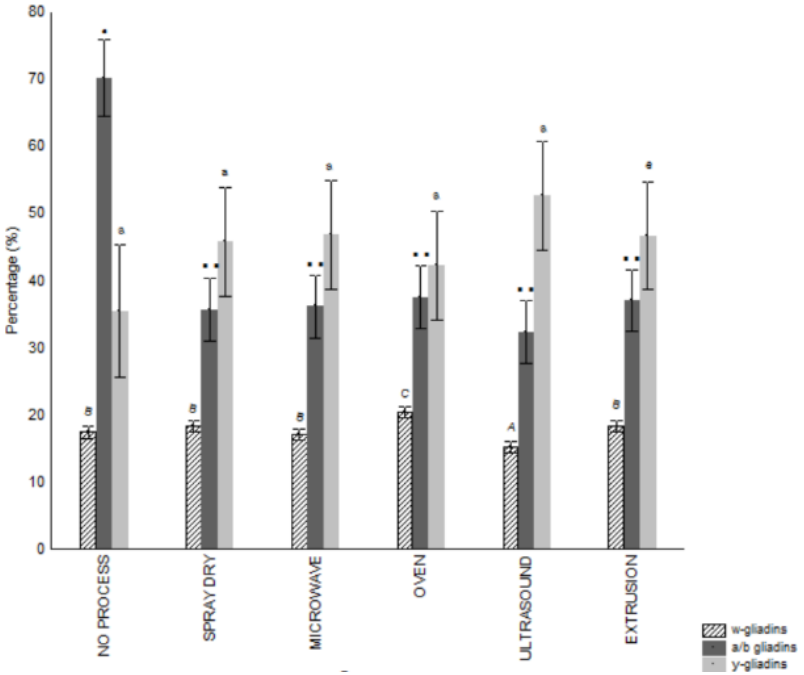
During heat and mechanical treatments gluten protein unfolds and protein crosslinking increases due to exposition of hydrophobic regions and free SH groups that immediately interact with each other, which leads to irreversible protein aggregation and formation of a three-dimensional network of high molecular wheat gluten aggregates (WANG et al., 2017), resulting in a high viscosity (CHANTAPET et al., 2013). As temperature increases, more functional groups were exposed, and increase interactions between these groups (WANG et al., 2017).

3.3.3.2 Gliadins

Gliadin distribution measured by RP-HPLC in wheat flour before and after treatments changed depending on the it (Figure 3.10).

It was possible to separate the gliadins in ω-, α/β- and γ-gliadin. The ω-gliadin increased ($p < 0,05$) after oven and extrusion processes. The α/β- gliadins decreased ($p < 0,05$) with all treatments but there were no differences between them. While the γ- gliadins were not affected ($p > 0,05$) by any treatment (Figure 3.10).

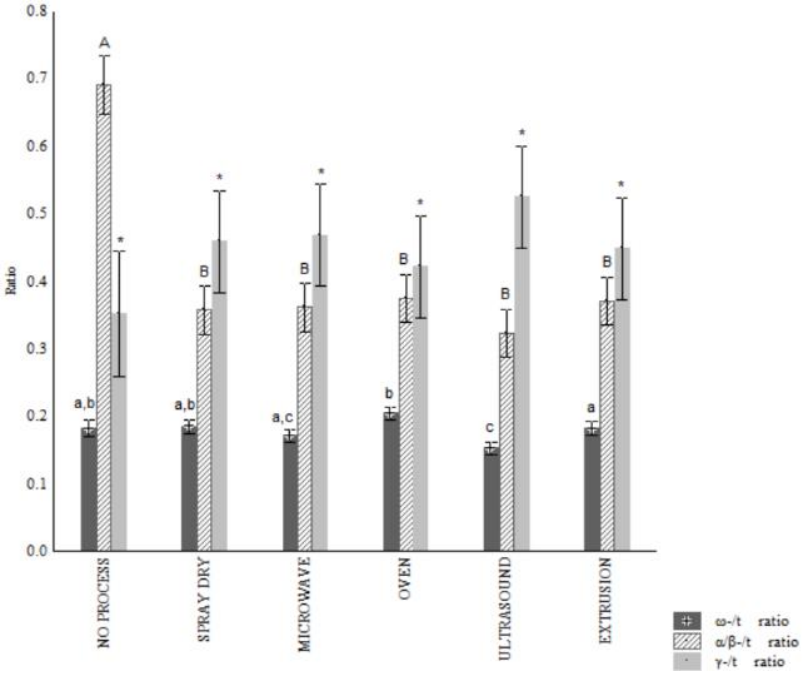
Figure 3. 10. Percentage of gliadin fraction in control flour before and after treatments.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour.

In relation to gliadin ratios, treatments affected the ω-/t and α/β-/t ratios. After ultrasound treatment the ω-/t ratio decreased, and increased after oven treatment ($p < 0,05$). Yet, the α/β-/t ratios decreased after all treatments with no difference ($p > 0,05$) between them (Figure 3.11).

Figure 3. 11. Relation of gliadins fraction and total gliadin in control flour before and after treatments.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation. NO PROCESS = Control Flour.

Leszczynska et al. (2003) reported that after microwave treatments the content of all gliadin fractions decreased, as we also observed. Differently from Zhu et al. (2011) where they reported that ultrasound decreased the size of wheat gluten, probably due to the breakdown of aggregates (MARCUZZO et al., 2010), in this work, we observed a decrease in α/β-gliadins

Similar gliadin distribution was noted in wheat from Argentina (RIBEIRO et al., 2016).

In baked products, gliadins are correlated with dough strength, mixing tolerance and loaf volume (RIBEIRO et al., 2016). During mixing and baking process the gliadins interchain SS bonds starts at 70 °C (LAMACCHIA; LANDRISCINA; D'AGNELLO, 2016).

With heat and mechanical work α-, β-, and γ–gliadins (S-rich) might be incorporated into the gluten polymer with intermolecular SS bonds, while ω-gliadins (S-poor) interact by hydrogen or other

noncovalent bonds (JOHANSSON et al., 2013), such as di- and iso-tyrosin bonds that are linked during the baking process, changing solubility and extractability (TILLEY et al., 2001), and that is why, the treatments affect gliadin distribution as well as glutenin.

The extractability of gliadins from control flour was higher than from treated samples. Related results have been reported with flour and bread, and S-S interaction explains this higher extractability, α- and γ-gliadins are more affected than are ω-gliadins (LAMACCHIA; LANDRISCINA; D'AGNELLO, 2016; WIESER, 1998).

Ultrasound treatment breaks down protein particles into smaller domains (MARCUZZO et al., 2010). Microwave heating can affect the gliadin structure leading to an increase or decrease in their immunoreactivity by promoting conformational and chemical changes in gliadin structure (LESZCZYNSKA et al., 2003).

Ribeiro et al. (2016) believe that is possible to make wheat flour non-toxic to celiacs and still with good baking qualities. On the other hand, a safe level of gluten is between 10 and 50 ppm (CATASSI et al., 2007) with is a gluten level so low, that is impossible to retain good baking qualities. Even when Lamacchia, Landriscina and D'agnello, (2016) achieved around 99 % decrease in toxicity of wheat seeds with reasonable baking qualities, this amount of gluten was still too high for most celiacs and could still cause damage to the intestinal villi. Because, the maximum ingestion of gluten per food allowed to is less than < 3 ppm (BRUINS SLOT et al., 2015).

Gliadins were primarily detected as toxic to celiacs, and there has been an increase in the development of methods for detection of traces of gliadin in heat-treated and non heat-treated foods. The current market includes several ELISA kits of antibodies directed against the epitopes of gliadin that are toxic to celiac people. It is also to be noted that except for HMW- GS, disulfide, tryptophan and tyrosine bonds may also exist in gliadins and/or LMW-GS which may affect dough properties (LI et al., 2016).

3.3.4 Digestibility and immunoreactivity by ELISA R5 and G12 Test

Besides the important and fundamental technological qualities of wheat gluten proteins, currently there is concern regarding their nutritional aspects and how treatment affect their digestibility.

Treatments had a significant effect on both digestibility and immunoreactivity. Digestibility decreased ($p < 0.05$) after spray-dry and ultrasound treatments, but was not affected by the other treatments.

Protein´s nutritional value depends on the quantity, digestibility and availability of essential amino acids. The extrusion process improves digestibility of proteins due to inactivation of protease inhibitors and other antiphysiological substances, but decreases the availability of some amino acids (SINGH; GAMLATH; WAKELING, 2007). In wheat flour, there is not necessary this inactivation, what explain why digestibility did not increase initiatively, neither after extrusion, neither after other heat treatments, except spray-dry and ultrasound, where digestibility decrease. Heat treatments leads to the unfolding of peptide chains, changes in hydrophobicity and susceptibility to the action of proteolytic enzymes, and influence digestibility and immunochemical reactivity, and here digestibility had a slight decrease after two treaments.

In vitro immunoreactivity to the R5 antibody decreased ($p < 0,05$) only in the spray-dried sample. Yet, i*n vitro* immunoreactivity using the G12 antibody decreased after spray-dry as well as after microwave treatment with the highest decrease occurring after spray-dry (Table 3.2, Figure 3.12), probably due to protein loss.

Table 3. 2. Percentage of digestibility of gluten (using pepsin) and levels of gliadin (measured by R5 and G12 ELISA immunoassay).

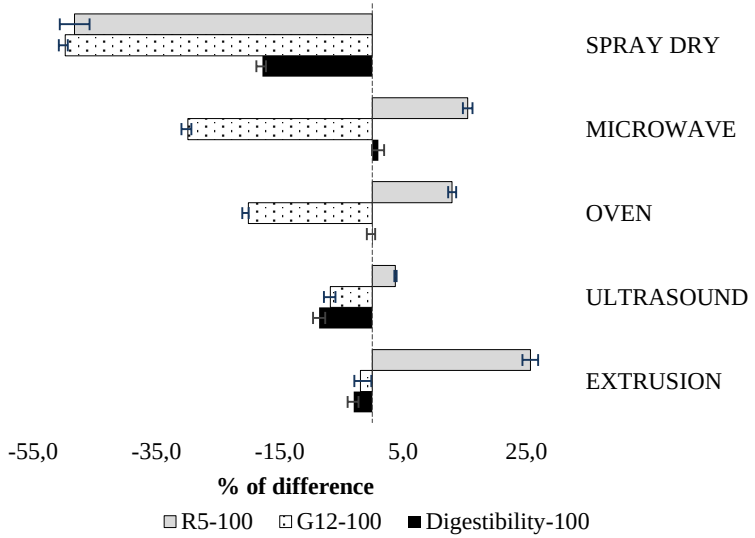
Process	Digestibility (%)	R5 (%)	G12 (%)
Control Flour	95.59 ± 0.54^{a}	9.64 ± 0.38^{a}	10.78 ± 0.44^{a}
Spray dry	78.59 ± 0.95^{c}	4.98 ± 1.13^{b}	5.41 ± 0.60^{c}
Microwave	96.52 ± 0.36^{a}	11.13 ± 0.07^{a}	7.56 ± 0.08^{bc}
Oven	95.70 ± 0.97^{a}	10.88 ± 1.34^{a}	8.61 ± 0.84^{ab}
Ultrasound	87.37 ± 0.76^{b}	10.00 ± 0.54^{a}	10.04 ± 1.74^{ab}
Extrusion	92.72 ± 6.03^{ab}	12.10 ± 1.02^{a}	10.58 ± 0.02^{ab}

Legend: Means ± standard deviations in the same column followed by a different letter are significantly different ($p \leq 0.05$).

There was positive correlation among ω-gliadins, γ-gliadins, and total gliadin contents and immune reactivity to the R5 ELISA test and no correlation in the case of α/β-gliadin. Also, the total protein content correlation with the R5 ELISA test is high, indicating that there is contribution of other non-gluten proteins that are toxic to celiacs (RIBEIRO et al., 2016). ω-gliadin contains epitopes that show a higher immunogenicity demonstrated by T cell–stimulatory sequences in celiacs (TYE-DIN et al., 2010). The results presented a low decrease in *in vitro*

immunoreactivity against R5 and G12, once that the most immunogenics proteins are γ- and ω-gliadin, that were slightly affected by treatments.

Figure 3. 12. In vitro immunoreactivity and digestibility of wheat flour subjected to different heat treatments.



Legend: Different letters indicate different means by Tukey test ($p < 0.05$). Vertical bars indicate standard deviation

In vitro immunoreactivity against G12 after microwave treatment was lower than the 99 % reduction reported by (LAMACCHIA; LANDRISCINA e D'AGNELLO, 2016), after microwave treatment of seeds. They associated this high decrease to denaturation of wheat proteins before gluten formation (LAMACCHIA; LANDRISCINA e D'AGNELLO, 2016).

The commercial antibodies used here are standard and bind to gluten-responsive DQ2/DQ8 T cells in celiacs (MORÓN et al., 2008; OSMAN et al., 2001). The R5 monoclonal antibody recognizes the QQPFP repetitive pentapeptide epitope (OSMAN et al., 2001) and is recommended by the *Codex Alimentarius*. The G12 monoclonal antibody recognizes the QPQLPY repetitive hexapeptide epitopes (MORÓN et al., 2008).

The literature shows that in autoimmune diseases triggered by gluten such as in celiac disease, gluten ataxia and dermatitis

herpetiformis, different epitopes are responsible for a particular set of manifestations (SILANO; VINCENTINI; DE VINCENZI, 2009). There are more than 50 T cell–stimulatory peptides in gluten proteins, with some similarities, such as hydrophobic residues at specific positions and the toxicity of ω-gliadin are higher (TYE-DIN et al., 2010), but also in HMW-GS and LMW-GS (MOLBERG et al., 2003). As a genetic disease, less than 10 % of genetic susceptible individuals develop clinical symptoms (FASANO, 2011). Also, 40 % of the general population have HLA-DQ2/8 genes, but the presence of these alleles does not always result in the development of celiac disease (HADITHI, 2007). The development of a genetic condition is being investigated by epigenetic, epigenitcs shows which environmental factors control gene expression (BROOKS et al., 2010; HEWAGAMA; RICHARDSON, 2009).

It was observed that *in vitro* immunoreactivity against R5 and G12 antibodies changes according with changes in glutenin and gliadin profiles (HMW-GS, LMW-GS, γ-, α/β-, and ω-gliadin). The changes in solubility and protein profiles observed, suggest that treatments applied modifies the structure/complexity of glutens proteins, probably masking or exposing epitopes and/or domains of glutenins and gliadins, leading to increased or decreased binding of R5 and G12 antibodies depending on the treatment. The antibodies bind to specific protein sequences which were affected by new covalent and non-covalent interactions (i.e. hydrophobicity) promoted by treatments.

Maybe the way the gluten proteins are presented to the individual is related to the disease development. Further research could clarify if together with genetic conditions, the way that gluten protein reaches the gut, defines how and if an autoimmune disease will be manifested.

3.4 CONCLUSION

The treatments affected solubility with exception of ultrasound, all treatment showed low solubility of polymercis and monomerics proteins. Also, treatments affected glutenins and gliadins profile, the amount of the HMW-GS decrease after spray-dry, oven and extrusion and the amount of the LMW-GS decrease too after spray-dry, microwave, oven and extrusion. Wheareas for gliadin, the amount of ω-gliadin increase after spra-dry and ultrasound treatments, and the amount of α/β- gliadin decrease after all treatments, while the treatment to do not affected γ-gliadins amount. Also, digestibility decrease after spray-dry and ultrasound. This changes with decrease of solubility and changes in

profile of proteins is results of rearrange of proteins during the treatments, resulting in a higher complex structure. And, finally, the potential celiac disease immune stimulatory epitopes were measured, and showed lower amount after spray-dry treatment by R5 monoclonal antibody and lower amounts after spray-dry and microwave treatment by G12 ELISA test in relation to control flour. At last, even with the alteration on the gluten structure and complexicity, these changes do not allow to produce a safe product to celiacs, the amount of the potential celiac disease immune stimulatory epitopes still were too high.

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4 ANCIENT AND MODERN WHEAT VARIETIES ARE TOXIC TO CELIACS – *IN VITRO* STUDY

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ABSTRACT

Modern wheat (*Triticum aestivum*) is a hexaploid resulting from hybridization of so called ancient wheat varieties. Due to the possibility that ancient wheats may be less toxic to celiacs, we tested pools of different ancient wheat using anti-bodies the recognize gluten proteins. Two diploid varieties possessing the AA genome - *T. monococcum ssp monococcum* and *T. urartu* and five tetraploid *T. turgidum* wheat varieties that show favorable agronomic characteristics and commercial appeal: *T. turgidum ssp durum* (AABB), *T. turgidum ssp polonicum* (AABB), *T. turgidum ssp turgidum* (AABB) and *T. turgidum ssp turanicum* (AABB). All wheat flours were extracted, purified, and separated using two-dimensional electrophoresis. Gels were, stained with Comassie blue or were transferred to nitrocellulose for Western blot analysis using an anti-gliadin and the Skerritt Antibody. All wheat varieties tested bound with both commercial antibodies used in ELISA kits, R5 and G12 that recognize T-cell stimulatory epitopes. Einkorn and durum species differ in toxicity depending of the sub-species. The results strongly indicate that it is not safe for all celiac to consume any kind of wheat, even though Einkorn, Kamut, Khorasan and/or Faro varieties, that have high commercial and gourmet appeal

Key-words: ancient grains, wheat, Celiac disease, *Triticum monoccocum, Triticum urartu, Triticum turgidum, Triticum aestivium*

4.1 INTRODUCTION

Wheat is the principal source of energy, protein, and fiber for the majority of the world's population (ABDEL-AAL; HUCL, 2014; SCHEUER et al., 2011). Wheat has been utilized as a food grain since the late Stone Age (6700 B.C.) and cultivated since 5000 B.C. (ABDEL-AAL; HUCL, 2014).

Wheat flour is unique in that when mixed with water the flour storage proteins form a continuous visco-elastic gluten network (SHEWRY et al., 2001). This allows the dough to retain gas resulting in the myriad of leavened products (e.g. bread) found throughout the world.

The protein network is composed of multiple subunits of prolamin, (gliadins in wheat) and glutelin (glutenins in wheat) (WIESER, 2007), that have a unique protein sequence that give rise to structure-function relationships that are important to dough rheology and other aspects of food technology (DUPONT et al., 2004).

Modern wheat (*Triticum aestivum*) is a hexaploid resulting from multiple hybridization events between different progenitor species, resulting in three subgenomes: A, B and D in modern wheat (*Triticum aestivium*) (EVERSOLE et al., 2014). Modern wheat has 21 pairs of chromosomes, with three homeologous sets of seven chromosomes in each of three subgenomes (A, B and D) (MAYER et al., 2014).

Gluten is a term used to describe the protein network formed when storage prolamins and glutenins proteins, the two-major group of protein in wheat endosperm are mixed with enough water to form dough. Gliadins are divided in γ-, α-, β- and ω-gliadin, and glutenins in high molecular weight (HMW-GS) and low molecular weight (LMW-GS) (JOHANSSON et al., 2013). ω-gliadins molecular weight (MW) is around 40 – 50 kDa while α/β-, γ-gliadins MW is around 28 – 35 kDa (WIESER, 2007). HMW-GS are 66-88 kDa and LMW-GS are of 32-45 kDa, are linked into polymers that range in size from about 150 kDa to over 1,500 kDa 32-45 kDa (DON et al., 2006).

Gliadins and glutenins are located at different loci on chromosomes within each genome (HE et al., 2005; RIBEIRO et al., 2016; WAGA et al., 2013).

Celiac disease (CD) is the most common wheat intolerance worldwide (IACOMINO et al., 2016). It is a complex immune-mediated disease (MURRAY, 1999). Genetic studies have implicated 43 predisposing loci that collectively explain around 50 % of the genetic variance in CD. The strong genetic association of CD with HLA-DQ2/8 has emphasized the pathogenic role of gliadin-specific adaptive immunity

mediated by HLA-DQ2/8-resctricted $CD4^+$ T lymphocytes (WITHOFF et al., 2016). Specific gliadin peptides are able to activate the cells involved in innate immunity, such as macrophages, dendritic cells, and cytotoxic intraepithelial lymphocytes, or to induce a direct toxic effect on enterocytes (GIANFRANI et al., 2015).

The gliadin fragments are digestion-resistant and are the primary trigger of the abnormal immune response in CD (DØRUM et al., 2016; RIBEIRO et al., 2016; TYE-DIN et al., 2010). Glutenin proteins can also elicit T-cell responses in CD patients (MOLBERG et al., 2003). The epitope with higher immunogenicity related with activation of gluten-specific T-cells in celiacs is the 33-mer, which is a fragment of ω-gliadin (TYE-DIN et al., 2010). The α-gliadins are proteins encoded by a gene family at the Gli-2 loci (Gli-A2, Gli-B2, and Gli-D2), while ω- and γ-gliadins are coded by clusters of genes at the Gli-1 loci (Gli-A1, Gli-B1, and Gli-D1) (GIL-HUMANES et al., 2010; RIBEIRO et al., 2016; ZHANG et al., 2015).

Currently available digestive enzyme supplements are ineffective in degrading immunogenic gluten epitopes (JANSSEN et al., 2015). So, the only effective medical treatment for CD patients is to strictly adhere to a gluten-free diet (COSNES et al., 2008; DON; KOEHLER, 2014).

With sustainable agriculture and special, nutritional appeals, the ancient wheats are being introduced in market in North America (with einkorn, emmer, faro, Kamut - Khorasan varieties) and in Europe (spelt variety) (ABDEL-AAL; HUCL, 2014). Additionally, speculation exists if these varieties could be less toxic to celiacs (MARIETTA; MURRAY, 2012), due to wheat domestication to improve farming and processing technologies (ABDEL-AAL; HUCL, 2014). These ancient wheat species were used as staple food for many thousands of years (ABDEL-AAL; HUCL, 2014).

Einkorn is the demonination of diploid (AA) hulled wheat grains such as *T. monococcum* and *T. urartu* (ABDEL-AAL; HUCL, 2014; KASARDA, 2013). Emmer varieties comprise tetraploid (AABB) hulled wheats, while no hulled tetrapolid (AABB) wheats are denominated durum, both derivated from *T. turgidum*. Spelt wheat is the hexaploid (AABBDD) wheat (KASARDA, 2013). Also, Kamut, Khorasan and Faro are taxonomically unclear. Faro is used to describe all hulled wheat (ABDEL-AAL; HUCL, 2014), but more accepted as emmer (KASARDA, 2013; VINCENTINI et al., 2009). Finally, Kamut® is comercial name of Khorasan wheat (http://www.kamut.com/) a hybrid between *T. polonicum* and *T. durum* (WIWART et al., 2013), but they

taxonomy is not clear (COLOMBA; GREGORINI, 2012; COLOMBA; VISCHI; GREGORINI, 2012).

The basic genome (AA) of bread wheat and other polyploid wheats, such as, *T. turgidum* (AABB), *T. timopheevii* (AAGG) and *T. zhukovskyI* (AAGGAmAm) has a central role in wheat evolution, domestication and genetic improvement (LING et al., 2013).

The diploid wheat species *Triticum monococcum* (AA) are among the most promising suitable candidates that are potentially tolerated by most celiacs, and present some breadmaking qualities (GIANFRANI et al., 2015). In a study with celiac patients, *T. monococcum* was well tolerated when compared to *T. aestivium* (ZANINI et al., 2015).

The first hybridization seems to have occurred several hundred thousand years ago, between wild einkorn *Triticum urartu* (AA genome) and *Aegilops speltoides* (SS genome) forming *Triticum turgidum* (AABB), ancestor of wild emmer and *Tritucum turgidum* ssp. *durum* (MAYER et al., 2014). *Aegilops speltoides*, the ancestor of the B genome (LING et al., 2013), is also known as small spelt in English, petit épeautre in French (ABDEL-AAL; HUCL, 2014).

The second hybridization between *T. turgidum* and *Aegilops tauschii* (DD genome) resulted in ancestral *T. aestivum* (AABBDD) (MAYER et al., 2014). *Aegilops tauschii* (the donor of the D genome) has high influence especially in the morphology and development of spike and seed (LING et al., 2013).

Because the only current therapy for CD is a strict gluten-free diet, researchers are seeking cereals or pseudo-cereals with low or null toxicity.

The goal of this study was to examine reactivity of modern and ancient wheat varieties using anti-gliadin antibodies to provide information on their potential toxicity to celiacs.

4.2 MATERIAL AND METHODS

Wheat seeds from different species were obtained from the National Small Grains Collection (NSGC) (Aberdeen ID, USA), a component of the National Plant Germplasm System (NPGS) of the United States Department of Agriculture - Agricultural Research Service (Table 4.1).

All analysis below were running in Grain Quality & Structure Research Unit (CGAHR) at United State Department of Agriculture (USDA).

Seeds (three pools of each sample were made according with sub-species) were ground using a Genogrinder (SPEX SamplePrep, Metuchen, NJ USA) and sieved through a 40 mesh sieve size with an opening of 425 µm.

Table 4. 1. Modern and ancient wheat varieties tested.

Variety	**Countries of origin**
Triticum monococcum ssp. Monococcum	US, Kenya, Hungary, Spain
T. urartu	Turkey, Syria
T. turgidum ssp. Durum	US, Spain
T. turgidum ssp. Polonicum	Australia, Argentina, Portugal, US
T. turgidum ssp. Turgidum	Australia, Portugal, UK
T. turgidum ssp. Turanicum	Spain, Hungary, Romania
T. aestivium ssp. Spelta	Switzerland, Hungary, Germany, Syria

Protein extraction was performed according to Larroque et al. (2000): 10 mg of sample was extracted with 1 mL of 0.5 % SDS-phosphate buffer, pH 6.9 using a vortex for 5 min followed by sonication for 15 seconds at 6 W and centrifuged at 12000 x g for 20 min. The supernatant was filtered through a 0.45 µm filter and immediately heated for 2 min at 80 °C.

To eliminate salt from the extract, the procedure of Vensel, Tanaka e Altenbach, (2014) was used. Briefly 1 volume of TCA stock (500 g TCA dissolved in 350 mL dH_2O) was added to 4 volumes of protein sample (e.g. 250 µL of TCA was added to 1 mL of sample) followed by incubation for 10 min at 4 °C the sample was centrifuged at 12,000 x g for 5 min and supernatant was discarded. The protein pellet was washed with 200 µL of cold acetone, followed by centrifugation at 12,000 x g for 5 min. This procedure was repeated twice and the pellet was dried at 95 °C until the acetone was evaporated.

Two-dimensional (2D) electrophoresis was performed using the ZOOM® IPGRunner™ System (Invitrogen - Life Technologies, Carlsbad, CA, USA). Prior to it the proteins were solubilized in rehydration buffer to a final volume of 140 µL then loaded on immobilized pH 3–10 gradient gel ZOOM® Strips. Isoelectrofocusing was performed at 200V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000V for 30 min. SERVA IEF Marker 3-10 (SERVA Electrophoresis GmbH, Heidelberg, Germany) was run as a pH standard. Prior to the 2D separation, each gel strip was incubated with equilibration buffer followed by alkylation buffer, all provide and prepared as

described by the ZOOM® kit. Proteins were then separated in the second dimension on NuPAGE® Novex 4-12 % acrylamide gels in MOPS buffer. Precision Plus Protein™ Kaleidoscope™ Prestained 10-250 kDa (Bio-Rad Laboratories Inc, CA, USA) were used as MW standard. The gels were either stained with Coomassie Colloidal Blue Staining kit (Invitrogen) or transferred for immunoblotting.

The separated proteins were electroblotted onto nitrocellulose membranes (Nitrocellulose Pre-Cut Blotting Membranes (0.45 µm porosity) (Invitrogen Life Technologies, Carlsbad, CA, USA) at 100 V for 60 min. To verify successful transfer of proteins from the 2D gel to the membranes, the membranes were stained with 0.1 % Ponceau Red in 0.5 % acetic acid (Sigma Aldrich, St. Louis MO USA). The membrane was blocked for 1 h at room temperature using 3 % nonfat milk in TBS pH 7.4 (Tris saline buffer 1X) for 1 h. The membranes were incubated with the primary antibodies described below for 1 h, then washed 5 times with 3 % nonfat milk in TBS for 5 min each. The blots were then incubated for 1 hour at room temperature with the secondary peroxidase-conjugated antibody for 1 h at room temperature. The membrane was extensively rinsed with 3% nonfat milk in TBS (6 × 5 min) and finally with TBS (3 times) before color development with TMB (Tetra Methyl Benzidine) to reveal proteins-antibody binding. The membranes were photographed individually and overlapped with others gels.

Antibodies and conjugates:

1 - Antibody anti-Gliadin - Gliadin Antibody Mouse Monoclonal [clone 4F3] (IgG2a,k) specific to wheat Gliadin (LS Bio - Life Span BioScience Inc., Seattle, WA, USA) diluted to 1:10,000 in 3% nonfat milk in TBS. The secondary antibody was horseradish peroxidase conjugated with goat anti-mouse Polyclonal to Mouse IgG Fab (Life Span BioScience Inc., Seattle, WA, USA) diluted to 1:3000 in 3% nonfat milk in TBS.

2 – Skerritt Antibody – The Skerritt antibody (clone 401.21) an IgG1 mouse mAb, developed against wheat gliadin and specific for ω-gliadin was kindly provided by Dr. Laura Allred (ELISA Technologies, Inc., Gainesville, FL USA). This antibody was developed by (SKERRITT; HILL, 1990) and following validation by the AOAC (SKERRITT; HILL, 1991) has been used in several commercial ELISA kits. diluted to 1:5000 in 3 % nonfat milk in TBS.

4.3 RESULTS AND DISCUSSION

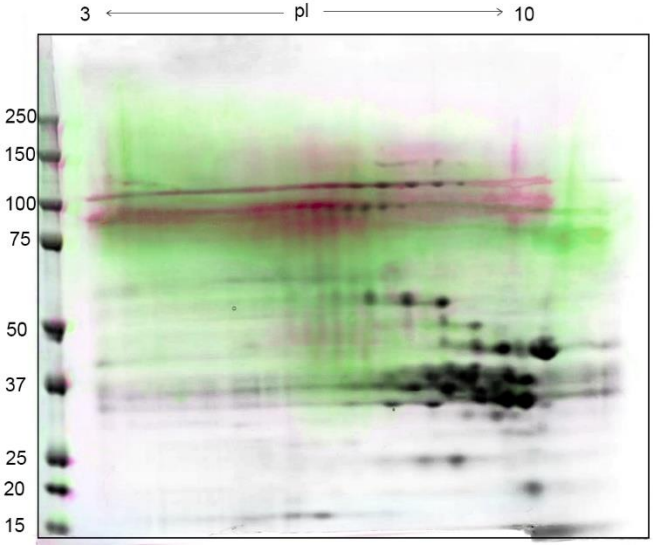
All wheat flours were extracted, purified, separated in 2 D gel, stained with Coomassie blue or were run a western blot with Gliadin antibody and Skerritt Antibody, and pictures of them were overlapped.

In order to determine the possibility of the wheat varieties being nontoxic to celiacs, were tested pools of different wheat varieties, two of them with AA genome - *T. monococcum* ssp. monococcum and *T. urartu.*

4.3.1 *T. monococcum* ssp. *monococcum*

Five varieties of *T. monococcum* ssp *monococcum* were mixed and showed low amounts of HMW-GS (67 – 100 kDa). The higher amount of protein has around 20 – 50 kDa, which is LMW-GS and coincides with the α/β- and γ-gliadin position (Figure 4.1). However, both antibodies seem to have more affinity with HMW-GS and gliadins in general.

Figure 4.1. Overlapping image of two-dimensional electrophoresis and respective western blot analysis of *T. monococcum* ssp *monococcum*.



Legend: Gel was stained with Coomassie, represented in black, the immunoblotting with Gliadin Antibody is in pink, and with Skerritt Antibody in green.

The Gliadin antibody binds with gliadin and HMW– GS. *Triticum monococcum* is a small grain, but with high endosperm protein

content and similar amino acid composition to modern wheat (ABDEL-AAL; HUCL, 2014).

The immunotoxicity of *T. monococcum* was previously tested and showed different intensity according with the lineage (GIANFRANI et al., 2015). The same authors concluded that the *T. monococcum* is less toxic than *T. aestivium*. According with Iacomino et al. (2016) (IACOMINO et al., 2016), *T. monococcum ID331* did not affect cell viability or induce cytoskeleton reorganization, nor did it inactivate zonulin release, but other *T. monococcum* did. Nevertheless, all *T. monococcum* have high toxicity against polyclonal celiacs intestinal T-cells (GIANFRANI et al., 2015). A high concentration of zonulin on gut cells induce an increase of permeability of the intestinal barrier, resulting in a worsening of the condition in celiacs (FASANO, 2011), due to a reorganization of intestinal cell cytoskeleton resulting in increased permeability (SILANO et al., 2012).

However, Zanini et al., 2015 demonstrated the toxicity of *T. monococcum* on histological and serological aspects of celiacs, even though it was tolerated by the majority of patients, leading the authors to recommend *T. monococcum* to patients with gluten sensitivity or for prevention of CD.

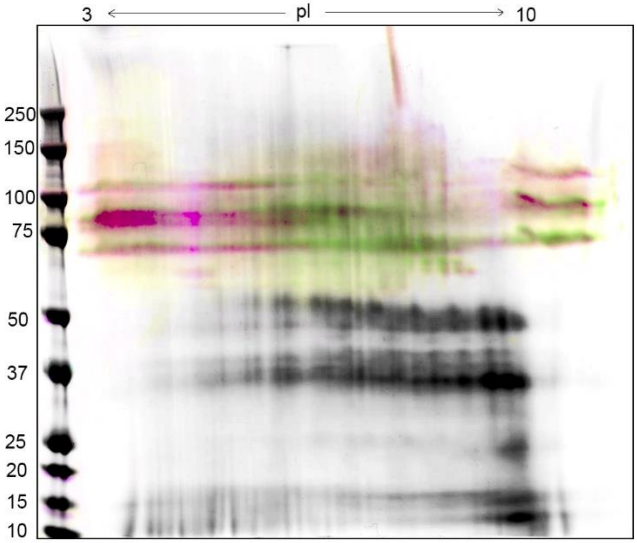
4.3.2 *T. urartu*

In 2D gel of *T. urartu* it is possible to see the proteins with molecular weight near 37 – 50 kDa, which represent LMW-GS and gliadins. On the other hand, the western blot of the gels show that the bound antibodies are in the HMW-GS region with weak binding or protein in the gliadin region (Figure 4.2).

Theses results confirm low toxicity of *T. urartu* as reported by Gell et al (2015), which when tested against G12 and R5 mAbs T cells stimulators to CD, showed no detectable stimulatory epitopes on T cells against G12 and very low against R5 (GELL et al., 2015). Also, the low cytotoxicity of *T. urartu* was reported in polyclonal T-cell lines from intestinal of celiacs (MOLBERG et al., 2005).

This can be explained due the lack of B and D genome in *T. urartu*, but on the other hand, the ω-gliadin from *T. aestivum* and *T. urartu* are closely related to each other (DUPONT et al., 2004), showing low toxicity to celiacs.

Figure 4. 2 Overlapping image of two-dimensional electrophoresis and respective western blot analysis of *T. urartu*.



Legend: The gel was stained with Coomassie, here represented in black, the immunoblotting with Gliadin Antibody is in pink, and with Skerrit Antibody in green.

Both, *T. monococcum* and *T. urartu* are known as einkorn, because they are diploid (HSAM; KIEFFER; ZELLER, 2001). However, the A genome on hexaploid wheat is derived from *T. urartu* (JING et al., 2007; LING et al., 2013). The A genome in *T. urartu* provides better grain yield and high disease resistance to modern wheat (ABDEL-AAL; HUCL, 2014).

Even though, einkorn has high protein content and is protected by the hulls against disease, it is poor in quality and presents low kernel and grain yields upon higher incidence of lodging (LONGIN et al., 2016).

Besides the agronomic traits, in relation to yield, as indicated by Gell et al. (2015) and Longin et al. (2016), einkorn wheat should be used for breeding new varieties with lower toxicity to celiacs and for nutritional improvement of modern wheat.

4.3.3 *T. turgidum*

Due to agronomic characteristics and commercial appeal, we used four *T. turgidum* wheat varieties as followed: *T. turgidum ssp durum* (AABB), *T. turgidum ssp polonicum* (AABB), *T. turgidum ssp turgidum* (AABB) and *T. turgidum ssp turanicum* (AABB).

The commercial appeal is that it is somewhat en vogue for gourmet cooking, is usually known as farro, but farro is the emmer, and emmer is one of two sub-species of *T. turgidum* (emmer and durum). Emmer wheat is not free-threshing, which means that there is a tightly adhering husk, or glume, that is difficult to remove from the grains. Durum wheat is free-threshing, that is, the seeds are readily released from the glumes (KASARDA, 2012).

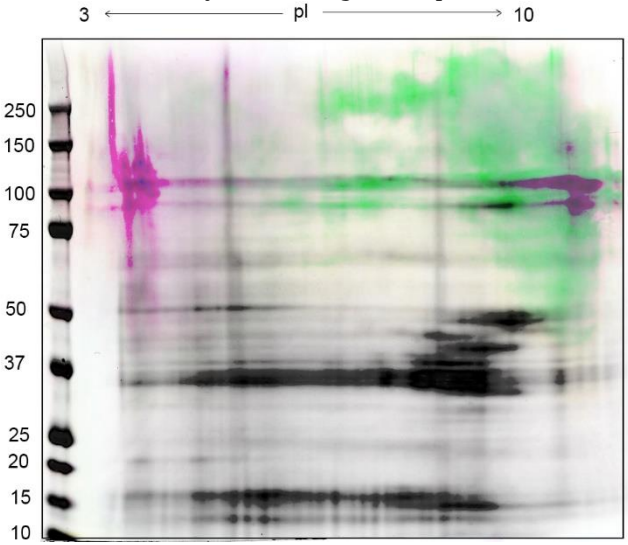
4.3.3.1 T. turgidum ssp *durum*

When T. turgidum ssp durum proteins were separated by 2D electrophoresis, it was possible to note an agglomerate of proteins with molecular weight near 37 – 50 kDa and pI higher than 7, which represents LMW-GS and possible γ- and ω-gliadins. But, the western blot of the gels demonstrated higher affinity against HMW-GS (75 – 100 kDa) with weak binding in the gliadin regions to the Gliadin Antibody and against HMW-GS only in the case of the Skerrit Antibody (Figure 4.3).

We were expecting that antibodies would bind in the ω-gliadin region once that (MAMONE et al., 2015) reported this occurrence using celiac sera and pasta made with *T. turgidum ssp durum* after *in vitro* digestion.

There are differences in content and type of gliadin between tetraploid and hexaploid wheat varieties (RIBEIRO et al., 2016).

Figure 4. 3. Overlapping image of two-dimensional electrophoresis and respective western blot analysis of *T. turgidum* ssp *durum*.



Legend: Gel was stained with Coomassie Blue, here represented in black, the immunoblotting with Gliadin Antibody is in pink, and with Skerrit Antibody in green.

According to Ribeiro et al. (2016) modern wheat varieties are no more toxic to celiacs than the ancient wheats, in fact, they concluded the opposite, the ancient varieties present the same or higher toxicity to celiacs than the modern wheat varieties, thus, “breeding practices did not negatively contribute to celiac disease-related toxicity and ultimately did not contribute to the increased prevalence of the disease during the latter half of the 20th century”.

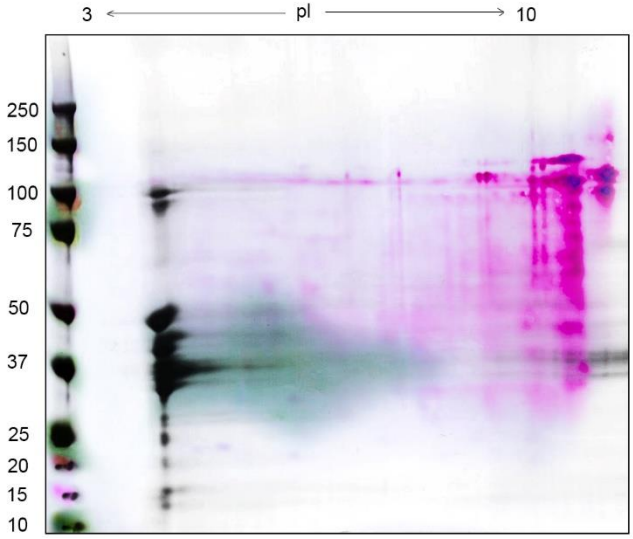
Durum wheat variety is the sencond most produce wheat around the world, because agronomic performance and quality trails of *T. tugidum ssp durum* were well established (LONGIN et al., 2016).

4.3.3.2 T. turgidum ssp *polonicum*

Proteins from *T. turgidum ssp polonicum* were not well separated by 2D gel but still, is was possible to note some tenuous stains in the HMW-GS and LMW-GS regions. The western blot results yielded stronger signals with high binding of Gliadin antibodies in the region of

HMW-GS, however, it almost did not show any binding against Skerrit Antibody (Figure 4.4).

Figure 4. 4 Overlapping image of two-dimensional electrophoresis and respective western blot analysis of *T. turgidum* ssp *polonicum*.

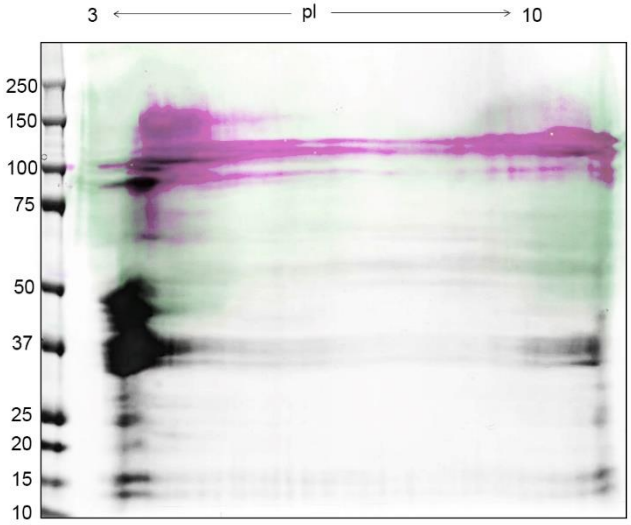


Legend: Gel was stained with Coomassie, here represented in black, the immunoblotting with Gliadin Antibody is in pink, and with Skerrit Antibody in green.

4.3.3.3 T. turgidum ssp *turgidum*

Gluten proteins from *T. turgidum ssp turgidum* revealed after 2D Gel separation and western blot behaved similarly to *T. turgidum ssp polonicum*. However, the Skerritt antibody binding in the upper part of the membrane corresponded to HMW-GS (Figure 4.5).

Figure 4. 5. Overlapping image of two-dimensional electrophoresis and respective western blot analysis of *T. turgidum* ssp *turgidum*.

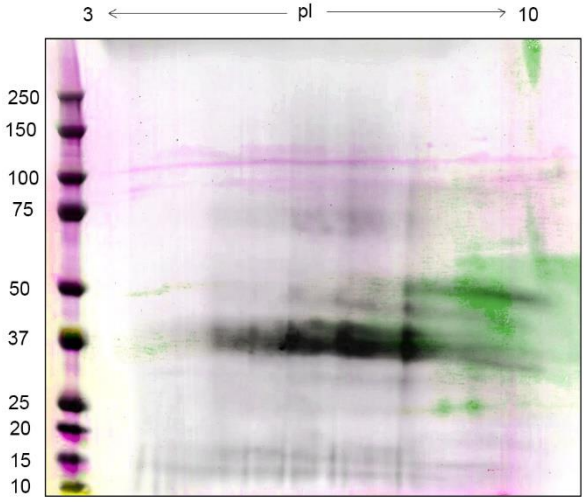


Legend: Gel was stained with Coomassie, here represented in black, the immunoblotting with Gliadin Antibody is in pink, and with Skerritt Antibody in green.

4.3.3.4 T. turgidum ssp *turanicum*

Gluten proteins from *T. turgidum ssp turanicum* separated by 2D Gels showed a high concentration of proteins with molecular weight around 40 kDa, that correspond to the region of LMW-GS and gliadin. When transferred to the western blot, Gliadin antibody bound in the LMW-GS region and Skerritt Antibody in LMW-GS region (Figure 4.6).

Figure 4. 6. Overlapping image of two-dimensional electrophoresis and respective western blot analysis of *T. turgidum* ssp *turanicum*.



Legend: Gel was stained with Coomassie, here represented in black, the immunoblotting with Gliadin Antibody is in pink, and with Skerritt Antibody in green.

Despite that all the wheat varieties tested did not have the D genome that expresses 33-mer toxic epitope, all samples slightly bound with antibodies in the gliadin region. This means that these varieties seem to be less toxic, and could potentially be used to breed with modern wheat to produce wheat with lower toxicity.

According to Ribeiro et al. (2016), the lack of D genome is the principal reason for the low toxicity. Our findings, support that the presence of the A and B genomes that express other gliadins, HMW-GS and LMW-GS also contribute in some way to celiac disease, as previously reported (GIANFRANI et al., 2015; RIBEIRO et al., 2016; TYE-DIN et al., 2010; VINCENTINI et al., 2009). Gianfrani et al. (2015) suggested that intestinal damage in celiacs is the result of a synergistic action on the stimulation of proinflammatory T lymphocytes.

Even though the appeal to use ancient wheats as low toxicity varieties exists, results indicate a higher toxicity in AA wheats than in the modern AABB wheats. Ribeiro et al. (2016) demonstrated high variability in the amount of T-cell stimulatory epitopes against R5 antibody among modern and ancient wheat varieties, showing that some modern wheats can be less toxic that old varieties.

Besides, some authors affirm that wheat has become increasingly toxic in recent years, our findings supported by Vincentini et al. (2009), deny this statement, in that old wheat varieties are also toxic. The same authors demonstrated that tetraploid ancient wheats have a heterogenic group of genotypes showing a widespread variability in eliciting immune reactions by T-cell of CD individuals.

Some researchers believe that newer cultivars bred from ancient and modern wheat could yield new wheat cultivars with low toxic sequences to celiacs and still keep good breadmaking or pasta-making qualities (MOLBERG et al., 2005; MOLBERG et al., 2005). In addition, the new breeds could be healthier with more disease resistance (WIWART et al., 2013) for example in the field, *T. turgidum ssp polonicum* has shown higher resistance to Fusarium head blight (caused by Fusarium fungi (WIWART et al., 2013).

From all *T. turgidum*, the durum has more economic importance but the other are rising due to commercial trends. Also, *T. turgidum* sub-species have elongated grains that are easy to thresh and have a significant resistance to drought (WIWART et al., 2013).

4.4 CONCLUSION

All wheat varieties tested contained gluten proteins recognized by anti-gluten Skerritt and Gliadin antibodies related to T-cell stimulatory epitopes, at distinct levels. Einkorn and durum species tested here differ in toxicity depending on the sub-species. Eikorn tested showed higher immunogenicity that durum varaities. So, it is not safe for all celiacs to consume the wheat varieties tested, that include ancients' varieties.

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5 COMPLIANCE WITH GLUTEN-FREE LABELLING REGULATION IN THE BRAZILIAN FOOD INDUSTRY

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ABSTRACT

Gluten is an important protein complex for baking products found in wheat, rye, and barley and some oat varieties. However, some people need to avoid these grains and their products because they result in gluten-related disorders. The only treatment for these individuals is to engage in a gluten-free diet. The objective of this work was to verify if the gluten content of several commercial food products sold in Brazil complied with their labeling. The Méndez ELISA R5 sandwich method was used to analyze 437 samples, and of these, 70 % were labeled as gluten-free, 26 % as containing gluten, and 4 % not labeled in relation to gluten. The results indicated that 89 % of the products labeled as gluten-free were correctly labeled and 11 % were not, which represented a risk for celiac people.

5.1 INTRODUCTION

When wheat flour is mixed with water to form dough, the proteins form a continuous gluten network, which confers viscoelasticity and enables the dough to be processed into bread and similar leavened food products (Shewry et al. 2001). The gluten network is composed of multiple subunits of prolamin and glutelin (Wieser 2007). Prolamins are storage proteins found in wheat (gliadin), barley (hordein), rye (secalin) (Shewry and Halford 2002), and some oat (avenin) varieties (Silano et al. 2014).

Celiac disease is an immune-mediated enteropathy that appears in patients with genetic predisposition (Murray 1999; Schuppan 2000; Sapone et al. 2012). Celiac disease includes symptomatic cases with either classical intestinal symptoms (chronic diarrhea, weight loss, and related symptoms) or nonclassical extraintestinal symptoms (such as anemia, osteoporosis, neurological disturbances, and dermatitis herpetiformis) (Murray 1999; Cosnes et al. 2008; Freitag et al. 2009; Sapone et al. 2012). In addition to celiac disease, there are other important cereal-related hypersensitivity reactions such as wheat allergy and gluten sensitivity (Sapone et al. 2012). Wheat allergy (WA) is defined as an adverse immunological reaction to wheat proteins, in which immunoglobulin E antibodies play a central role in the pathogenesis of these diseases (Battais et al. 2005). It is now becoming clear that besides celiac disease and wheat allergy, there are cases of gluten reactivity that involve neither allergic nor autoimmune mechanisms, termed nonceliac gluten sensitivity (NCGS). The symptoms in NCGS may resemble those associated with celiac disease but with a prevalence of extraintestinal symptoms, such as behavioral changes, bone or joint pain, muscle cramps, leg numbness, weight loss, and chronic fatigue (Catassi et al. 2013).

The only effective therapy is to assume a gluten-free diet (Cosnes et al. 2008; Lewis et al. 2011; Sapone et al. 2012; Catassi et al. 2013), except for WA that is necessary avoid all kinds of wheat proteins (Scibilia et al. 2006; Catassi et al. 2013). The increase in the diagnostic rate of gluten-related disorders and consequent adherence to a gluten-free diet by individuals affected, as well as by their healthy family members and nonceliac gluten-free dieters, leads to increase the gluten-free market. The healthy family members of a celiac individual usually adopt the gluten-free diet as well, owing to a social issue, to help the celiac member keep on the diet (Moore 2014), because many celiac individuals have difficulties in following a diet without gluten owing to peer pressure (do Nascimento et al. 2014). Also, following a gluten-free diet is a trend in nutrition that became a popular way to lose weight; however, it is not true (Gaesser and Angadi 2012, 2015; Jones 2012).

Many of the gluten-free products currently available present low technological and nutritional quality, especially when compared with the wheat-containing counterparts (Mariotti et al. 2009), and the low technology applied to gluten-free manufacture can lead to contamination during the process. Additionally, some gluten-free products labeled as such may contain gluten owing to contamination with gluten-containing items during harvest, storage, processing, or transport. For people who have a gluten-restricted diet, consumption of food products with gluten worsens their pathologic and nutritional state (Marcílio et al. 2005).

Great improvement in food labeling has been seen on a global basis over the past 10 years. However, considerable controversy exists regarding the definition of gluten-free food products. The term gluten-free varies from country to country. According to the U.S. Food and Drug Administration (FDA), any product labeled as gluten-free should contain less than 20 mg/kg of gluten; however, it is not mandatory to label any food item regarding the presence or absence of gluten in the United States (FDA 2013). According to the *Codex Alimentarius* Commission (2008), food that contains less than 20 mg/kg of gluten should be labeled as gluten-free, above 20 and up to 100 mg/kg of gluten as reduced gluten content, and above 100 mg/kg of gluten as containing gluten. For countries in the European Community it is obligatory to label all food products regarding the presence of gluten, and they follow *Codex Alimentarius* (Commission of the European Communities 2009). In 2002, Brazilian health authorities defined the resolution that all food and beverage products must be labeled regarding the presence or absence of gluten from wheat, barley, rye, oats, and their derivatives (Brazil 2002, 2003a, 2003b). Recently, the Brazilian government launched a new clinical trial protocol and therapeutic guidelines regarding celiac disease (Brazil 2015) to help in the correct diagnosis of celiac disease and to monitor trends in nutrition. The National Health Council recently released a note with the official recommendation that nonceliacs do not follow a gluten-free diet such as otherwise healthy individuals were adopting to lose weight (Conselho Regional de Nutrição 2014).

The *Codex Alimentarius* and the Working Group on Prolamin Analysis and Toxicity recommend the enzyme-linked immunoassay (ELISA) that uses the R5 monoclonal antibody (Mendez method) as the best method for gluten determination. This method is specific for the glutamine-glutamine-proline-phenylalanine-proline (QQPFP) amino acid sequence and represents a valuable tool for the quality control of gluten-free foods (Osman et al. 2001).

The aim of this study was to verify if food products commercialized in Brazil were correctly labeled in relation to the presence or absence of gluten by using the ELISA R5 method.

5.2 MATERIALS AND METHODS

5.2.1 Samples

A total of 437 samples representing a major cross section of products for the gluten-free market were analyzed. The samples were submitted by food companies, predominantly those manufacturing gluten-free products, to the Cereal Science and Technology Laboratory of the Food Science Department at the Federal University of Santa Catarina, Brazil, through the extension program for gluten determination. Products were labeled as containing gluten, gluten-free, or not labeled. Unlabeled samples were products not commercially available as quality control or products in the development phase.

The samples were categorized as shown in Table 5.1. To avoid contamination, sample preparation and gluten determination were done in an isolated room used solely for that purpose.

5.2.2 Gluten determination

Samples were received and stored according to conditions recommended by the company requesting analysis. Before analysis, all samples were homogenized with an A11 basic analytical mill (IKA-Werke, Staufen, Germany). Heat-processed products or soy-containing products were extracted with the Mendez cocktail supplied with the ELISA kit (López et al. 2002) followed by the addition of 80% (v/v) aqueous ethanol to a final concentration of 60% ethanol. Other samples were extracted directly with 60% (v/v) ethanol according to the manufacturers' instructions. The extracted samples were analyzed with the R5 antibody Transia Plate prolamins (PR0320, Diffchamb, Lyon France) (AACC International Approved Method 38-50.01).

Each sample was extracted in two replicates and was analyzed in duplicate and at two different dilutions.

A second-degree polynomial standard curve was plotted by using reference samples (1.56, 3.12, 6.25, 12.5, and 25 ng/mL of gliadin) to calculate gliadin concentration, and the results were multiplied by 2 to convert to gluten.

Table 5. 1. Description of Each Food Category.

Code	Description
FFA	Flours, *farofas* (toasted manioc flour mixtures), and starches
SNK	Snacks
CM	Cookies and muffins
CC	Instant chocolate powder and candies
MIX	Baking mixtures and instant products
PAS	Pasta
GR	Grains
PC	Potato chips
CER	Maize flakes and granola
SS	Seasonings, herbs, and spices
OUT	Other items not falling into one of the above categories

5.2.3 Statistical Analysis

We used different frequency distribution graphs according to category, label, and gluten level and according to label and legislation. For a better comparison of the results, these were grouped into two ranges of gluten content: samples with less than 20 mg/kg and samples with more than 20 mg/kg (Codex Alimentarius Commission 2008).

5.3 RESULTS AND DISCUSSION

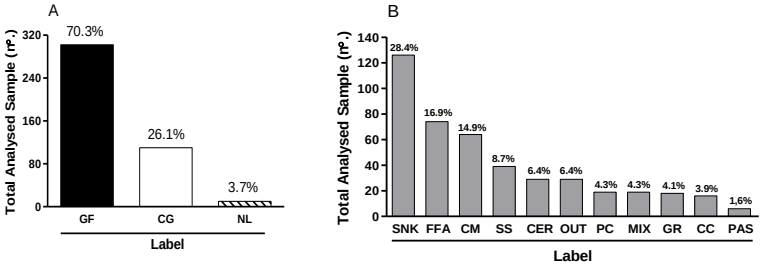
5.3.1 Sample profile analysis

Because these were samples sent by companies for gluten determination, it was possible to outline a profile of the companies based on the Brazilian legislation on gluten. The majority of samples were labeled as gluten-free and thus targeted consumers who adhere to a gluten-free diet. The second major group was composed of samples labeled as containing gluten, which were sent for analysis to verify if these foods were contaminated with gluten, even when they were manufactured with gluten-free ingredients. Finally, we received some samples that were not labeled, which were sent by companies to verify the presence of an ingredient that could contaminate all final products or which represented a new product to be released in the market as gluten-free (Fig. 1A). Some of these samples could be products not yet launched in the market but being tested to detect possible gluten contamination. It is an indication of

the interest of the food companies in offering more gluten-free products but safe food products according to gluten regulations.

Gluten-free products have been witnessing strong growth even among nonceliac patients, with more new product introductions in snacks and alcoholic beverages (Gaesser and Angadi 2015; Markets and Markets 2015). The high demand for variety has led to an exponential increase in the number of food companies that produce gluten-free products (SEBRAE 2014; ACELBRA 2015). Data are not available about the gluten-free only market in Brazil, but the specialty food market has risen 211.5 % from 2009 to 2014, with sales increasing from US$ 50.3 million to US$ 156.7 million (Leite 2015). On the other hand, in the United States the gluten-free market totaled US$ 8.8 billion in 2014 and was expected to rise at least 20 % in 2015 (Economist 2014). The European region is projected to be the fastest-growing market in the near future owing to the health concerns in the region (Markets and Markets 2015).

Figure 5. 1. Frequency distribution graphs: A, label and B, category of samples analysed.



Legend: GF = Gluten-Free; CG = Contain Gluten; NL = Not Label. SNK = snacks; FFA =flours, *farofas* (toasted manioc flour mixtures) and starches; CM = cookies, and muffins; SS = seasoning, herbs and spices; CER = corn flakes and granola; OUT = others; PC = potato chip; MIX = baking mixture and instant products; GR = grains; CC = instant chocolate powder and candies; and PAS = pasta.

According to the National Health Council of Brazil, approximately 1 % of the Brazilian population are celiac (i.e., around two million celiacs). The high demand has resulted in labeling regulations to give more security for gluten-free consumers. The first regulation was about labeling food (Brazil 2003a) and the second about pharmaceutical drugs (Brazil 2003b); in 2014, it was mandated for all schools in Brazil to provide

adequate food for students with specific health conditions, such as celiac disease (Brazil 2014).

From all food categories analyzed, categories with the greatest number of submissions were 1) snacks, 2) flours, *farofas* (toasted manioc flour mixtures), and starches (FFA), and 3) cookies and muffins (Fig. 1B), suggesting that these are the main food products that are targeted to celiacs. Snacks, muffins, and cookies do not require preparation and are easy to eat; therefore, they are widely consumed. In Brazil, snacks are made basically from maize, which naturally does not contain gluten, so individuals who discover they have a gluten-related disorder and need dietary change do not need to replace this food item to maintain a gluten-free diet. Snacks are generally inexpensive and good tasting, which are the most common issues of gluten-free foods from celiac people (do Nascimento et al. 2014).

Celiacs look for safe food products with good sensorial and nutritional qualities and low price. Lack of any of these attributes forces them to prepare their own food at home. This explains the high number of FFA samples. Grains, seeds, flours, and starch can help them obtain adequate dietary fiber (Gaesser and Angadi 2015). According to do Nascimento et al. (2013), rice flour, cassava starch, maize starch, and soy flour are some of the ingredients most frequently used in gluten-free food products.

A smaller number of products in the categories seasonings, herbs, and spices (SS), grains, and pasta that naturally should not have gluten were submitted for analysis. These products, including grains and pasta, are manufactured without wheat, rye, and barley. However, in the case of the SS category ACELBRA, the Brazilian Celiac Association, recommends that celiac patients do not eat processed seasonings owing to possible contamination.

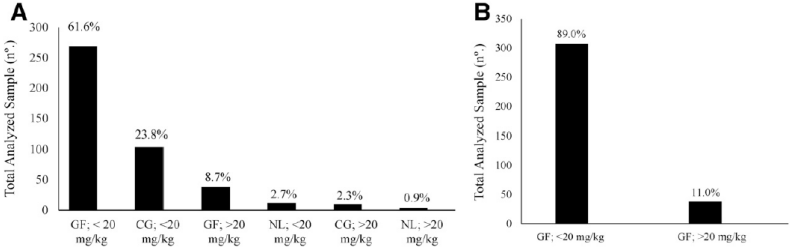
5.3.2 Compliance with labeling regulation

A gluten-free diet consists of a combination of naturally gluten-free foods (e.g., fruit, vegetables, rice, and unprocessed meat) and specially manufactured gluten-free versions of wheat-based foods such as bread and pasta (Singh and Whelan 2011). To determine if the food is gluten-free, the ELISA R5 method is cost effective.

Most of the total samples were correctly labeled as gluten-free (Fig. 5.2A). When we look just to food products labeled as gluten-free, 89 % of samples were correctly labeled (Fig. 5.2B). This means that the number of samples wrongly labeled was 11 %, which represents a risk for celiac

people. Other authors reported a higher amount of inaccurate labeling in 2010 in Brazil (Plaza 2010) and Canada (Koerner et al. 2011), with better results in the United States (Sharma et al. 2015). To decrease this amount of mislabeling it is necessary to adopt good manufacturing practices and quality control as well as testing the raw materials and final products for the presence of gluten. Cross contamination of naturally gluten-free or manufactured gluten-free food products can happen in the field and during harvest, transport, storage, and processing (Murray 1999; Sharma et al. 2015).

Figure 5. 2. Frequency distribution graphs: A, label and legislative category; and B, percentagem sample labeled gluten-free with more than 20 mg/kg.



Legend: GF = gluten-free; CG = Countain Gluten; NL = Not Labeled.

On the other hand, some samples were labeled as containing gluten although the level was below 20 mg/kg (Fig. 2A); thus, these samples could be labeled as gluten-free. Some companies prefer to label their samples as containing gluten rather than adopt manufacturing practices to avoid contamination with gluten, because this would result in higher expenses that will affect the final price to consumers. Changing the label from containing gluten to gluten-free without contamination control could be dangerous, because food can be contaminated at several places in the manufacturing chain. If a contamination issue results, the company faces product recalls and potential lawsuits in Brazil (Brazil 2002) and in other countries in the case of exported food products.

There were gluten-free labeled products in all categories, because the aim of this analysis was to verify whether there was or was not gluten contamination in food products, which most likely occurred during the processing (Table II).

In our study, we found that the cookies and muffins category contained the largest number of food products labeled as gluten-free with a gluten level below 20 mg/kg (Table 5.2). This category represents the most common gluten-free items available at the supermarkets, and these

products are preferably manufactured with rice and maize flour (Singh and Whelan 2011; do Nascimento et al. 2014).

Of the total samples analyzed, snacks represent the largest category; however, most of the samples were labeled as containing gluten, even when gluten analysis showed values below 20 mg/kg (Table 5.2). Snacks are made with maize, which is a naturally gluten-free cereal; however, usually in Brazil farmers practice a maize/wheat and soybean/wheat rotation system, which means that naturally gluten-free cereals are planted, harvested, stored, and processed in the same field with the same farm machinery used for wheat production (Arenhardt et al. 2015), and this can result in contamination (Murray 1999). Thus, it is easier to label these foods as containing gluten than to follow measures to avoid contamination, because this would result in added cost to the company and to final consumers.

Table 5. 2. Frequency Distribution of Gluten Level According to Category and Label.

Category (%)	<20 mg/kg			≥20 mg/kg		
	GF	CG	NL	GF	CG	NL
SNK	6.2	21.1	…	0.2	0.9	…
FFA	10.3	1.4	1.8	2.5	0.7	0.2
CM	12.8	…	…	2.0	…	…
SS	6.2	…	0.5	2.0	…	…
OUT	5.0	0.7	…	0.2	0.5	…
CER	5.7	…	…	0.7	…	…
PC	4.3	…	…	…	…	…
MIX	3.0	0.5	0.2	0.7	…	…
GR	3.4	0.2	…	…	0.2	0.2
CC	3.2	…	0.2	0.2	…	0.2
PAS	1.4	…	…	…	…	0.2

[a] Data are expressed as total of samples analyzed (% of frequency). Categories are defined in Table I. GF = gluten-free; CG = contains gluten; and NL = not labeled.

Contamination led to mistakes on labeling; in our study the main products wrongly labeled were found in the categories cookies and muffins, FFA, and SS (Table II), indicating failures in manufacturing practices and quality control. In almost all categories we found food products wrongly labeled, products that were labeled as gluten-free but contained above 20 mg/kg of gluten. This presents a big challenge to celiac consumers, who need to avoid gluten and try to buy safe products

or ingredients to make food at home. These companies should adopt measures to avoid gluten contamination; although inspection of gluten-free ingredients and good manufacturing practices increase the production price, according to Lee et al. (2007) and Worosz and Wilson (2012) the final gluten-free food products are more expensive, so producing them is an investment. And this investment can be reverted in final products more expensive (Singh and Whelan 2011) that gluten-free dietarians are used to pay, once the sensorial qualities improve (do Nascimento et al. 2014).

5.4 CONCLUSIONS

These results show for the celiac population in Brazil that companies are testing their food products to verify if labeling is correct to provide products that are safe and to accurately identify potential gluten levels. However, if 89% of gluten-free food products are correctly labeled, this means that 11% represent a risk for the health of the celiac population. To improve this amount, routine auditing is necessary to verify the correct labeling about gluten in food products and adopt good manufacturing practices.

Brazil is becoming a global economic player, so it is important to be aligned with global legislation concerning gluten presence and ensure that the label can be trusted. This results in greater confidence in both the global market and consumers and represents one more step toward health maintenance for celiacs.

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6 THESIS CONCLUSION

Wheat gluten proteins subjected to common processing conditions were analyzed for protein solubility and chromatographic profiles demonstrated modification of structure/complexity as well as immunotoxicity.

Similarly, treatments affected glutenins and gliadins profile, the amount of the HMW-GS decrease after spray-dry, oven and extrusion and the amount of the LMW-GS decrease too after spray-dry, microwave, oven and extrusion. Wheareas for gliadin, the amount of ω-gliadin increase after spra-dry and ultrasound treatments, and the amount of α/β-gliadin decrease after all treatments, while the treatment to do not affected γ-gliadins amount. Likewise, digestibility decrease after spray-dry and ultrasound.

This changes is results of rearrange of proteins during the treatments, resulting in a higher complex protein structure.

And, finally, the potential celiac disease immune stimulatory epitopes decrease after spray-dry treatment by R5 monoclonal antibody and decrease after spray-dry and microwave treatment by G12 ELISA test in relation to control flour.

At last, even with the alteration on the gluten structure and complexicity, these changes do not allow to produce a safe product to celiacs, the amount of the potential celiac disease immune stimulatory epitopes still were too high.

All wheat varieties tested contained proteins which were recognized by anti-gluten antibodies including Skerritt and Gliadin antibodies related to T cell stimulatory epitopes, at different levels. Einkorn and durum species differ in toxicity depending of the sub-species.

So, it is not safe for all celiacs to consume any kind of wheat, even though ancient wheat grain varieties seem to have high commercial and gourmet appeal.

These results show for the celiac population in Brazil that companies are testing their food products to verify if labeling is correct to provide products that are safe and to accurately identify potential gluten levels. However, if 89 % of gluten-free food products are correctly labeled, this means that 11 % represent a risk for the health of the celiac population. It is necessary auditing to verify the correct labeling about gluten in food products and adopt good manufacturing practices.

7 FINAL CONSIDERATIONS

For futher studies it is suggested in vivo study with mice C57BL/6j to test if alteration on gluten protein promoted by treatment result in a different via of celiac disease manifestation.

Also, with animals, with the ways, age stage and levels of treated or do not treated flour lead a different manifestation of celiac disease.

Another important suggestion is determining in celiac patients an epigenetic study to start to elucidate which environmental factors affect the manifestation of the celiac disease, once that not all DQ2/8 people are celiac.

Finally, sit is reasonable think about develop a method to determine individual level of gluten tolerance to celiacs by western blott.