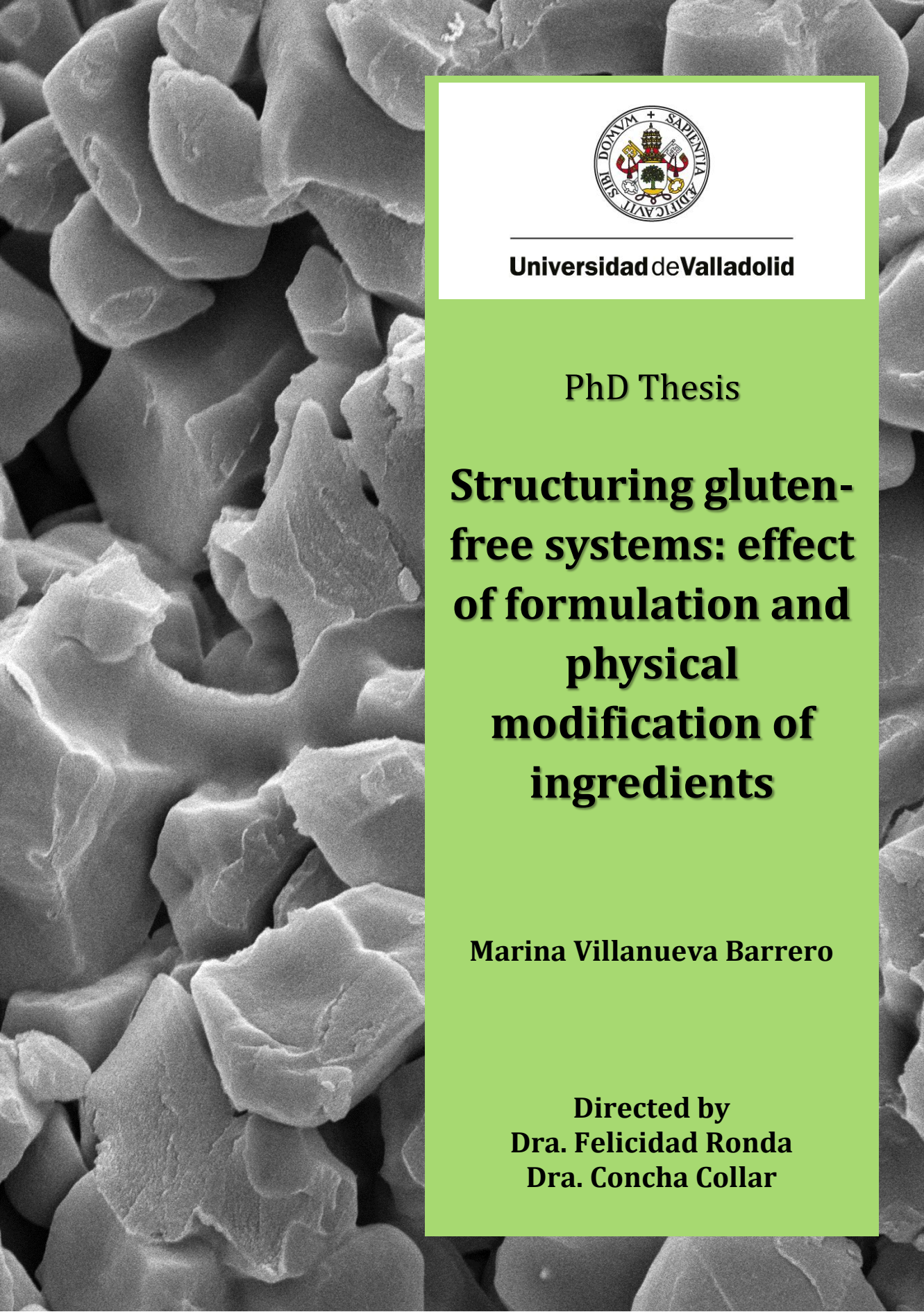

Universidad de Valladolid

PhD Thesis

Structuring gluten-free systems: effect of formulation and physical modification of ingredients

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PROGRAMA DE DOCTORADO EN CIENCIA E INGENIERÍA AGROALIMENTARIA Y DE BIOSISTEMAS

TESIS DOCTORAL:

Structuring gluten-free systems: effect of formulation and physical modification of ingredients

Presentada por Marina Villanueva Barrero para optar al grado de Doctor/a por la Universidad de Valladolid

Dirigida por:

Felicidad Ronda
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AUTORIZACIÓN DE LOS DIRECTORES DE LA TESIS PARA SU PRESENTACIÓN

Las Dras. Dña. Mª Felicidad Ronda Balbás y Dña. Concepción Collar Esteve, como directoras de la Tesis Doctoral titulada "STRUCTURING GLUTEN-FREE SYSTEMS: EFFECT OF FORMULATION AND PHYSICAL MODIFICATION OF INGREDIENTS" realizada por Dña. Marina Villanueva Barrero en la Escuela Técnica Superior de Ingenierías Agrarias de la Universidad de Valladolid dentro del Programa de Doctorado en Ciencia e Ingeniería Agroalimentaria y de Biosistemas, autorizan su presentación dado que reúne las condiciones necesarias para su defensa.

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"Decir de los cazadores que estamos, todos, un poco de la cabeza, no es ninguna novedad. Lo que un cazador es capaz de hacer por una perdiz, no puede imaginarlo más que otro cazador. El cazador con afición está dispuesto a todo"

Miguel Delibes, Las perdices del domingo

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RESUMEN

La creciente demanda de productos libres de gluten ha favorecido el desarrollo de numerosos productos de panadería buscando imitar las características de calidad de sus homólogos elaborados con trigo. A pesar de ello la mayoría de los productos sin gluten existentes en el mercado actual son de calidad física y sensorial sensiblemente inferior y presentan importantes carencias nutricionales, en concreto un balance proteína/almidón significativamente más bajo que en los panes de trigo. La mayoría de los estudios realizados para la obtención de productos sin gluten se han basado en la sustitución de harina de trigo por harinas sin gluten (principalmente de arroz), mezcla de almidones de diferentes orígenes (maíz, arroz, patata, tapioca, etc.) y distintos hidrocoloides (polímeros capaces de mimetizar la estructura del gluten).

Esta tesis doctoral aborda el estudio de diferentes mecanismos de estructuración de sistemas sin gluten. Por una parte, la creación de estructura mediante la incorporación de proteínas exógenas y el efecto de la adición de ácidos orgánicos, una alternativa tecnológica que encierra un evidente interés físico-químico y nutricional. Por otro lado, el cambio en las propiedades tecnológicas de las matrices sin gluten –harina, almidones o mezclas de almidones y proteínas-, mediante el empleo de tratamientos hidrotérmicos asistidos con microondas, siendo éste un método novedoso y apenas investigado.

Para ello, se han estudiado fundamentalmente dos tipos de matrices: sistemas panarios (masas de pan y panes) y geles alimentarios. El pan es un producto de elevado consumo diario que se adecua muy bien a la adición de sustancias de interés nutricional ya que representa un aporte significativo en la dieta diaria. De la misma forma, los geles sirven de base para numerosos productos ya que permiten modificar la textura, estabilidad y elasticidad de los mismos.

La primera parte de este estudio se ha centrado en la evaluación de la capacidad estructurante de las proteínas modulada por el pH de las masas y su dependencia del tipo de almidón utilizado. La incorporación de proteínas conduce a la formación de una fase proteica continua y su adición a los productos sin gluten aumenta la consistencia de las masas, además de mejorar

la estructura de los productos resultantes y/o su calidad organoléptica. En matrices en base almidón la incorporación de proteínas exógenas a la formulación aumenta el valor nutricional del producto en función de la cantidad y calidad proteica. La adición de una mezcla de ácidos orgánicos, ácido acético y láctico, en dosis similares a los producidos de forma natural por las masas madre, está justificada por las características que aportan. En panes elaborados con almidón de arroz fortificados con proteína, la acidificación produjo una mejora en la calidad física aportando mayor volumen y menos dureza a los panes resultantes además de mejorar la conservación del pan durante su almacenamiento. Los resultados obtenidos del análisis sensorial reforzaron el empleo de acidificantes en masas ya que no se obtuvo una reducción en los atributos sensoriales evaluados, incluidos el sabor y el olor de los panes, comparados con sus equivalentes sin acidificar.

Se estudiaron las propiedades térmicas de gelatinización de las mezclas de almidón de arroz, patata o tapioca con albúmina de huevo o aislado de proteína de soja en función del pH del medio. Se seleccionó un pH tamponado a 4.5. Los resultados obtenidos se compararon con los correspondientes a un medio acuoso, sin acidificar, cuyo pH fluctuó entre 4.94 y 7.34 según el almidón y la proteína. También se estudiaron las propiedades reológicas de los geles obtenidos a partir de estas mezclas a dos temperaturas de formación (90º y 120ºC). Las propiedades térmicas y reológicas dependieron del origen del almidón, el tipo de proteína añadida y el pH de las dispersiones. Se obtuvieron geles mucho más débiles a 120 ºC en comparación con los procesados a temperaturas más bajas, siendo los elaborados con almidón de arroz los más sensibles al aumento de la temperatura y los de patata los más resistentes. En general, las proteínas desempeñaron el papel de agentes estructurantes de los geles a ambas temperaturas, como también revela el análisis de microestructura, aunque el efecto fue más marcado en los geles preparados a 120ºC. La acidificación, por el contrario, ejerció un efecto debilitante, más pronunciado para el almidón de patata que para los demás almidones, muy probablemente debido al mayor contenido en grupos fosfato en este almidón. Cabe destacar que la acidificación a pH 4.5 redujo la temperatura de gelatinización del almidón de tapioca independientemente de la presencia de proteínas.

La segunda parte del trabajo se centró en estudiar y comprobar la viabilidad técnica del tratamiento hidrotérmico asistido con microondas, como mecanismo

estructurante en matrices de harina de arroz y de mezclas almidón-proteína. Este tratamiento demostró cambios en la cristalinidad de los almidones y la morfología de los gránulos que influyó en su hinchamiento y solubilidad y en las propiedades de empastado, funcionales y térmicas de las muestras.

En matrices con harina de arroz, el contenido inicial de humedad y el tiempo de tratamiento con microondas fueron factores clave sobre las modificaciones observadas en las harinas. Con mayores niveles de humedad, se observó un aumento de la temperatura de gelatinización, la disminución de la entalpía de gelatinización y el aumento de la recristalización de la amilopectina después de siete días de almacenamiento. Además, se observaron temperaturas de empastado más altas y valores de picos de viscosidad más bajos que con la harina nativa. Los cambios producidos en las propiedades térmicas y estructurales y el retraso en la temperatura de gelatinización, podrían ser los responsables del aumento de la consistencia de las masas y de que los panes elaborados aumenten de volumen y mejoren su textura.

Por último, se realizó un estudio hidrotérmico asistido con microondas sobre mezclas almidón-proteína. Se mezclaron almidones de arroz o patata con un 5% de caseinato de calcio o aislado de proteína de soja. Los resultados obtenidos evidenciaron la viabilidad del tratamiento para modular las propiedades de hidratación y de empastado de las mezclas y su adecuación al uso de destino. Las propiedades reológicas de los geles elaborados a partir de estos almidones se vieron fuertemente afectados por la presencia y tipo de proteína y por el tratamiento con microondas. El efecto más marcado se observó sobre el almidón de patata.

Con el fin de atender la demanda de un sector actualmente muy importante de la población, las estrategias estudiadas, tanto el empleo de acidificantes de masas de almidón fortificadas con proteína, como de nuevos procesos tecnológicos como es la modificación física de las harinas o mezclas almidón-proteína mediante tratamiento hidrotérmico asistido con microondas, han demostrado su eficacia para modificar las propiedades de los sistemas y por lo tanto, la calidad tecnológica, organoléptica y nutricional de los panes libres de gluten.

ABSTRACT

The increasing demand for gluten-free (GF) products has encouraged the development of numerous bakery products that seek to imitate the quality characteristics of their wheat counterparts. Nevertheless, most of the GF products on the current market are of significantly poorer physical and sensory quality and have significant nutritional deficiencies, particularly a significantly lower protein/starch balance than wheat breads. Most of the studies carried out to obtain GF products have been based on the replacement of wheat flour by GF flours (mainly rice flour), a mixture of starches from different sources (corn, rice, potato, tapioca, etc.) and/or different hydrocolloids (polymers mimicking the gluten structure).

This Doctoral Thesis deals with the study of strategies for structuring GF systems, by using two different approaches: first, the creation of structure through the incorporation of exogenous proteins with/without acidification, a technological alternative involving both physical-chemical and nutritional interest, and in second place the investigation of the structuring ability of microwave-assisted hydrothermal treatments of GF matrices -flour, starches or starch/protein mixtures-, which appears as a novel and barely research method.

To accomplish the goals, two types of matrices have been studied: bread systems (bread doughs and breads) and food gels. Bread is a highly consumed food constituting a suitable vehicle of nutrient-dense ingredients since it represents a significant contribution to the daily diet. Gels are used as a base for many food products, since they confer texture, stability and elasticity to food matrices.

The first part of this study was focused on the evaluation of the structuring ability of proteins added to different starches under modulating pH conditions. The incorporation of proteins leads to the formation of a continuous protein phase and their addition to GF products increases the consistency of the doughs, in addition to improving the structure of the resulting products and/or their organoleptic quality. In starch-based matrices, the addition of exogenous proteins to the formulation increases the nutritional value of the product as a function of the quantity and quality of added protein. The addition of a blend of organic acids, acetic and lactic acid, in similar doses to those naturally present

in sourdoughs, is justified by the characteristics they provide. In rice starch breads fortified with protein, acidification resulted in an improvement of physical quality, providing greater volume and softness to the resulting breads, and improving bread preservation during storage. The results obtained from the sensory analysis reinforced the use of acidifiers in doughs since no reduction was obtained in the sensory attributes evaluated, including the taste and smell of the breads, compared to their non-acidifying counterparts.

The thermal gelatinization properties of mixtures of rice, potato or tapioca starch with egg albumin or soy protein isolate were studied dependent on the pH of the medium. A buffer pH of 4.5 was selected. The results obtained were compared with those of a non-acidified aqueous medium, whose pH fluctuated between 4.94 and 7.34 depending on the starch and protein type. The rheological properties of the gels obtained from these mixtures at two different temperatures (90º and 120ºC) were also studied. The thermal and rheological behaviour depended on the starch source, the type of protein added and the pH of the dispersions. Much weaker gels were obtained at 120ºC compared to the gels prepared at lower temperatures; the rice starch being the most sensitive to increasing gelification temperature and potato gels the most resistant. In general, proteins behaved as structure enhancing agents at both temperatures, as also revealed the microstructure analysis, although the effect was more significant in gels prepared at 120ºC. In contrast, acidification weakened the structure of the gels, especially for potato starch most likely due to its higher phosphate content. It should be stressed that acidification at pH 4.5 reduced the gelatinization temperature of tapioca starch regardless the presence of protein.

The second part of the research focused on studying and testing the technical feasibility of microwave-assisted hydrothermal treatment as structuring agent in rice flour and starch-protein matrices. The treatment provided changes in the starch crystallinity and morphology of the granules that influenced their swelling and solubility and the pasting, functional and thermal properties of the samples.

In rice flour matrices, the initial moisture content and microwave treatment time were key factors in the changes observed in the flours. At higher moisture levels, an increase in gelatinization temperature, a decrease in gelatinization enthalpy and an enhancement in the amylopectin recrystallization after seven days of storage took place. In addition, higher pasting temperatures and lower

peak viscosity values than with native flour were observed. The changes in thermal and structural properties and the delay in the gelatinization temperature could be responsible for the increase of the dough consistency and the increase in the volume and the improvement of texture of breads.

Finally, a microwave-assisted hydrothermal study was carried out on starch-protein mixtures. Rice or potato starch was mixed with 5% calcium caseinate or soy protein isolate. The results obtained showed the feasibility of the treatment to modulate the hydration and pasting properties of the mixtures. The rheological properties of the gels made from these starches were strongly affected by the presence and the type of protein and by microwave treatment. The most marked effect was observed on potato starch.

In order to meet the demands of an important sector of the population nowadays, the strategies studied were effective in modifying the properties of the systems and, therefore, the technological, organoleptic and nutritional quality of GF systems.

INTRODUCCIÓN

INTRODUCTION

INTRODUCCIÓN

1. La enfermedad celiaca y otras patologías asociadas al gluten

La enfermedad celiaca (EC) caracterizada por la intolerancia de por vida al gluten está siendo reconocida cada vez más como una enteropatía autoinmune (Niewinski, 2008). Es un trastorno del tracto gastrointestinal en el que la ingestión de gluten conduce al daño de la mucosa del intestino delgado, en individuos con susceptibilidad genética. Una amplia gama de otras enfermedades, principalmente autoinmunes, pueden estar asociadas con la EC. Un ejemplo es la diabetes tipo 1, en la que entre el 2 y el 5% de los pacientes también sufren de EC. Otras enfermedades asociadas son, entre otras, el tiroiditis autoinmune, la hepatitis autoinmune y la osteoporosis (Matthias et al., 2011). La prevalencia de la enfermedad celíaca es aproximadamente del 1% en la población general y estudios recientes indican una tendencia creciente durante las últimas décadas por razones que actualmente no están claras (Sapone et al., 2012). Hoy en día, el único tratamiento disponible para los pacientes con EC es la dieta libre de gluten (SG).

Además, existen otras patologías asociadas con la ingesta de gluten, que parecen estar aumentando en importancia. Entre ellas, la alergia a las proteínas del trigo, definida como una reacción inmunológica adversa y la sensibilidad al gluten, una patología de intolerancia al gluten que excluye la alergia a las proteínas del trigo y la enfermedad celiaca, ya que ningún mecanismo alérgico ni autoinmune puede ser identificado (Sapone et al., 2011).

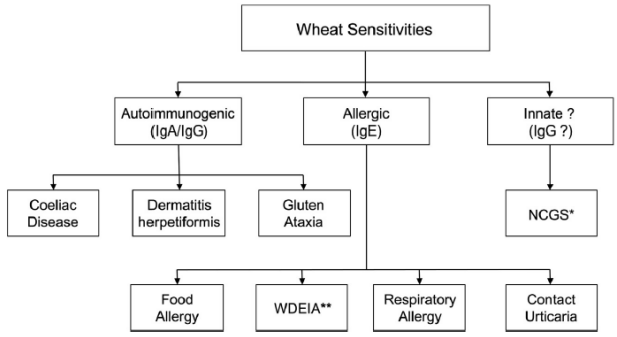

Figura 1. Clasificación de enfermedades relacionadas con el trigo (adaptado de Sapone et al., 2012); *Sensibilidad al gluten no celiaca; ** Anafilaxia dependiente de cereales inducida por ejercicio.

Seguir una dieta SG no es una tarea fácil ya que los granos que contienen gluten, especialmente el trigo, son los ingredientes principales en alimentos culturalmente populares como el pan, la pasta o las galletas. Otros cereales como la cebada, el centeno y trigos ancestrales como el korasan (Kamut®) o la espelta, no están exentos de gluten.

Actualmente, el mercado de los productos SG está experimentando un crecimiento debido principalmente al aumento del número de pacientes diagnosticados y por un nuevo nicho de mercado que forman los consumidores que opcionalmente evitan el gluten (Capriles, dos Santos, & Arêas, 2016). Para individuos con patologías asociadas al gluten, el segmento del mercado de alimentos SG es realmente importante (Sapone et al., 2012), pero a pesar de ello, todavía tienen problemas para encontrar productos SG debido a los altos precios, variedad y disponibilidad, además de que los existentes carecen de propiedades físicas y sensoriales adecuadas.

2. Productos sin gluten

2.1. Importancia del gluten en los productos horneados

El maíz y el arroz son los principales ingredientes utilizados para la elaboración de los productos de panadería SG. Los productos elaborados con estos cereales tienen un sabor similar, lo que limita las posibilidades de elección de los consumidores. La harina de arroz es una de las más adecuadas para la elaboración de productos GF, ya que es natural, hipoalergénica y de sabor suave. Proporciona una alta cantidad de carbohidratos digeribles pero una baja cantidad de proteínas (prolaminas), por lo que se necesita de otros componentes para reforzar las masa y el contenido nutricional del producto final (Mandala & Kapsokefalou, 2011). En los últimos años, ha habido un gran interés por los panes SG, cuyas formulaciones implican principalmente la incorporación de almidones de diferentes orígenes, otras proteínas diferentes al gluten como las proteínas lácteas, incluso la adición de gomas y/o sus combinaciones (Mariotti, Lucisano, Ambrogina Pagani, & Ng, 2009). La elaboración de productos horneados de buena calidad es un proceso complejo que comprende varios pasos, desde la mezcla, el moldeado, la fermentación y por último, la cocción. La matriz de proteína de gluten es un factor clave en panificación. Además de contribuir a la capacidad de absorción de agua de la masa, el gluten proporciona extensibilidad, elasticidad y cohesividad a la masa de pan, permitiendo que el gas producido durante la fermentación se mantenga

retenido durante el desarrollo de la masa, dando lugar a panes de alta calidad y bien desarrollados (Wieser, 2007).

La eliminación del gluten en los productos de panadería tiene efectos perjudiciales en cuanto a la calidad de los productos, las características nutricionales y la aceptación del consumidor. En panificación SG, la retención deficiente de gas y el bajo volumen de pan resultante, con una suavidad pobre de miga, son los principales retos identificados. La falta de gluten también conduce a una masa líquida en lugar de una masa propiamente dicha, lo que resulta a su vez en un pan horneado con una textura desmenuzable, con color deficiente y defectos de calidad post horneado (Naqash, Gani, Gani, & Masoodi, 2017).

Los panes SG se caracterizan por ser elaborados con receta heterogénea, siendo una combinación de almidón y harina, así como proteínas, fibras, grasas e hidrocoloides (Brites, Trigo, Santos, Collar, & Rosell, 2010). Los almidones más utilizados en la panificación SG son el almidón de maíz y la fécula de patata, pero también los almidones de tapioca, trigo y arroz, entre otros (Masure, Fierens, & Delcour, 2016). Sin embargo, estos almidones tienen un potencial mínimo de formación de estructura y, por lo tanto, se utilizan frecuentemente junto con proteínas e hidrocoloides (Capriles & Arêas, 2014).

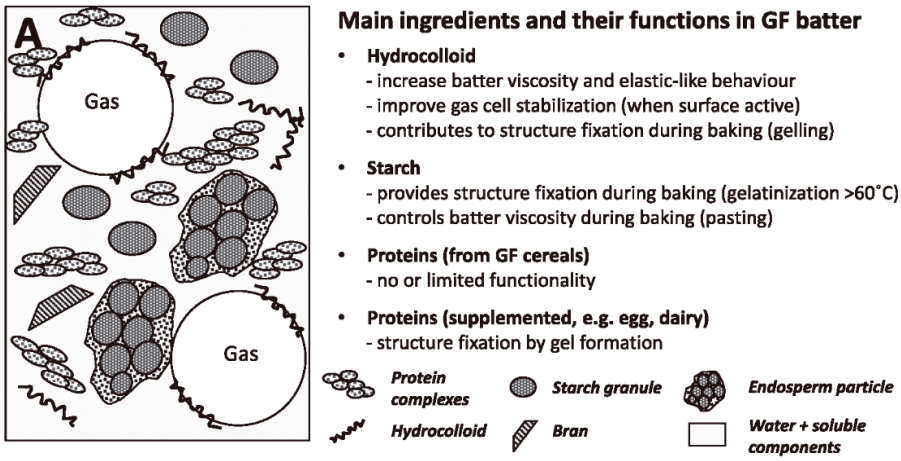

Figura 2. Principales ingredientes y su función en las masas sin gluten (Renzetti & Rosell, 2016).

3. Mecanismos de estructuración de sistemas sin gluten

3.1. Sistemas almidón-proteína-ácido

El almidón y sus propiedades: su papel en los productos sin gluten

Como ingrediente básico en la industria de alimentos procesados, el almidón juega un papel importante ya que proporciona viscosidad, textura y consistencia a muchos productos alimenticios. El almidón es un componente importante de los alimentos vegetales y de la dieta humana, y constituye la principal materia prima de una gran parte de los alimentos que consumimos. Por lo tanto, el origen botánico, el tamaño, la forma y la estructura del almidón, que afectan a las propiedades físico-químicas y funcionales, son cruciales.

Tabla 1. Propiedades de algunos almidones

Almidón	Rango de Temperatura de Gelatinización (ºC)	Forma del gránulo	Tamaño del gránulo (µm)
Trigo	51-60	Lenticular Redondo	20-35 2-10
Maíz	62-72	Poliédrico	5-25
Arroz	68-78	Poligonal	3-8
Patata	58-66	Elíptico	15-100
Tapioca	52-64	Semi esférico	5-35

Adaptado de Berlitz et al., 2009 y Biliaderis, 2009.

Como se muestra en la Tabla 1 y en la Figura 3, los gránulos de almidón se presentan en todas las formas y tamaños (esferas, elipses, polígonos, túbulos irregulares), y sus dimensiones oscilan entre 0,1 y 100 µm, dependiendo de la fuente botánica (Pérez, Baldwin, & Gallant, 2009).

De forma natural, los gránulos de almidón contienen dos tipos principales de polisacáridos, amilosa y amilopectina, independientemente de la fuente o tejido de la planta. Ambos son polímeros de α-D-glucosa conectados por enlaces (1-4) en cadenas más cortas o más largas. La amilopectina, el componente principal de la mayoría de los almidones, consiste en un gran número de cadenas más cortas que están unidas en su extremo reductor por un eslabón (1-6), lo que hace que este polisacárido sea muy grande y esté ampliamente ramificado. La amilosa consiste en una sola cadena o en unas pocas cadenas largas, lo que hace que la molécula sea lineal o ligeramente ramificada. El contenido de amilosa de la mayoría de los almidones oscila entre 20-30% (Bertoft, 2017).

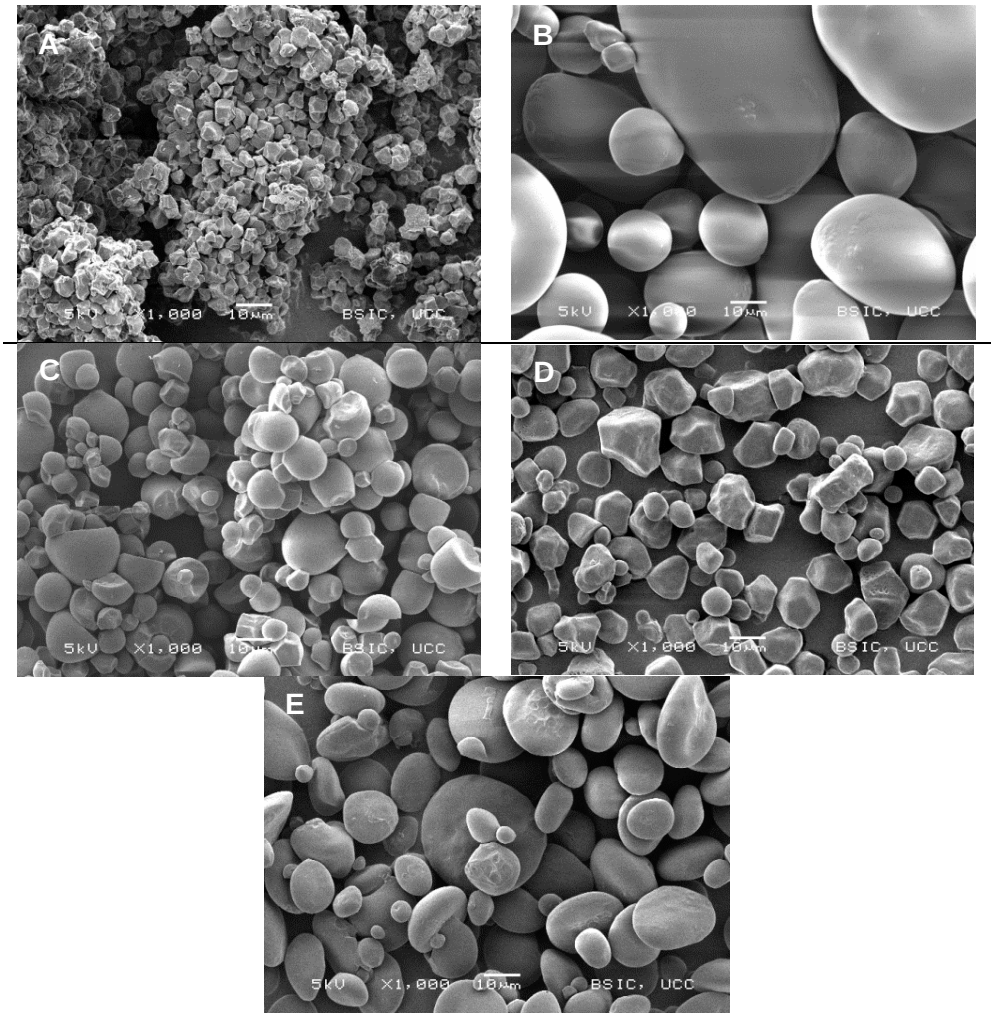

Figura 3. Microestructuras de diferentes almidones A) Almidón de arroz, B) Almidón de patata, C) Almidón de tapioca, D) Almidón de maíz y E) Almidón de trigo. Adaptado a partir de Horstmann, Axel, & Arendt, 2018.

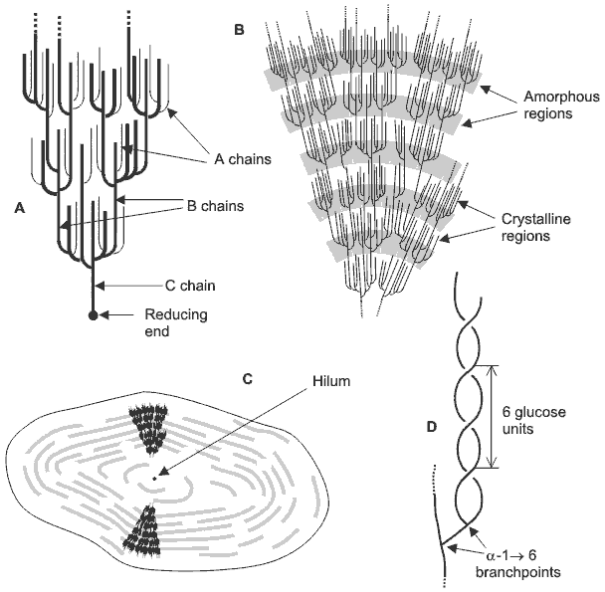

Figura 4. Estructura del gránulo de almidón. A) Características principales del modelo de *cluster* propuesto por primera vez por Robin en 1974. B) La organización de las regiones amorfas y cristalinas de la estructura generando las capas concéntricas que contribuyen a los "anillos de crecimiento" visibles por microscopía óptica. C) La orientación de las moléculas de amilopectina en una sección transversal de un gránulo entero. D) La probable estructura de doble hélice ocupada por las cadenas vecinas y que da lugar al alto grado de cristalinidad del gránulo (Coultate, 2016).

El almidón es un material semicristalino. Los gránulos de almidón, dependiendo de su origen botánico y composición, exhiben dos tipos de patrones de difracción de rayos X, que se asocian con dos formas polimórficas cristalinas: el tipo A, que se encuentra principalmente en almidones de cereales, y el tipo B, que se observa en tubérculos y almidones con alto contenido de amilosa (Lopez-Rubio, Flanagan, Gilbert y Gidley, 2008). Ambas estructuras están compuestas por conjuntos ordenados de hélices dobles, y la variación polimórfica se basa en las diferencias de empaquetamiento de las dobles hélices dispuestas hexagonalmente; la estructura B es más espaciosa, acomodando mayores cantidades de agua (Biliaderis, 2009). Otro polimorfismo cristalino se observa a menudo en almidones granulares, el llamado tipo V, que, en contraste con la naturaleza doblemente helicoidal de las estructuras cristalinas A y B, se ha descrito que está formado por hélices de amilosa simples, algunas de las cuales están unidas a lípidos endógenos (Lopez-Rubio et al., 2008).

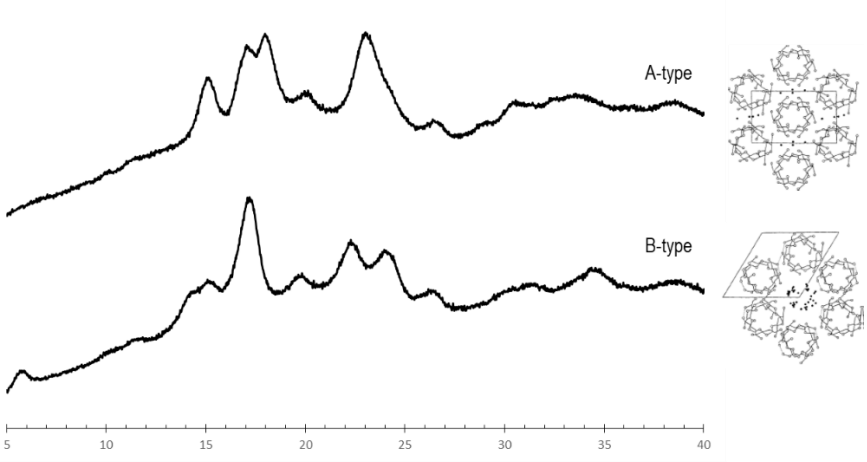

Figura 5. Difractogramas de rayos X de almidones de tipo A y tipo B. Representación de la disposición de dobles hélices (sección transversal) en tipo A y tipo B.

Propiedades del almidón

Cuando los gránulos de almidón se calientan en exceso de agua, los gránulos se hidratan, se hinchan y se transforman en una pasta. La estructura granular colapsa debido a la fusión de los cristales, el desenrollamiento de las dobles hélices y la ruptura de los enlaces de hidrógeno. Estos cambios se denominan gelatinización del almidón y se acompañan de la pérdida de birrefringencia característica de los gránulos. Al enfriarse, las cadenas de almidón disgregadas se retraen gradualmente en estructuras parcialmente ordenadas que difieren de las de los gránulos nativos. Se trata de un proceso irreversible (Wang, Li, Copeland, Niu y Wang, 2015). Este fenómeno es causado principalmente por la amilosa, ya que la amilopectina, debido a su organización altamente ramificada, es menos propensa a la retrogradación (Ai & Jane, 2015).

El punto de inicio de la gelatinización y el rango sobre el cual ocurre se rige por la concentración de almidón, el método de observación, el tipo de gránulo y las heterogeneidades dentro de los diferentes gránulos (Atwell et al., 1988). La gelatinización se produce en un rango de temperatura, y generalmente se presenta como temperatura de inicio (To), temperatura máxima (Tp) y temperatura de finalización (Te). La calorimetría de barrido diferencial (DSC) se utiliza a menudo para determinar la temperatura de gelatinización del almidón basándose en la transición térmica de la fusión de la región cristalina del almidón durante el calentamiento. Durante el proceso de fabricación y consumo de alimentos en base almidón se forman geles que al someterlos a grandes

deformaciones, pueden causar deformaciones irreversibles o fallos estructurales por fractura (Tabilo-Munizaga y Barbosa-Cánovas, 2005).

La retrogradación es un proceso continuo, que inicialmente implica una rápida recristalización de las moléculas de amilosa seguida de una lenta recristalización de las moléculas de amilopectina. La retrogradación de la amilosa determina la dureza inicial de un gel, la viscosidad y la digestibilidad de los alimentos procesados. Se considera que el desarrollo a largo plazo de la estructura del gel y la cristalinidad del almidón procesado, que intervienen en el proceso de formación del pan y los bizcochos, se debe a la retrogradación de la amilopectina (Wang et al., 2015).

El empastado es también una propiedad importante del almidón para la aplicación en alimentos porque permite aumentar la viscosidad. El empastado se define como el fenómeno posterior a la gelatinización en la disolución del almidón. Implica hinchazón granular, exudación de los componentes moleculares granulares y, finalmente, la desintegración total de los gránulos (Atwell et al., 1988).

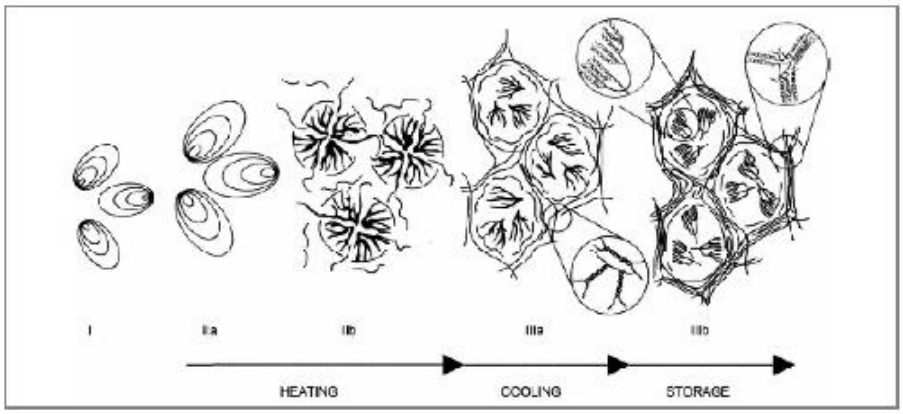

Figura 6. Representación esquemática de los cambios que se producen en una mezcla de almidón y agua durante el calentamiento, el enfriamiento y el almacenamiento. (I) Gránulos nativos de almidón; (II) Gelatinización, asociada con hinchazón (a) y lixiviación de la amilosa y rotura parcial de los gránulos (b), que resulta en la formación de una pasta de almidón; (III) Retrogradación: formación de una red de amilosa (gelificación/retrogradación de amilosa) durante el enfriamiento de la pasta de almidón; (a) y formación de moléculas de amilopectina ordenadas o cristalinas (retrogradación de amilopectina) durante el almacenamiento; (b) (Wang y cols., 2015).

La hinchazón de gránulos, la gelatinización, el empastado y la retrogradación son aspectos importantes de la funcionalidad del almidón, y son cruciales en los

productos horneados. La ausencia de gluten aumenta el papel del almidón en la estructura y textura de los panes SG (Witczak, Ziobro, Juszczak y Korus, 2016). De hecho, durante la cocción, el almidón se une al agua y crea una estructura permeable al gas que influye, por lo tanto, en la reología de la masa, la retención de agua y la estructura final y la calidad de los panes SG (Houben, Höchstötter, & Becker, 2012). Las propiedades de los productos finales e intermedios están estrechamente relacionadas con el tipo de almidón utilizado en términos de origen, especie, tamaño de las partículas, contenido de amilosa/amilopectina, tratamiento del almidón y combinación de diferentes fuentes de almidón y otros ingredientes (Witczak et al., 2016).

Además, a pesar de que varias técnicas reológicas, incluyendo ensayos de oscilación, de tensión, medidas de deformación y de deformación-relajación progresiva se han utilizado ampliamente para evaluar las propiedades mecánicas fundamentales del gluten, el uso de la reología en los estudios del comportamiento reológico de la masa SG sólo se ha aplicado durante la última década (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007). Las propiedades reológicas fundamentales y empíricas de las masas informan sobre las interacciones entre los ingredientes y la creación de estructuras a nivel macromolecular y macroscópico, respectivamente. Además, los atributos de calidad de los panes, como el volumen y la textura, pueden correlacionarse con las propiedades reológicas de la masa (Pérez-Quirce, Collar y Ronda, 2014).

Proteínas

Para mejorar las propiedades finales (físicas, texturales y nutricionales) de los productos en base almidón, la incorporación de proteínas exógenas es otra gran alternativa considerando que las proteínas son conocidas como modificadores macromoleculares de estructura y textura en los alimentos (Rao & Lopes da Silva, 2007). Este enriquecimiento es aún más importante para las formulaciones SG que generalmente tienen un equilibrio pobre de proteínas y almidones. Además, las proteínas desempeñan un papel importante en la formulación de la masa y pueden modificar el comportamiento del almidón, especialmente al cambiar la disponibilidad de agua.

Se pueden añadir proteínas de diferentes orígenes para aumentar el valor nutricional y funcional de los productos SG. La incorporación de proteínas conduce a la formación de una fase continua (Moore, Schober, Dockery y Arendt, 2004), y se añaden a las aplicaciones SG (Crockett, Ie y Vodovotz, 2011) para aumentar el módulo elástico mediante enlaces cruzados, mejorar la calidad

percibida mediante la mejora del color marrón y el sabor debido a la reacción de Maillard, mejorar la estructura en el proceso de formación de gel y ayudar en la formación de espuma (Moore, Dal Bello y Arendt, 2008). El resultado es un pan con mayor volumen, una mejor regularidad de la miga y mejores características sensoriales (Moore et al., 2008).

La selección de las proteínas utilizadas en una formulación sin gluten es una cuestión crítica (Mandala & Kapsokefalou, 2011). Las proteínas lácteas tienen un alto nivel nutricional. Tienen gran poder de hincharse y son capaces de construir una red fuerte. El caseinato es un buen emulsionante y es capaz de estabilizar una masa/pasta. Las moléculas hidrofóbicas de caseinatos están ligadas a su estado agregado como complejos de caseinatos o micelas de caseinatos. Además de los beneficios funcionales, la adición de proteínas de la leche y aminoácidos esenciales como la lisina, la metionina y el triptófano también aumentan el nivel nutricional de los productos de panadería SG (Kenny, Wehrle, Stanton y Arendt, 2000). Durante la elaboración de la masa, la adición de ingredientes lácteos aumenta la capacidad de ligado con agua, reduce la viscosidad de la masa y hace que la masa se comporte de forma más plástica (Gallagher, Gormley y Arendt, 2004). En el pan final, aumentan el volumen y mejoran la textura, el sabor, el color de la corteza y la vida útil (Houben et al., 2012). Los aislados de proteína de soja, también con mayor contenido de lisina, aumentan el valor nutricional del pan a la vez que aumentan el módulo elástico, lo que resulta en una mayor retención de gas y volumen del pan, y mejora la retención de agua en los panes resultantes. Otros autores también afirmaron que la adición de aislado de proteína de soja a un pan de almidón de tapioca y arroz con la incorporación del hidrocoloide HPMC, redujo la estabilidad de la masa al suprimir la funcionalidad de HPMC, alterar la distribución del agua dentro de la masa, debilitar las interacciones de HPMC con la matriz de almidón y reducir la estabilidad de la espuma (Crockett et al., 2011). Su funcionalidad depende de parámetros como el valor del pH, la fuerza iónica y la temperatura, lo que lo convierte en un buen ingrediente para modificar las propiedades de las matrices.

Las legumbres también se consideran un buen suplemento para los alimentos a base de cereales, ya que tanto las proteínas de las legumbres como las de los cereales son complementarias en aminoácidos esenciales. Los cereales son deficientes en el aminoácido esencial lisina, mientras que las legumbres tienen un alto contenido de este aminoácido. Por otro lado, las proteínas de cereales complementan las proteínas de legumbres en el aminoácido esencial metionina (Marco y Rosell, 2008).

Como sustituto del gluten, también se pueden utilizar proteínas de huevo. Debido a su actividad en las zonas fronterizas, actúan como agentes espumantes, estabilizadores de migas y proporcionan una buena forma a los productos panificables. Excepto la ovomucina, todas las demás proteínas de la clara de huevo intervienen en el proceso de formación de gel. Estas proteínas permiten crear estructura y son capaces de dar estabilidad a la masa. Además, pueden aumentar la capacidad de retención de gas conectando los gránulos de almidón.

Acidificación

En general, la masa madre es una mezcla de harina y agua que se mezcla y luego se fermenta con bacterias ácido-lácticas (LAB). Por lo general, las LAB son cepas heterofermentativas que producen ácido láctico y acético en la mezcla, lo que da como resultado un sabor agrio de la masa. El proceso de acidificación afectado por la aplicación de las levaduras se utiliza principalmente para mejorar la calidad física, el sabor, el aroma y el envejecimiento de los panes de trigo (Komlenić, Slačanac, & Jukić, 2008).

La masa es muy sensible a los cambios en la fuerza iónica y el pH que pueden tener un impacto directo en los componentes de la masa (Clarke, Schober y Arendt, 2002). La caída del valor del pH causada por los ácidos orgánicos producidos influye en el comportamiento viscoelástico de la masa (Wehrle & Arendt, 1998). Como reportaron Komlenić et al. (2008) quienes estudiaron la reología de la masa de trigo y la calidad del pan, la adición de ácido láctico no tenía influencia ni en el volumen ni en la firmeza del pan. Para modificar la textura de los alimentos en base almidón, se ha probado la acidificación de los sistemas alimentarios a base de arroz añadiendo ácido acético o láctico (Blanco, Ronda, Pérez y Pando, 2011) y sus mezclas (Ronda, Villanueva y Collar, 2014; Villanueva, Mauro, Collar y Ronda, 2015), o su producción a partir de bacterias ácido-lácticas en la fermentación de la masa (Moore et al., 2008).

La acidificación también tuvo un efecto sobre sistemas complejos como las masas SG a base de arroz (Jekle & Becker, 2012; Ohishi, Kasai, Shimada, & Hatae, 2007; Ronda et al., 2014), mejorando la calidad de los productos finales de panadería (Blanco et al., 2011; Jayaram, Cuyvers, Verstrepen, Delcour, & Courtin, 2014; Villanueva et al., 2015). Además, los ácidos acético y láctico confieren a los panes SG unas propiedades sensoriales aceptables en términos de olor y sabor, ya sea cuando se producen mediante un cultivo iniciador o cuando se añaden como ingredientes en las formulaciones del pan, lo que provoca un retraso en el envejecimiento (Moore et al., 2008).

3.2. Tratamiento físico

Como ya se ha mencionado, la fuente de almidón, la composición, la estructura y las diferentes propiedades hacen que los almidones sean útiles para diversas aplicaciones que contribuyen a diferentes funcionalidades. Sin embargo, la mayoría de ellos están limitados debido a su inestabilidad con las condiciones de temperatura, cizallamiento y pH. Por lo tanto, los almidones nativos a menudo se modifican para desarrollar propiedades específicas tales como solubilidad, textura, adhesión y tolerancia al calor, de manera que sean adecuados para aplicaciones industriales (Ashogbon y Akintayo, 2014).

Las modificaciones físicas (tratamientos de calor-humedad, *annealing*, pre-gelatinización, tratamientos de altas presiones, radiación y sonicación) y químicas (reticulación, sustitución, hidrólisis ácida y oxidación) mejoran en gran medida las propiedades de los almidones nativos y amplían la gama de aplicaciones del almidón en los alimentos (Hoover, 2010). La modificación física es simple, barata, y está ganando importancia debido a la ausencia de agentes químicos exógenos. Además, los almidones modificados físicamente se consideran materiales naturales de alta seguridad que pueden ser etiquetados como "etiqueta limpia" (Jacobs & Delcour, 1998).

La clasificación de los almidones y féculas de diferentes fuentes botánicas basada en modificaciones físicas depende de si la integridad molecular de los almidones y féculas se destruye o se preserva después de la modificación. El primero abarca todos los procesos de pre-gelatinización (secado en tambor, secado por aspersión y cocción por extrusión) en los que se pierde el orden granular del almidón junto con la despolimerización parcial de los componentes del almidón y el segundo son los procesos hidrotérmicos, en los que se preserva la integridad molecular de los almidones. Los tratamientos hidrotérmicos implican la incubación de gránulos de almidón en exceso de agua/contenido de agua intermedio (*annealing*) o a bajos niveles de humedad (tratamiento de calor-humedad) durante un cierto período de tiempo, a una temperatura superior a la temperatura de transición vítrea pero inferior a la temperatura de gelatinización (Ashogbon & Akintayo, 2014).

Tratamientos de calor-humedad

El tratamiento de calor-humedad (heat-moisture tretament, HMT) de los almidones se define como una modificación física que implica el tratamiento de gránulos de almidón a bajos niveles de humedad (<35% de humedad en peso) durante un cierto período de tiempo (15 min-16 h) y a una temperatura (84-120ºC) superior a la temperatura de transición vítrea (Tg) pero inferior a la

temperatura de gelatinización. Bajo las condiciones anteriores, se ha demostrado que los cambios en el patrón de rayos X, cristalinidad, interacciones de la cadena de almidón, hinchazón de gránulos, lixiviación de amilosa, viscosidad, parámetros de gelatinización, retrogradación y susceptibilidad a la hidrólisis de ácido y α-amilasa se producen en almidones de cereales, tubérculos y leguminosas (Hoover, 2010).

En general, los tratamientos HMT proporcionan cambios tales como la disminución de la solubilidad del almidón, el poder de hinchamiento, la lixiviación de la amilosa y la viscosidad máxima, pero el aumento de la temperatura de empastado. El grado en que se producen estos cambios en las características fisicoquímicas de los almidones depende principalmente de la modificación de la estructura semicristalina de los gránulos de almidón (Zia-ud-Din, Xiong y Fei, 2017).

Actualmente, las harinas y almidones sometidos a este tratamiento tienen importantes aplicaciones en la industria alimentaria y muchos estudios han indicado los efectos del tratamiento HMT (Beta & Hwang, 2018; Collar, 2017; Puncha-Arnon & Uttapap, 2013; Sun, Han, Wang, & Xiong, 2014; Xiao, Liu, Wei, Shen, & Wang, 2017) y sus aplicaciones en diferentes productos (Chandla, Saxena, & Singh, 2017; Collar & Armero, 2018; Fathi, Aalami, Kashaninejad, & Sadeghi Mahoonak, 2016; Kim, Oh, & Chung, 2017; Krupa, Rosell, Sadowska, & Soral-ŚMietana, 2010).

Tratamiento de calor-humedad mediante microondas

Los métodos convencionales de HMT, que incluyen el ajuste del contenido inicial de humedad del almidón al nivel deseado, seguido de un calentamiento a alta temperatura (la mayoría de las veces en contenedores sellados) durante largos períodos de tiempo, a menudo son difíciles de adaptar a procesos a gran escala. Métodos alternativos de procesamiento de HMT utilizan irradiación de microondas para el tratamiento rápido de estos ingredientes (BeMiller & Huber, 2015).

La radiación de microondas (MW) puede suministrar energía con alta eficiencia dependiendo principalmente de las propiedades dieléctricas de una muestra tratada. Las MW son ondas electromagnéticas con frecuencias entre 1 y 300 GHz donde las moléculas polares e ionizables (agua y sales minerales, principalmente) pueden absorber eficientemente. La potencia y la frecuencia de la fuente de microondas, el contenido de humedad del almidón y la duración del tratamiento son variables importantes.

La absorción de energía tiene lugar a nivel molecular, produciendo un rápido aumento de la temperatura de todo el volumen de la muestra, lo que diferencia significativamente el MW y el calentamiento térmico convencional. Se han realizado estudios de impacto del tratamiento de los MW sobre las propiedades fisicoquímicas, estructurales y funcionales de los almidones de cereales y leguminosas, pero el efecto sobre las harinas se ha estudiado poco hasta la fecha.

En general, los cambios producidos derivados de los tratamientos HMT asistidos por microondas se refieren a los siguientes aspectos:

- una pérdida de humedad con mayores cambios a mayores contenidos de humedad inicial
- una disminución de la susceptibilidad a la digestión por las amilasas y un aumento del contenido de almidón resistente (RS) y de almidón de digestión lenta (SDS) (con mayores cambios en el caso de los almidones tipo *waxy*)
- aumento de la viscosidad máxima, de la viscosidad de caída y de la viscosidad final en el caso de los almidones no waxy, y disminución de la viscosidad final en el caso de los almidones *waxy*
- aumenta el poder de hinchado (swelling powder, SP) y disminuye y la solubilidad
- aumentos en la temperatura de inicio de gelatinización y la temperatura de pico de gelatinización; disminuciones en la entalpía (ΔH) de gelatinización
- cambios en los patrones de difracción de rayos X del tipo B al tipo A+B cuando se utilizaron almidones del tipo B con poco o ningún cambio en el grado de cristalinidad; y un aumento del contenido de dobles hélices.

Por lo tanto, la modificación funcional de los ingredientes puede tenerse en cuenta en la panificación SG para aumentar la consistencia de la masa, aumentar el volumen específico y reducir la dureza típica de estos panes.

INTRODUCTION

1. Celiac disease and other pathologies associated to gluten

Celiac disorder (CD) characterized by the life-long intolerance to gluten is increasingly being recognized as an autoimmune enteropathy (Niewinski, 2008). It is a disorder of the gastrointestinal tract in which the ingestion of gluten leads to damage of the small intestinal mucosa by an immune mediated mechanism, in individuals that have genetic susceptibility towards gluten. A wide range of other conditions can be associated with the CD. This group is mainly comprised of other autoimmune diseases. One example is type 1 diabetes where 2–5% of patients also suffer from CD. Other associated diseases are, among others, autoimmune thyroiditis, autoimmune hepatitis and osteoporosis (Matthias et al., 2011). The prevalence of CD affecting approximately 1% of the general population and recent studies indicate a trend toward a rising prevalence of CD during the last several decades for reasons that are currently unclear (Sapone et al., 2012). Currently, the only effective therapy for CD patients is the GF diet.

In addition, other pathologies are associated with the intake of gluten, which seem to be increasing in importance. Among them, wheat protein allergy, defined as an adverse immune reaction and sensitivity to gluten, a pathology of gluten intolerance that excludes allergy to wheat proteins and celiac disease since no allergic or autoimmune mechanism can be identified (Sapone et al., 2012).

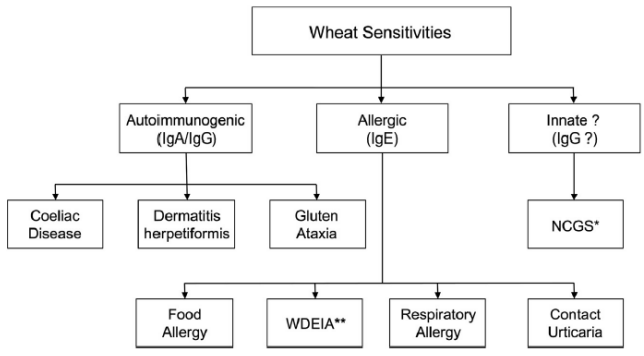

Figure 1. Spectrum and classification of wheat-related disorders (adapted from Sapone et al., 2012); *Non-coeliac gluten sensitivity; **Wheat-dependent exercise-induced anaphylaxis.

Following a gluten-free diet is not an easy task since grains containing gluten, especially wheat, are the main ingredients in culturally popular foods such as bread, pasta or biscuits. Other cereals such as barley, rye and ancestral wheats such as khorasan (Kamut®) or spelt are not GF.

Currently, the market for GF products is experiencing a significant growth due mainly to the increase in the number of gluten-release patients diagnosed and to the merging of a new niche market for consumers who optionally avoid gluten (Capriles, dos Santos, & Arêas, 2016). For individuals with pathologies associated with gluten, the segment of the GF food market is really important (Sapone et al., 2012). Despite this, they still have problems finding GF products due to high prices, low variety and availability, and the poor physical and sensory characteristics they exhibit.

2. Gluten-free products

2.1. Technology importance of gluten in bakery products

Maize and rice are the main ingredients used for preparing GF bakery products. Maize and rice products are similar in taste, thus offering a consumer's limited choice. Rice flour is one of the most suitable cereal flour for preparing GF products because it is natural, hypoallergenic, and has a bland taste. It provides a high amount of digestible carbohydrates but a low amount of proteins (prolamins), thus indicating the need for other components to reinforce the batter matrix and the nutritional content of the final product (Mandala & Kapsokefalou, 2011). There has been increasing interest in new GF breads, whose formulations mainly involve the incorporation of starches of different origin, other non-gluten proteins such as dairy proteins, gums, and their combinations (Mariotti, Lucisano, Ambrogina Pagani, & Ng, 2009). The production of baked goods is a complex process involving several steps from mixing, molding, proofing, to final baking. Gluten protein matrix is a key factor in breadmaking. Besides contributing to the water absorption capacity of the dough, gluten provides extensibility, elasticity and cohesiveness to bread dough allowing the fermentation gas to be occluded and maintained in the liquid phase during the dough development, leading to well-developed high-grade breads (Wieser, 2007).

The elimination of gluten in baked products results in deleterious effects in terms of quality attributes of products, nutritional characteristics, and

consumer acceptance. In GF breadmaking deficient gas retention and the resulting low loaf volume with poor crumb softness, are the major identifiedchallenges. The lack of gluten also leads to a liquid batter instead of dough, which in turn results in baked bread with a crumbling texture, poor colour and post baking quality defects (Naqash, Gani, Gani, & Masoodi, 2017).

GF breads are characterized by a heterogeneous recipe, being a combination of starch and flour, as well as proteins, fibers, fats and hydrocolloids (Brites, Trigo, Santos, Collar, & Rosell, 2010). The most commonly used starches in GF bread-making are maize starch and potato starch but also starches from tapioca, wheat and rice among other (Masure, Fierens, & Delcour, 2016). However, these starches have minimal structure-building potential and, thus, are frequently used along with proteins and hydrocolloids (Capriles & Arêas, 2014).

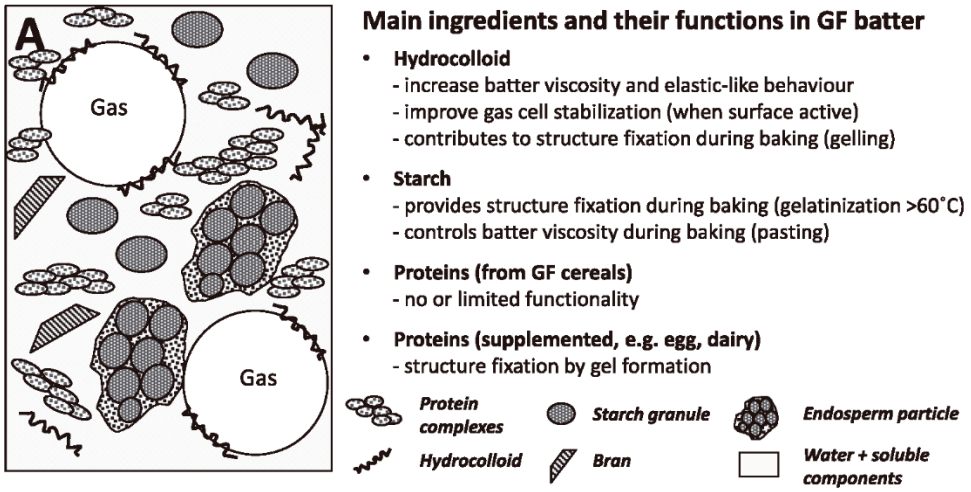

Figure 2. Main ingredients and their functions in gluten-free batter (Renzetti & Rosell, 2016).

3. Structuring mechanisms in gluten-free systems

3.1. Starch-Protein-Acid system

Starch and its properties: Its role in gluten-free products

As a basic ingredient in processed food industry, starch plays an important role as a nutritive stabilizer to provide viscosity, texture and consistency of many food products. Starch is both a major component of plant foods and it is an

important constituent of the human diet and constitute the raw material of a large proportion of the food. Therefore, the botanical origin, the size, shape and structure, which affects physicochemical and functional properties, are crucial.

Table 1. Properties of Certain Starches

Source of Starch	Gelatinization Temperature Range (ºC)	Granule Shape	Granule Size (μm)
Wheat	51-60	Lenticular Round	20-35 2-10
Maize	62-72	Polyhedral	5-25
Rice	68-78	Polygonal	3-8
Potato	58-66	Elliptical	15-100
Tapioca	52-64	Semi spherical	5-35

Adapted from Berlitz et al., 2009 and Biliaderis, 2009.

As shown Table 1 and Figure 3, starch granules occur in all shapes and sizes (spheres, ellipsoids, polygons, platelets, irregular tubules); their long dimensions range from 0.1 to at least 100 μm, depending on the botanical source (Pérez, Baldwin, & Gallant, 2009).

Most naturally, occurring starch granules contain two principal types of polysaccharides, amylose and amylopectin regardless of the plant source or tissue. Both are polymers of α-D-glucose connected by (1-4)-linkages into shorter or longer chains. Amylopectin, the major component of most starches, consists of a large number of shorter chains that are bound together at their reducing end side by a (1-6)-linkage, which makes this very large polysaccharide extensively branched. Amylose consists only of either a single or a few long chains, thus making the molecule linear or slightly branched. The amylose content of most starches is 20-30% (Bertoft, 2017).

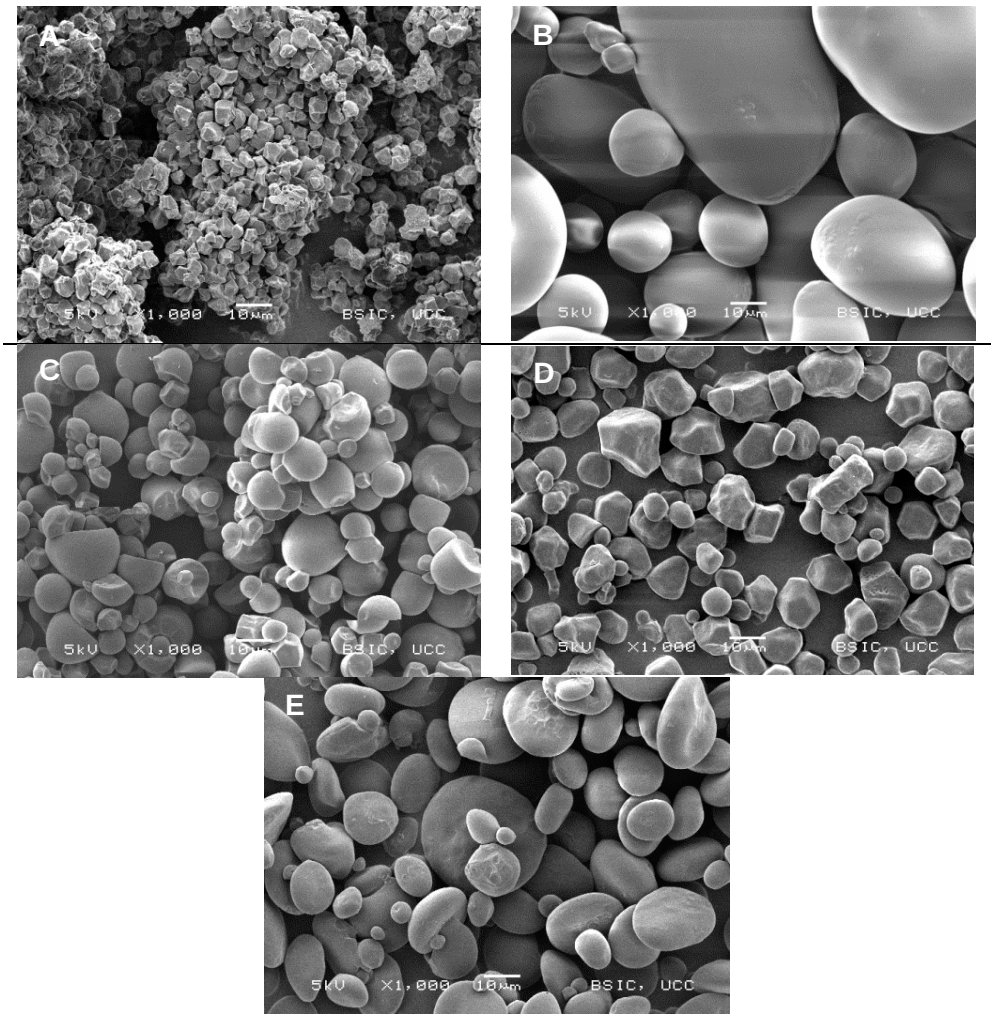

Figure 3. Microstructure of different starches A) Rice starch, B) Potato starch, C) Tapioca starch, D) Corn starch and E) Wheat starch. Adapted from Horstmann, Axel, & Arendt, 2018.

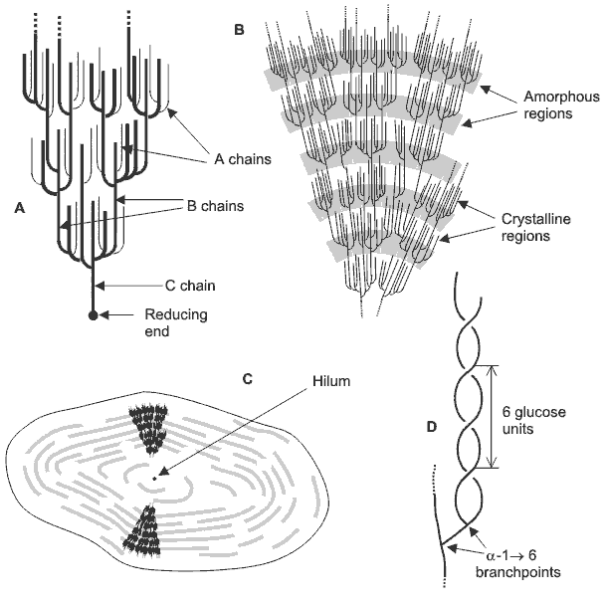

Figure 4. Structure of the starch granule. A) The essential features of the cluster model first proposed by Robin in 1974. B) The organization of the amorphous and crystalline regions (or domains) of the structure generating the concentric layers which contribute to the "growth rings" visible by light microscopy. C) The orientation of the amylopectin molecules in a cross-section of an idealised entire granule. D) The likely double helix structure taken up by neighbouring chains and giving rise to the extensive degree of crystallinity in the granule (Coultate, 2016).

The starch is a semi-crystalline material. Starch granules, depending on their botanical origin and composition, exhibit two types of X-ray diffraction patters, which are associated with two crystalline polymorphic forms: the A-type mainly found in cereal starches and the B-type form observed in tubers and high amylose starches (Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008). Both structures are composed of ordered arrays of double helices, and the polymorphic variation is based on packing differences of the hexagonically arranged chain duplexes; the B structure is more spacious, accommodating larger amounts of water (Biliaderis, 2009). Another crystal polymorph is often observed in granular starches, the so-called V-type, which, in contrast with the double helical nature of the A and B crystal structures, has been described to arise from single amylose helices some of which are complexed with endogenous granular lipids (Lopez-Rubio et al., 2008).

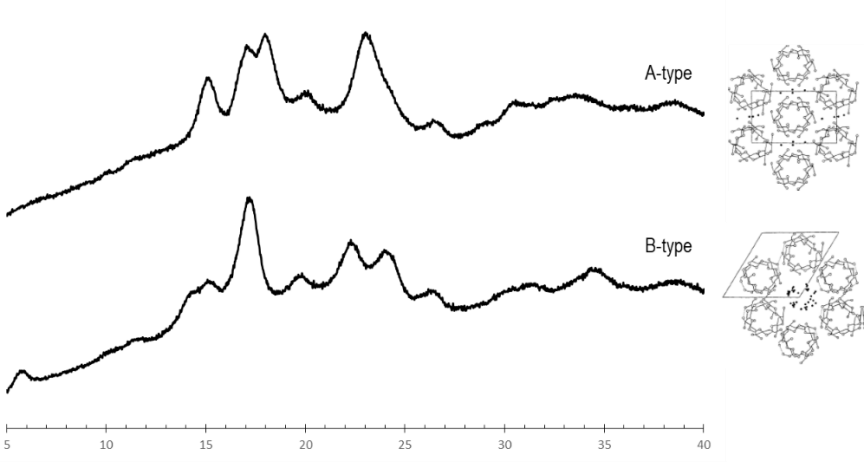

Figure 5. X-ray diffraction patterns of A type and B type starches. Unit cells and arrangement of double helices (cross section) in A-amylose and B-amylose.

Starch properties

When starch granules are heated in excess water, starch granules become hydrated, swell, and are transformed into a paste. The granule structure collapses due to melting of crystallites, unwinding of double helices, breaking of hydrogen bonds. These changes are referred to as starch gelatinization and are accompanied by the loss of characteristic birefringence of granules. On cooling, the disaggregated starch chains retrograde gradually into partially ordered structures that differ from those in native granules. It is an irreversible process (Wang, Li, Copeland, Niu, & Wang, 2015). This occurrence is primarily caused by the amylose, since amylopectin, due to its highly branched organization, is less prone to retrogradation (Ai & Jane, 2015).

The point of initial gelatinization and the range over which it occurs is governed by starch concentration, method of observed, granule type and heterogeneities within the granule population under observation (Atwell et al., 1988). Gelatinization occurs over a temperature range, and usually presented as onset temperature (To), peak temperature (Tp) and completion temperature (Te). Differential scanning calorimetry (DSC) is often used to determine gelatinization temperature of starch based on the thermal transition of starch crystallite melting during heating. During thermal processing-manufacturing and consumption of starchy foods, hydrocolloid gels are formed and they are

subjected to large deformations that may cause either irreversible deformation or structural failure due to fracture (Tabilo-Munizaga & Barbosa-Cánovas, 2005).

Retrogradation is an ongoing process, which initially involves rapid recrystallization of amylose molecules followed by a slow recrystallization of amylopectin molecules. Amylose retrogradation determines the initial hardness of a starch gel and the stickiness and digestibility of processed foods. The long-term development of gel structure and crystallinity of processed starch, which are involved in the staling of bread and cakes, are considered to be due to retrogradation of amylopectin (Wang et al., 2015).

Pasting is also an important starch property for food application because it increases viscosity. Pasting is defined as the phenomenon following gelatinization in the dissolution of starch. It involves granular swelling, exudation of the granular molecular components, and eventually, the total disruption of the granules (Atwell et al., 1988).

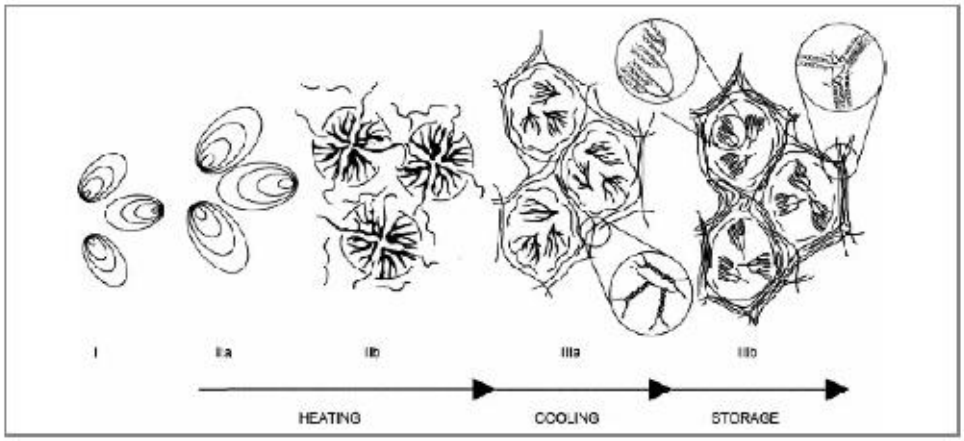

Figure 6. Schematic representation of changes that occur in a starch-water mixture during heating, cooling and storage. (I) Native starch granules; (II) Gelatinization, associated with swelling (a) and amylose leaching and partial granule disruption (b), resulting in the formation of a starch paste, (III) Retrogradation: formation of an amylose network (gelation/amylose retrogradation) during cooling of the starch paste (a) and formation of ordered or crystalline amylopectin molecules (amylopectin retrogradation) during storage (b) (Wang et al., 2015).

Granule swelling, gelatinization, pasting and retrogradation are important aspects of starch functionality, and these are crucial in baked products. The absence of gluten increases the role of starch in providing structure and texture to GF breads (Witczak, Ziobro, Juszczak, & Korus, 2016). In fact, during baking, starch binds water and creates a gas-permeable structure influencing, therefore, dough rheology, water retention and final structure and quality of the gluten-free breads (Houben, Höchstötter, & Becker, 2012). The properties of final and intermediate products are closely linked to the type of starch used in terms of origin, species, particle size, amylose/amylopectin content, starch treatment and combination of different starch sources and other ingredients (Witczak et al., 2016).

In addition, despite several rheological techniques, including oscillation, stress relaxation, creep and creep-recovery measurements have been used extensively for assessing fundamental mechanical properties of gluten, the use of dynamic rheometry in studies of GF-dough rheological behaviour has only been applied over the last decade (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007). Fundamental and empirical rheological properties of doughs inform about interactions among ingredients and the creation of structure at macromolecular and macroscopic levels, respectively. In addition, quality attributes of breads such as volume and texture can be correlated with dough rheological properties (Pérez-Quirce, Collar, & Ronda, 2014).

Proteins

In order to improve the final properties (physical, textural and nutritional) of starchy products, incorporation of exogenous proteins is another great alternative considering that proteins are known as structure and texture macromolecular modifiers in foods (Rao & Lopes da Silva, 2007). Such enrichment is even more important for GF formulations that usually have a poor protein/starch balance. In addition, proteins play an important role in dough formulation may modify starch behaviour, especially by changing water availability.

Proteins from different sources can be added to increase both nutritional and functional values of GF products. Protein incorporation leads to the formation of a continuous protein phase (Moore, Schober, Dockery, & Arendt, 2004), and

are added to GF applications (Crockett, Ie, & Vodovotz, 2011) to increase elastic modulus by cross linking, to improve perceived quality by enhancing Maillard browning and flavour, to improve structure with gelation and to aid in foaming (Moore, Dal Bello, & Arendt, 2008). These result in bread with increased loaf volume, improved crumb regularity and improved sensory characteristics (Moore et al., 2008).

The selection of the proteins used in a gluten-free formulation is a critical issue (Mandala & Kapsokefalou, 2011). Milk proteins have a high nutritional level. They pretend to swell in high level, and they able to build up a network. Caseinate is a good emulsified and is able to stabilize a dough/batter. The hydrophobic caseinate molecules is linked to their aggregated status as caseinate complex or caseinate micelles. Next to functional benefits, the addition of milk proteins and essential amino acids like lysine, methionine and tryptophan also increase the nutritional level of the GF bakery products (Kenny, Wehrle, Stanton, & Arendt, 2000). During dough production, the addition of dairy-based ingredients increases the water-binding capacity, lowers the dough stickiness and make dough behave more plastic (Gallagher, Gormley, & Arendt, 2004). In the final bread, they increase the volume and improve the texture, taste, crust colour and shelf life (Houben et al., 2012). Soybean protein isolates, also higher in lysine content, increases the nutritional value of rice cassava bread and increases elastic modulus, resulting in enhanced gas retention and loaf volume, and improves water binding in the bread loaves. Other authors stated that the addition of soybean protein isolate to an HPMC treated rice cassava bread reduced dough stability by suppressing HPMC functionality, altering water distribution within the dough, weakening HPMC interactions with the starch matrix and reducing foam stability (Crockett et al., 2011). Its functionality depends on parameters such as pH value, ionic strength and temperature, which makes it a good ingredient to modify the properties of the matrices.

Legumes are also considered a good supplement for cereal-based foods since both legume and cereal proteins are complementary in essential amino acids. Cereals are deficient in the essential amino acid lysine, while legumes have a high content of this amino acid. On the other hand, cereal proteins complement legume proteins in the essential amino acid methionine (Marco & Rosell, 2008).

As a gluten replacer, egg proteins can also be used. Due to their border areas activity, they act as foaming agents, as crumb stabilizers and provide a good shape in breadmaking products. Except ovomucin, all other egg white proteins are responsible for the gel-forming process. These phenomena form the protein structure and are able to give stability to the dough. They can increase the gas-binding capacity by connecting the starch granules.

Acidification

In general, sourdough is a mixture of flour and water that is mixed and then fermented with lactic acid bacteria (LAB). Usually, LAB are heterofermentative strains which produce lactic and acetic acid in the mixture, resulting in a sour taste of dough. The acidification process affected by the application of sourdoughs is mainly used to improve the physical quality, taste, flavour and ageing of wheat breads (Komlenić, Slačanac, & Jukić, 2008).

Dough is very sensitive to changes in ionic strength and pH that can have a direct impact on the constituents of dough (Clarke, Schober, & Arendt, 2002). The drop of pH value caused by the produced organic acids influences the viscoelastic behaviour of dough (Wehrle & Arendt, 1998). As reported by Komlenić et al. (2008) who studied the wheat dough rheology and bread quality, lactic acid addition had no influence either on bread specific volume or bread firmness. To modify the texture of starchy foods, acidification of rice-based food systems has been tested by adding acetic or lactic acid (Blanco, Ronda, Pérez, & Pando, 2011) and their blends (Ronda, Villanueva, & Collar, 2014; Villanueva, Mauro, Collar, & Ronda, 2015), or their production from lactic acid bacteria in sourdough fermentation (Moore et al., 2008).

Acidification also had an effect on complex systems such as rice based GF doughs (Jekle & Becker, 2012; Ohishi, Kasai, Shimada, & Hatae, 2007; Ronda et al., 2014), imparting the quality of the final baked products (Blanco et al., 2011; Jayaram, Cuyvers, Verstrepen, Delcour, & Courtin, 2014; Villanueva et al., 2015). Moreover, acetic and lactic acids confer acceptable sensorial properties to GF breads in terms of odour and taste, either when produced by a starter culture or added as ingredients in bread formulations, leading to staling retardation (Moore et al., 2008).

3.2. Physical treatment

As already mentioned, the starch source, composition, and structure, and the different properties make starches useful for various applications contributing to different functionalities. However, most of them are limited due to their instability with temperature, shear, and pH conditions. Therefore, native starches are often modified to develop specific properties such as solubility, texture, adhesion, and heat tolerance, so as to be suitable for industrial applications (Ashogbon & Akintayo, 2014).

Physical (heat-moisture treatment, annealing, pre-gelatinization, high-pressure treatment, radiation, and sonication) and chemical (cross-linking, substitution, acid hydrolysis, and oxidation) modifications greatly improve the properties of native starches and extend the range of starch applications in food (Hoover, 2010). The physical modification is simple, cheap, and are gaining prominence because of the absence of exogenous chemical agents. In addition, physically modified starches are considered natural materials with high safety that can be labelled as "clean label" (Jacobs & Delcour, 1998).

Classification of starches from different botanical sources based on physical modifications depends on whether the molecular integrity of the starches are destroyed or preserved after the modification. The former encompasses all the pre-gelatinization processes (drum drying, spray drying, and extrusion cooking) where starch granular order is loss together with partial depolymerization of starch components and the latter are the hydrothermal processes, where the molecular integrity of the starches are preserved. Hydrothermal treatments involve incubation of starch granules in excess water/intermediate water content (annealing) or at low moisture levels (heat-moisture treatment) during a certain period of time, at a temperature above the glass transition temperature but below the gelatinization temperature (Ashogbon & Akintayo, 2014).

Heat-moisture treatment

Heat-moisture treatment (HMT) of starches is defined as a physical modification that involves treatment of starch granules at low moisture levels (<35% moisture w/w) during a certain time period (15 min–16 h) and at a temperature (84–120ºC) above the glass transition temperature (Tg) but below the gelatinization temperature. Under the above conditions, changes in X-ray pattern, crystallinity, starch chain interactions, granule swelling, amylose

leaching, viscosity, gelatinization parameters, retrogradation, and susceptibility towards acid and α-amylase hydrolysis have been shown to occur in cereal, tuber, and legume starches (Hoover, 2010).

In general, HMT provides changes such as decrease of starch solubility, swelling power, amylose leaching, and peak viscosity but increase in the pasting temperature. The degree to which these changes in the physicochemical characteristics of starches occur is mainly dependent on the modification in the semicrystalline structure of starch granules (Zia-ud-Din, Xiong, & Fei, 2017).

Currently, the flours and starches subjected to this treatment have important applications in the food industry and many studies have indicated the effects of HMT (Beta & Hwang, 2018; Collar, 2017; Puncha-Arnon & Uttapap, 2013; Sun, Han, Wang, & Xiong, 2014; Xiao, Liu, Wei, Shen, & Wang, 2017) and their applications in different products (Chandla, Saxena, & Singh, 2017; Collar & Armero, 2018; Fathi, Aalami, Kashaninejad, & Sadeghi Mahoonak, 2016; Kim, Oh, & Chung, 2017; Krupa, Rosell, Sadowska, & Soral-ŚMietana, 2010).

Microwave assisted heat-moisture treatment method

Conventional HMT methods, which involve adjustment of the starch moisture content to the desired level followed by high-temperature heating (most often within sealed containers) for lengthy periods of time, are often difficult to adapt to large-scale processes. Alternative HMT processing methods use of microwave irradiation for rapid HMT treatment (BeMiller & Huber, 2015).

Microwave (MW) radiation can deliver energy with high efficiency depending mainly on dielectric properties of a treated sample. MW are electromagnetic waves with frequencies between 1 and 300 GHz that polar and ionizable molecules (water and mineral salts, mainly) may absorb efficiently. Wattage and frequency of the microwave source, moisture content of the starch and duration of the treatment are important variables.

The energy absorption takes place at a molecular level, producing a rapid increase in the temperature of all the sample volume, which significantly differentiates MW and conventional thermal heating. MW treatment impact studies on the physicochemical, structural and functional properties of cereals and legumes starches were carried out, but the effect on flours has been so far little studied.

In general, the results of microwave-assisted HMT concern the following aspects:

- a loss of moisture with greater property changes at higher moisture contents
- a decrease in susceptibility to digestion by amylases and increases in the contents of resistant starch (RS) and slowly digestibly starch (SDS) (with greater changes for waxy starches)
- increases in peak, trough/hot-paste, and final viscosities for non-waxy starches and decreases in the same for waxy starches
- both increases and decreases in swelling powder (SP) and solubility
- increases in To and Tp; decreases in the ΔH of gelatinization
- changes in X-ray diffraction patterns from the B type to the A+B type when B-type starches were used with little or no change in the degree of crystallinity; and an increase in double-helix content.

Therefore, the functional modification of ingredients can be taken into account in GF breadmaking for increasing dough consistency, increasing the specific volume and reducing the typical hardness of these breads.

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OBJETIVOS & PLAN DE TRABAJO

OBJECTIVES & WORK PLAN

OBJETIVOS Y PLAN DE TRABAJO

El objetivo principal de esta Tesis Doctoral ha sido el estudio de diferentes estrategias de estructuración de sistemas sin gluten. Por una parte, mediante reformulación, incorporando mezclas de ácidos orgánicos y proteínas exógenas a matrices de almidón, y por otra parte mediante la aplicación de tratamientos físicos (tratamiento hidrotérmico asistido con microondas) a diferentes sistemas sin gluten sencillos (basados en almidón+proteína) y sistemas panarios (basados en harina de arroz).

Para conseguir este objetivo principal, se plantearon los siguientes objetivos particulares, agrupados según el tipo de estrategia de estructuración:

A) ESTRUCTURACIÓN MEDIANTE REFORMULACIÓN: EFECTO DE LA ACIDIFICACIÓN Y FORTIFICACIÓN PROTEICA SOBRE MATRICES DE ALMIDÓN, GELES, MASAS PANARIAS, Y PANES SIN GLUTEN (SECCIÓN I)

1. Estudiar el impacto de la acidificación y de la incorporación de proteínas exógenas sobre la viscoelasticidad y propiedades de empastado de masas basadas en diferentes almidones.
2. Investigar el efecto de la acidificación y de la incorporación de proteínas exógenas sobre la calidad de panes de almidón de arroz.
3. Conocer la importancia del pH (4.5) y de la incorporación de proteínas a matrices de almidón sobre las propiedades térmicas del binomio proteína-almidón y la reología de los geles mixtos.

B) ESTRUCTURACIÓN MEDIANTE TRATAMIENTOS FÍSICOS DE LA HARINA: IMPACTO DEL TRATAMIENTO HIDROTÉRMICO ASISTIDO CON MICROONDAS SOBRE LA MODULACIÓN DE LAS PROPIEDADES TECNO-FUNCIONALES DE SISTEMAS MODELO Y SISTEMAS PANARIOS. (SECCIÓN II)

4. Estudiar las modificaciones físicas de la harina de arroz mediante tratamiento hidrotérmico asistido con microondas, y su impacto específico sobre la microestructura, propiedades de empastado y cristalinidad del sistema.
5. Definir los efectos de la modificación física de mezclas de almidón+proteína mediante tratamiento hidrotérmico asistido con

microondas sobre las propiedades funcionales, propiedades de empastado y reología de los geles.

6. Cuantificar los efectos de la harina de arroz modificada físicamente sobre las propiedades reológicas de las masas y sobre la calidad de los panes resultantes.

Para alcanzar el objetivo 1 se planteó el estudio del efecto de la adición de la mezcla de ácido acético y láctico, en dosis similares a las producidas de forma natural por las masas madre (0.5 g/100 g de mezcla almidón+proteína), en matrices de diferentes almidones (maíz, patata, tapioca y trigo) enriquecidas con proteínas exógenas. Se seleccionaron proteínas de caseinato de calcio y aislado de proteína de soja en dosis de 5 g/100 g de mezcla almidón+proteína. En este primer trabajo se ha efectuado un estudio detallado de la reología y de las propiedades de empastado de las masas. Los factores de estudio fueron tipo de almidón, tipo de proteína y presencia de ácido. Este estudio se recoge en el *Capítulo I* de la presente memoria.

También en cumplimiento del objetivo 1 se abordó el estudio de la acidificación y la fortificación de la masa de almidón de arroz con diferentes proteínas: albúmina de huevo, caseinato de calcio, proteína de guisante y aislado de proteína de soja. En este trabajo, se ha evaluado el efecto de la adición de dos dosis de proteína, 5 y 10 g/100 g de mezcla almidón+proteína sobre las propiedades reológicas y de empastado de las matrices. Estos primeros trabajos permitieron seleccionar la fuente de almidón de mayor interés tecno-funcional. Este estudio se recoge en el *Capítulo II* de la memoria.

Para dar cumplimiento al objetivo 2 de la Tesis Doctoral y atendiendo a los resultados de los estudios anteriores, se planificaron ensayos para estudiar el efecto de la acidificación y del enriquecimiento proteico (albúmina de huevo, caseinato de calcio, proteína de guisante y aislado de proteína de soja) a las dos dosis de proteína previamente evaluadas (5 y 10 g/100 g de mezcla almidón+proteína) sobre la calidad de los panes sin gluten. Se estudió el efecto sobre la evolución de las masas durante la fermentación, las propiedades térmicas de las masas y las características físicas y sensoriales de los panes resultantes. Este estudio, recogido en el *Capítulo III* de la memoria, permitió

seleccionar las fuentes de almidón y proteína de mayor interés en base a su desempeño en la producción de pan sin gluten.

Para llevar a cabo el objetivo 3, se establecieron las propiedades térmicas de mezclas almidón-proteína y las propiedades reológicas de geles elaborados a diferentes temperaturas (90 y 120ºC) con almidón de arroz, patata y tapioca y adición de dos proteínas: albúmina de huevo y aislado de proteína de soja. En esta ocasión se decidió estudiar el efecto de acidificación de las mezclas fijando el pH a 4.5, ya que durante la fermentación en las masas acidificadas enriquecidas con proteína (en dosis de 5 g/ 100 g en base almidón+proteína) se alcanzaron valores de pH entre 4.3 y 4.8 para los almidones estudiados. Se hizo uso de la microscopía laser confocal con el fin de analizar las diferencias obtenidas en los geles a las temperaturas estudiadas. Este trabajo se llevó a cabo durante mi estancia en la Universidad de Tesalónica (Grecia) bajo la supervisión de los profesores Dr. Biliaderis y Dra. Lazaridou y queda recogido en el *Capítulo IV* de la memoria

El objetivo 4 se abordó mediante el estudio de las propiedades de absorción de energía microondas por parte de la harina de arroz en función de su humedad y tiempo de tratamiento. La evolución de la temperatura durante el tiempo de tratamiento, considerado un parámetro importante del que dependen los cambios que se produzcan, también se evaluó. Al ser un trabajo novedoso, del que apenas existen publicaciones al respecto, se estudió el efecto del tratamiento sobre la estructura de la harina de arroz y sus propiedades térmicas y de empastado. Este trabajo queda recogido en el *Capítulo V* de la memoria.

Para dar cumplimiento al objetivo 5 se planificaron experiencias de modificación física mediante tratamiento hidrotérmico asistido con microondas de mezclas almidón-proteína. Para ello se estudiaron los almidones de arroz y patata con mezclas de caseinato de calcio y aislado de proteína de soja en dosis del 5 g/100 g en base almidón+proteína. Se estudiaron las propiedades funcionales, propiedades de empastado y la reología de los geles resultantes con el fin de establecer qué modificaciones se producían en estas matrices de almidón enriquecidas con proteína. Este estudio queda recogido en el *Capítulo VI* de la memoria.

El objetivo 6 se abordó mediante el estudio del efecto de la adición de harina de arroz tratada mediante radiación de microondas (a dos diferentes niveles de humedad inicial, 20 y 30%), en dosis del 30 y 50%, a masas de pan y evaluación

de la calidad de los panes resultantes. Se estudió la reología de las masas, las propiedades de empastado de las masas y la calidad física de los panes. Estos estudios quedan recogidos en el *Capítulo VII* de la memoria.

Todas las líneas de investigación y capítulos correspondientes, llevaron consigo un análisis estadístico de los resultados, que representó una herramienta de gran utilidad para la comparación y discusión de los resultados obtenidos. La descripción de los métodos empleados y de los resultados derivados se recoge en los respectivos subapartados de cada capítulo de esta Tesis Doctoral.

OBJECTIVES AND WORK PLAN

The main objective of this Doctoral Thesis was the study of different structuring strategies of GF systems. The first approach involved reformulation, by incorporating blends of organic acids and exogenous proteins into starch matrices, and the second approach applying physical treatments (hydrothermal treatment assisted by microwaves) to different model GF systems (based on starch+protein) and bread systems (based on rice flour).

To achieve this main objective, the following specific objectives, divided according to the type of structuring strategy, were set:

A) STRUCTURING BY REFORMULATION: EFFECT OF ACIDIFICATION AND PROTEIN FORTIFICATION ON STARCH MATRICES, GELS, DOUGHS, AND GLUTEN-FREE BREADS (SECTION I)

1. To study the impact of acidification and the incorporation of exogenous proteins on the viscoelasticity and pasting properties of doughs based on different starches.
2. To investigate the effect of acidification and the incorporation of exogenous proteins on the quality of rice starch breads.
3. To know the role of pH (4.5) and the exogenous proteins incorporated into starch matrices on the thermal properties of protein-starch mixtures and the rheology of mixed gels.

B) STRUCTURING BY PHYSICAL TREATMENTS OF FLOURS: IMPACT OF HYDROTHERMAL TREATMENT ASSISTED BY MICROWAVES AS A TOOL ON THE MODULATION OF THE TECHNO-FUNCTIONAL PROPERTIES OF MODEL AND BREAD SYSTEMS (SECTION II)

4. To study the physical modifications of rice flour by means of hydrothermal treatment assisted by microwaves, and its specific impact on the microstructure, pasting properties and crystallinity of the system.
5. To define the effects of the physical modification of starch+protein mixtures by hydrothermal treatment assisted by microwaves on the functional properties, pasting properties and rheology of the gels.

6. To quantify the effects of physically modified rice flour on the rheological properties of the doughs and on the quality of the resulting breads.

In order to achieve objective 1, the effect of the addition of the mixture of acetic and lactic acid, in similar doses to those naturally produced by the sourdoughs (0.5 g/100 g of starch+protein mix), in matrices of different starches (corn, potato, tapioca and wheat) enriched with exogenous proteins was studied. Calcium caseinate proteins and soy protein isolate were selected in doses of 5 g/100 g of starch+protein mixture. In this first work, a detailed study of the rheology and pasting properties of the doughs was carried out. The factors studied were type of starch, type of protein and presence of acid. This study is included in *Chapter I* of this document.

Also in compliance with objective 1, the study of acidification and fortification of the dough of rice starch with different proteins was approached: egg albumin, calcium caseinate, pea protein and soy protein isolate. In this work, the effect of the addition of two doses of protein, 5 and 10 g/100 g of starch+protein mixture on the rheological and pasting properties of the dough was evaluated. These preliminary studies allowed the selection of the source of starch with the most promising techno-functional interest. This study is included in *Chapter II* of this document.

In order to fulfil objective 2 of this Doctoral Thesis and taking into account the previous results, the effect of acidification and protein enrichment (egg albumin, calcium caseinate, pea protein and soy protein isolate) at the two previously evaluated protein doses (5 and 10 g/100 g of starch + protein mixture) on the quality of GF breads were evaluated. The effect on the dough evolution during proof, dough thermal properties and physical and sensory characteristics of the resulting breads was studied. This study, included in *Chapter III* of this Thesis, allowed the selection of the sources of starch and protein of most interest based on their performance on the production of GF bread.

To carry out objective 3, the thermal properties of the starch-protein mixtures and the rheological properties of gels made at different temperatures (90 and 120ºC), with rice, potato and tapioca starch and the addition of two proteins were established: egg albumin and soy protein isolate. Firstly, it was decided to

study the effect of acidification of the mixtures by setting the pH at 4.5, since during fermentation in the enriched protein acidified doughs (at a dose of 5 g/100 g starch+protein), pH values between 4.3 and 4.8 were reached for the starches tested. Confocal laser microscopy was used to analyze the differences obtained in the gels at both temperatures. This work was carried out during my stay at the University of Thessaloniki (Greece) under the supervision of Professors Dr. Biliaderis and Dr. Lazaridou and is included in *Chapter IV* of this document.

Objective 4 was approached by studying the microwave energy absorption properties of rice flour as a function of its moisture content and treatment time. The evolution of temperature during treatment time, considered an important parameter on which the changes occurring depend, was also evaluated. As it is an innovative work, the effect of the treatment on the structure of rice flour and its thermal and pasting properties was analysed. This study is included in *Chapter V* of this Doctoral Thesis.

In order to achieve objective 5, experiences of physical modification by microwave-assisted hydrothermal treatment of starch-protein mixtures were planned. For this purpose, rice and potato starches with calcium caseinate mixtures and soy protein isolate in 5 g/100 g doses on a starch+protein basis were studied. The functional properties, pasting properties and the rheology of the resulting gels were examined in order to establish what modifications were made to these protein-enriched starch matrices. This study is included in C*hapter VI* of this document.

Objective 6 was addressed by studying the effect of the addition of rice flour treated by microwave radiation (at two different initial moisture levels, 20 and 30%), at doses of 30 and 50%, to bread doughs and the evaluation of the quality of the resulting breads. The rheology of the doughs, the pasting properties of the doughs and the physical quality of the breads were studied and are included in *Chapter VII* of this Thesis.

All the lines of research and their corresponding chapters included a statistical analysis of the results, which represented a very useful tool for the comparison and discussion of the results obtained. The description of the methods used and the derived results are included in the respective subsections of each chapter of this Doctoral Thesis.

LISTADO DE ARTÍCULOS

LIST OF PAPERS

LISTADO DE ARTÍCULOS

El contenido de esta tesis doctoral se divide en siete capítulos constituidos por los artículos publicados (o en proceso de publicación) en revistas científicas de alto impacto. Estos capítulos se corresponden con los objetivos anteriormente planteados.

I. Villanueva, M., Pérez-Quirce, S., Collar, C., Ronda, F. 2018. Impact of acidification and protein fortification on rheological and thermal properties of wheat, corn, potato and tapioca starch-based gluten-free bread dough. LWT – Food Science and Technology, 96, 446-454.

II. Ronda, F., Villanueva, M., Collar, C. 2014. Influence of acidification on dough viscoelasticity of gluten-free rice starch-based dough matrices enriched with exogenous protein. LWT – Food Science and Technology, 99, 12-20.

III. Villanueva, M., Mauro, R.R., Collar, C., Ronda, F. 2015. Acidification of protein-enriched rice starch dough: effects on breadmaking. European Food Research and Technology, 240, 783-794.

IV. Villanueva, M., Ronda, F., Moschakis, T., Lazaridou, A., Biliaderis, C.G. 2018. Impact of acidification and protein fortification on thermal properties of rice, potato and tapioca starches and rheological behaviour of their gels. Food Hydrocolloids, 79, 20-29.

V. Villanueva, M., Harasym, J., Muñoz, J.M., Ronda, F. 2018. Microwave absorption capacity of rice flour. Impact on the radiation on rice flour microstructure, thermal and viscometric properties. Journal of Food Engineering, 224, 156-164.

VI. Villanueva, M., De Lamo, B., Harasym, J., Ronda, F. 2018. Microwave radiation and protein addition modulate the functionality and gel rheological characteristics of rice and potato starches. Carbohydrate Polymers, 201, 374-381.

VII. Villanueva, M., Harasym, J., Muñoz, J.M., Ronda, F. 2018. Microwave assisted heat moisture treatment of rice flour improves the viscoelastic behavior of doughs and its bread-making performance. Submitted to Food Hydrocolloids.

SECCIÓN I

SECTION I

ESTRUCTURACIÓN MEDIANTE REFORMULACIÓN: EFECTO DE LA ACIDIFICACIÓN Y FORTIFICACIÓN PROTEICA SOBRE MATRICES DE ALMIDÓN, GELES, MASAS PANARIAS Y PANES SIN GLUTEN.

CAPÍTULO I

CHAPTER I

IMPACTO DE LA ACIDIFICACIÓN Y ENRIQUECIMIENTO PROTEICO SOBRE LAS PROPIEDADES REOLÓGICAS Y TÉRMICAS DE MASAS SIN GLUTEN A PARTIR DE ALMIDÓN DE MAÍZ, PATATA, TAPIOCA Y TRIGO.

Villanueva, M., Pérez-Quirce, S., Collar, C., Ronda, F. 2018. Impact of acidification and protein fortification on rheological and thermal properties of wheat, corn, potato and tapioca starch-based gluten-free bread dough. LWT – Food Science and technology, 96, 446-454.

Impact of acidification and protein fortification on rheological and thermal properties of wheat, corn, potato and tapioca starch-based gluten-free bread doughs

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Abstract

The study of new gluten-free (GF) foods is necessary since consumers intolerant to gluten are more and more frequently diagnosed. The study evaluated the impact of acidification -with acetic+lactic blend at 0.5 g/100 g level- and protein fortification -with caseinate (CA) or soy-protein isolate (SPI)- on the rheological features of wheat, corn, potato and tapioca starch-based bread doughs. Oscillatory and creep-recovery tests were carried out to characterise their viscoelastic behaviour, and thermomechanical tests were performed to assess their visco-metric performance. Dough stickiness was also measured. The acid blend had a modulator effect on dough rheological properties that depended on both the type of protein and the source of the starch. Proteins structured and strengthened the doughs especially those made with SPI-potato starch and CA-wheat starch mixtures. Acidification decreased G' and G'' moduli until 70% with respect to unacidified doughs. The effect was much more marked in protein-fortified doughs. A significant increase in all pasting viscosities was observed with protein addition, particularly in the case of CA. In general, protein addition decreased dough stickiness whereas the opposite effect was noted with the presence of acid. Acidification of protein-enriched starch matrices modulate

dough rheological properties which are of relevance in GF products development.

Keywords: Acetic acid; Gluten-Free Doughs; Lactic acid; Proteins; Rheology

1. Introduction

The development of products for consumers with gluten-related disorders constitutes a prioritized and challenging topic in starch-based goods area. In addition to diagnosed patients, also people looking for non allergenic ingredients contribute to a growing GF market category; therefore the risen variety of offered items seems to be an imperious need.

Understanding the rheological characteristics of food materials is of key importance in designing new products. In breadmaking applications, the rheological properties of doughs affect both dough handling ability and breadmaking process (Hoseney & Smewing, 1999), and hence final bread characteristics (Ronda, Pérez-Quirce, & Villanueva, 2017). Fundamental and empirical rheological properties of doughs also inform about interactions among ingredients and the creation of structure at macromolecular and macroscopic levels, respectively (Ronda, Villanueva, & Collar, 2014).

Gluten protein matrix is a key factor in breadmaking. Besides contributing to the water absorption capacity of the dough, gluten provides extensibility, elasticity and cohesiveness to bread dough allowing the fermentation gas to be occluded and maintained in the liquid phase during the dough development, leading to well-developed high-grade breads (Wieser, 2007). The elimination of gluten in baked products results in deleterious effects in terms of quality attributes of products, nutritional characteristics, and consumer acceptance (Naqash, Gani, Gani, & Masoodi, 2017). The most commonly used starches in GF bread-making are maize starch and potato starch but also starches from tapioca, wheat and rice among other (Masure, Fierens, & Delcour, 2016). However, these starches have minimal structure-building potential and, thus, are frequently used along with proteins and hydrocolloids (Capriles & Arêas, 2014). Proteins and polysaccharides are present together in many kinds of food systems, and both types of food macromolecules contribute to the structure, texture and stability

of food through their thickening or gelling behaviour and surface properties (Doublier, Garnier, Renard, & Sanchez, 2000). The incorporation of proteins in GF matrices is focused on the nutritional enhancement and on the improvement of bread final characteristics (physical and textural).

Inter- and intra-molecular interactions established between exogenous proteins and starch molecules, main responsible for dough structuring, certainly depend on dough pH (Houben, Höchstötter, & Becker, 2012; Ronda et al., 2014). Acidification through lactic and acetic acid addition confers suitable properties to final breads either when produced by the exogenous microflora or added to breadmaking matrices Acidification improved the odour and taste of fresh bread and increased the protease and amylase activities that led to retarded staling during storage (Moore, Dal Bello, & Arendt, 2008). Acidification by acetic acid and lactic acid addition have shown to provide a significant impact in protein-enriched rice starch-based doughs properties (Ronda et al., 2014) and in the quality and shelf-life of rice starch-based breads fortified with CA, SPI and pea protein isolate (Villanueva, Mauro, Collar, & Ronda, 2015). Taken into account the importance of other starches, as potato, tapioca, corn and wheat, on the development of GF products, the study of the effect of acidification on protein-enriched doughs made with theses starches seems timely.

In GF products, starch becomes the primary structural element due to the lack of gluten, mainly during the baking stage, when the batter temperature reaches starch gelatinization values. However, starches from different sources differ markedly on water binding capacity which affects dramatically dough consistency and dough development during fermentation, and the quality of the final products (Ronda et al., 2017). With this in mind, the aim of the present study was to evaluate the impact of the addition of 0.5 g/100 g (starch+protein) of acetic + lactic acid mixture to different GF bread doughs made with maize, potato, tapioca or wheat starches fortified with CA or SPI (at 5 g/100 g (starch+protein) level) on the viscoelasticity, stickiness and pasting properties of bread doughs.

2. Material and methods

2.1. Materials

Corn, potato and wheat starches were supplied from Ferrer Alimentación S.A. (Barcelona, Spain), and tapioca starch from Cargill S.L. (Brenntag, Sevilla, Spain).

Salt, sugar (Azucarera, Toro, Spain) and sunflower oil Coosur Premium (Jaen, Spain) were purchased from the local market. Hydroxy-propyl-methyl-cellulose (HPMC, Methocel-K4M-Food-Grade) was provided as a gift by Dow Chemical (Midland, USA). Proteins used in GF formulations were: soybean protein isolate (SPI) Supro 500-E IP given by Proveedora hispano-holandesa S.A. (Barcelona, Spain) and calcium caseinate (CA) by Armor proteines (Saint-Brice-en-Coglès, France). Acetic acid and lactic acid of analytical grade from Panreac (Barcelona, Spain) were used. Distilled water was used to prepare all the suspensions to study the pasting profiles and tap water was used to make GF doughs.

2.2. Methods

Dough preparation

A straight dough process was performed in duplicate per formulation, using the following formula on a 100 g starch (or starch+protein) basis: 6 g oil, 5 g sucrose, 1.5 g salt, 2.0 g HPMC and 75 g water. CA and SPI were added at 0 or 5 g/100 g (starch + protein basis) levels and doughs were supplemented with (0.1 + 0.4) g/100 g (starch + protein basis) of acetic+lactic acid when acid-treatment was applied. The experimental design resulted in 24 different combinations (Table 1). GF dough-making was achieved by blending first solid ingredients and oil in a kitchen-aid professional mixer KPM5 (Michigan, USA) at speed 2. Then water was added and hand mixed. Finally the dough was mixed with dough hook at a speed 4 for 8 min. Acid blend, when added, was diluted in a small part of water and adjusted to the dough before the mixer was powered on.

2.3. Dough measurements

pH and total titratable acidity of doughs

Total titratable acidity (TTA) was measured on ten grams of dough blended with 100 mL of a solution of acetone in water (5 mL/100 mL) under constant stirring. The titration was carried out against 0.1 mol/L NaOH until a final pH of 8.5. The results were expressed as milliequivalents of lactic acid/g of dough. This measurement was taken in triplicate on unyeasted doughs.

Table 1. Randomized experimental design

Formula	Starch	Protein	Acetic/Lactic Acid*
1	Potato	SPI	0.1/0.4
2	Wheat	0	0.1/0.4
3	Potato	SPI	0
4	Corn	SPI	0
5	Corn	SPI	0.1/0.4
6	Corn	CA	0
7	Tapioca	SPI	0
8	Wheat	0	0
9	Tapioca	SPI	0.1/0.4
10	Corn	0	0.1/0.4
11	Corn	CA	0.1/0.4
12	Potato	0	0
13	Tapioca	CA	0
14	Tapioca	0	0.1/0.4
15	Potato	CA	0.1/0.4
16	Wheat	SPI	0.1/0.4
17	Wheat	CA	0
18	Tapioca	CA	0.1/0.4
19	Tapioca	0	0
20	Potato	CA	0
21	Corn	0	0
22	Wheat	CA	0.1/0.4
23	Wheat	SPI	0
24	Potato	0	0.1/0.4

Protein: 0: without protein, CA: With 5g/100g Calcium caseinate, SPI: With 5g/100g soybean protein isolate. *g/100g with respect to starch or starch+protein basis

Fundamental rheological tests

Oscillatory and creep–recovery tests were carried out with RheoStress-1 rheometer (Thermo Haake, Karlsruhe, Germany) with parallel plate geometry (60 mm diameter) of serrated surface and with 3-mm gap. The excess of dough was removed, and vaseline oil was applied to cover the exposed sample surfaces. All measurements were done at 25 °C. Before each assay the dough was allowed 10 min for relaxation. Frequency sweeps were carried out from 10 to 1 Hz in the linear viscoelastic region (LVR). A constant stress value of 1 Pa was chosen for the frequency sweeps of all doughs. Stress sweeps were carried out from 0.1 to 100 Pa at 1 Hz. From the curves, the maximum stress beyond which the dough structure was broken, τmax, was established. Frequency sweep data were fitted

to the power law model as in previous works (Ronda et al., 2014). Within the applied frequency range, the mechanical spectra fitted the power law model with R2 values above 0.99.

Creep tests were performed by imposing a step of shear stress in the LVR and outside the linear viscoelastic region (OLVR). For the creep study in the LVR, a constant shear stress of 1 Pa was applied for 150 s, while in the recovery phase the stress was suddenly removed and the sample was allowed for 300 s to recover the elastic (instantaneous and retarded) part of the deformation. For the OLVR study, a constant shear stress of 50 Pa was applied for 60 s and the sample was allowed to recover for 180 s after removing the load. Each test was performed in triplicate. The data from creep tests were modelled to the 4-parameter Burgers model (Ronda et al., 2014).

Dough stickiness

Stickiness was measured by following the procedure proposed by Grausgruber, Hatzenbichler, & Ruckenbauer (2003). A texturometer TA-XT2 from Stable Microsystem (Godalming, UK) provided with a SMS/Chen-Hoseney device where the sample was placed, and a methacrylate 25 mm cylinder (P/25P) as compression cell, were used. The positive maximum force (adhesive force), was used to measure stickiness. Six replicates were made for each dough.

Pasting properties

Viscometric profiles of formulated doughs from different starch sources and proteins in acidified/no acidified medium were obtained by using a Rapid-Visco-Analyser (RVA-4, Newport Scientific, Warriewood, Australia) and profile Standard 1. Freeze-dried dough samples (Collar, 2003) were transferred (3.0 g for corn and wheat starches, 2.5 g for tapioca starch and 2.0 g for potato starch of 14 g/100 g moisture basis) into canisters and 25 ± 0.1 mL of distilled water were added and processed following standard method. The pasting temperature (PT), peak time (P-time), peak viscosity (PV), trough viscosity (TV), breakdown (BD), final viscosity (FV) and setback viscosity (SB) were calculated from the pasting curve using Thermocline v. 2.2 software. All measurements were performed in duplicate.

2.4. Statistical analysis

Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) was used for non-linear regressions and multi-factor analysis of variance. LSD (Least Significant

Difference) test was used to evaluate significant differences ($p < 0.05$) between samples.

3. Results and discussion

3.1. pH and total titratable acidity of doughs

The pH of unacidified and protein-free matrices varied depending on the starch source, and followed the order: Tapioca (pH=5.9) < Corn (pH=6.1) < Potato (pH=6.5) < Wheat (pH=6.8) (Fig. 1a). Protein presence systematically increased the dough pH value while the acetic-lactic blend provided a decrease ~ 2.5 units. The type of protein and the starch source also affected the pH of the dough through the significant ($p<0.05$) (protein x starch x pH) 3rd order interactive effect (Fig.1a). Dough pH increased with protein presence between 3 % (for wheat and potato starch doughs) and 18% (for tapioca starch dough) depending on the starch source. Acidification of control matrices reduced significantly ($p<0.05$) the pH from 6–6.7 to 3.4–3.6. However, acidification of protein-enriched doughs only decreased pH to 4.3–4.8. The buffering effect of proteins, responsible for the lower effect of acidification on dough pH, was previously reported by Villanueva et al. (2015) for rice starch-based doughs. Fig.1a shows the buffering effect was significantly higher for CA than SPI regardless the starch source used for dough formulation; consequently, the pH of acidified CA-enriched doughs was higher than those of SPI-enriched doughs.

The TTA of control doughs (unacidified and protein-free doughs) varied significantly ($p<0.05$) depending on the starch sources (Fig.1a): Wheat (0.0028 meq/g) < Tapioca (0.0039 meq/g) < Corn (0.0077 meq/g) < Potato (0.0100 meq/g). Acid addition increased the TTA of doughs from 0.008 meq/g to 0.034 meq/g on average. Protein addition increased dough TTA but the increase depended on starch source and protein type as denoted by the significant ($p<0.05$) 3rd order interaction depicted in Fig.1a. The increase was always higher for CA than SPI, in coherence with the higher buffering effect of the former, also responsible for the lower decrease of pH in acidified doughs in CA presence.

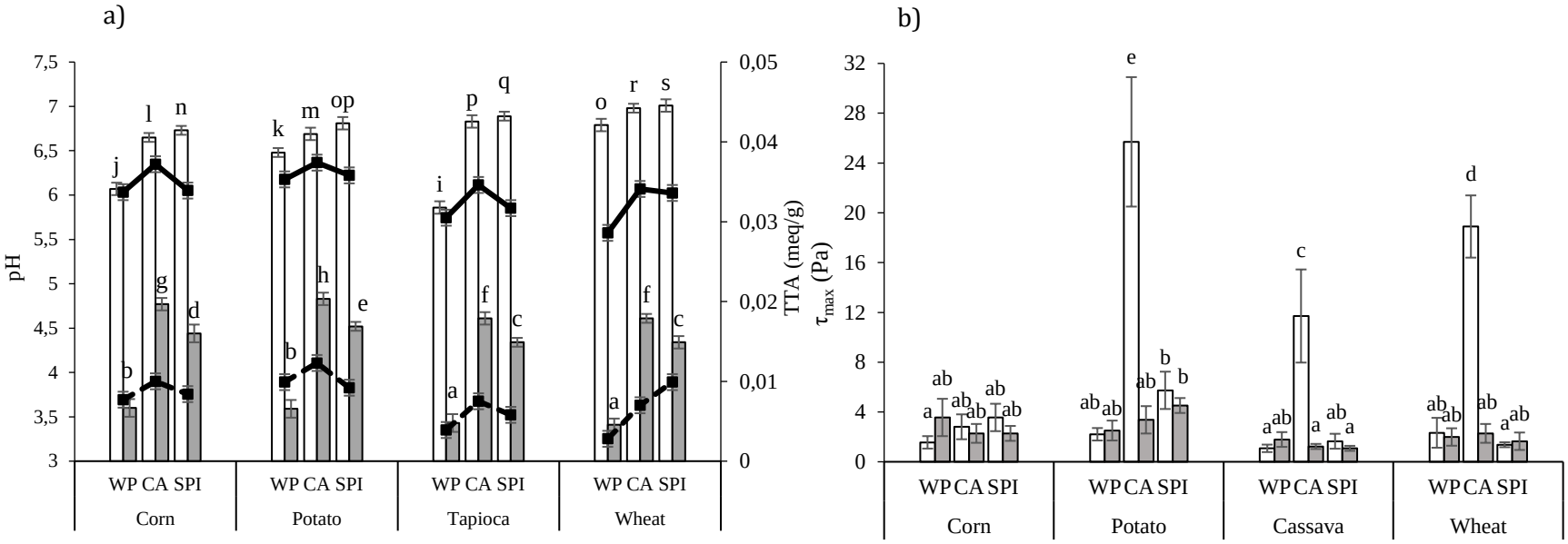

Figure 1. pH and TTA values (a) and maximum stress values, τ_{max} (b) recorded for samples with different starch source, type of protein and acid addition. WP: doughs without protein, CA: doughs with 5% calcium caseinate, SPI: doughs with 5% soy protein isolate. Void bars (principal axes) and discontinue lines (secondary axes) correspond to doughs without acid addition, filled bars and continuous lines correspond to acidified doughs. Error bars represent the mean standard deviation. Different letters within each graph mean statistically significant differences between means ($p<0.05$).

3.2. Dynamic oscillatory rheology

The stress sweep tests provided the τmax value or maximum stress doughs were able to stand before breaking their structure (Fig.1b). The τmax values of all doughs were around 2–4 Pa (without significant differences among them) with the exception of unacidified CA-enriched doughs made with potato, wheat or tapioca starches (maize starch doughs were not affected by CA addition). The τmax of these doughs were much higher: 26, 19 and 12 Pa respectively. This could be due to the organization of casein micelles that form large supramolecular entities further considered as spherical particles. They are covered by κ-casein, which stabilizes them in the suspension through steric and electrostatic repulsions. Moreover, the hairy surface prevents neutral polymers from adsorbing on the micelles (Bourriot, Garnier, & Doublier, 1999) and Ca+2 ionic interactions, which partially can replace the behaviour of disulphide bridges, could deliver similar rheological characteristics to gluten systems (Stathopoulos & O'Kennedy, 2008). The acid blend addition counteracted the CA stabilization effect and led to similar τmax values than protein-free matrices.

Table 2 shows the single effects and Fig.2a the 3rd order interactive effects of factors studied on viscoelastic parameters obtained from frequency sweeps. Viscoelastic behaviour of dough samples corresponded to solid-like systems with storage modulus values (G'_1) higher than loss modulus (G''_1), slight frequency dependence (low a and b exponents), and values for ($\tan \delta_1$) under 1, in good accordance with earlier results found for acidified rice starch doughs enriched with proteins that included SPI and CA proteins (Ronda et al., 2014). The slight dependence of the moduli on angular frequency (a and b values ranged 0.13–0.37) and the values of phase shift tangent (tan δ) varying in the range 0.33–0.68 are characteristics of the systems called pseudo-gels. This is in agreement with earlier observations in GF doughs (Witczak, Korus, Ziobro, & Juszczak, 2010). Starch source affected significantly ($p<0.001$) the viscoelastic moduli. The highest G_1' and G_1'' moduli were obtained for potato starch doughs (17300 Pa and 9400 Pa on average, respectively) while the lowest values were observed for wheat starch (3000 Pa and 1700 Pa) (Table 2). Factors related to the botanical origin of starch responsible for starch swelling such as amylose/amylopectin ratio, molecular weight of amylose and amylopectin, their distribution within the granule, granule size, the lipid content and other minor components (such as minerals and salts) play a crucial role (Waterschoot, Gomand, Fierens, & Delcour, 2015). The incorporation of proteins also affected

markedly dough consistency. Proteins raised both viscoelastic moduli, G1' and G1'', leading to averaged increases of 145 and 130% respectively with respect to the values of non-protein added-doughs. Other authors also concluded that proteins such as soy proteins affected rice dough consistency since they are the main components involved in water absorption (Marco & Rosell, 2008). The increase in rice based dough consistency was also previously reported as result of SPI and CA addition (Ronda et al., 2014; Matos & Rosell, 2014).

Table 2. Single effects on pH. acidity and the rheological properties from oscillatory tests of gluten-free bread doughs made with starches from different sources, without or with protein (5 g calcium caseinate or soy protein isolate per 100 g of starch+protein) with or without acid addition (acetic+lactic acid 0.1+0.4 g/100 g starch+protein)

Variable	Unit	Mean	Level	Starch		Protein		Acid	
pH of the medium									
pH		5.43	1	5.38	b	4.90	a	6.65	b
			2	5.48	c	5.74	c	4.21	a
			3	5.32	a	5.63	b		
			4	5.52	d				
SE				0.004		0.003		0.003	
TTA	meq/g	0.0209	1	0.0218	b	0.0190	a	0.0079	a
			2	0.0233	c	0.0225	c	0.0339	b
			3	0.0190	a	0.0210	b		
			4	0.0193	a				
SE				0.0002		0.0001		0.0001	
Dynamic Oscillatory Rheometry									
G'_1	Pa	7763	1	5803	c	3942	a	9990	b
			2	17309	d	9205	b	5537	a
			3	4959	b	10143	c		
			4	2982	a				
SE				162		138		111	
a		0.30	1	0.28	a	0.31	b	0.30	a
			2	0.31	b	0.33	c	0.30	a
			3	0.29	a	0.27	a		
			4	0.33	c				
SE				0.01		0.004		0.003	
G''_1	Pa	4126	1	2741	b	2196	a	5332	b
			2	9443	c	5411	c	2920	a
			3	2590	b	4771	b		
			4	1731	a				
SE				84		72		58	
b		0.23	1	0.25	b	0.25	b	0.22	a
			2	0.19	a	0.24	b	0.24	b
			3	0.23	b	0.21	a		
			4	0.27	c				
SE				0.01		0.01		0.005	

tan δ	0.53	1	0.47	a	0.56	b	0.54	a
		2	0.55	c	0.57	c	0.53	a
		3	0.52	b	0.47	a		
		4	0.58	d				
SE			0.01		0.005		0.01	
c	-0.07	1	-0.03	c	-0.06	b	-0.08	a
		2	-0.12	a	-0.09	a	-0.06	b
		3	-0.07	b	-0.06	b		
		4	-0.06	b				
SE			0.01		0.01		0.004	

Starch level: 1: corn, 2: potato, 3: tapioca, 4: wheat; Protein level: 1: without protein, 2: Calcium caseinate, 3: Soya protein isolate; Acid level: 1: without acid addition, 2: with acid addition. Within each parameter, different letters in the corresponding column mean statistically differences between means at $p<0.05$. TTA: total titratable acidity. G'_1. G''_1. and $(\tan \delta)_1$ represent the elastic and viscous moduli and the loss tangent at a frequency of 1 Hz. The a, b and c exponents quantify the dependence degree of dynamic moduli and the loss tangent with the oscillation frequency. SE: Pooled standard error

The results could be explained by the creation of a robust crosslinked structure in doughs by added proteins, especially in the case of SPI by glycinin and its high water retention ability (Crockett, Ie, & Vodovotz, 2011). On the opposite, dough acidification always decreased both viscoelastic moduli as was also previously concluded for rice starch (Ronda et al., 2014). The ANOVA study showed that all the 2nd order and 3rd order effects significantly ($p<0.01$) affected G_1', G_1'' and $\tan \delta_1$. This means that the effect of the protein type depended on both the starch source and the pH of the dough. As can be seen in Fig.2a SPI provided the most strengthening effect in potato starch doughs, with increases up to 250% in G_1' with respect to the protein-free dough. Important increases in G' and G'' were also found by Patraşcu, Banu, Vasilean, & Aprodu (2016) when added SPI to potato starch systems. However, in the case of wheat starch was the CA-protein who had the highest effect on dough consistency leading to increases in G_1' and G_1'' of 320% while SPI only led to an increase of 105%. The effect of both proteins was similar in the case of corn and tapioca starch doughs (Fig.2a). The effect of dough acidification on viscoelastic moduli was always greater in the case of protein-enriched doughs. The acidification of protein-free doughs only provided a significant ($p<0.05$) effect in the case of potato, with slight decreases in G_1' and $G_1''$of 14 and 18% respectively. However, in presence of protein, the decrease in the elastic modulus, G1', was 41 and 74% for SPI- and CA-enriched wheat doughs with respect to the non-acidified counterparts.

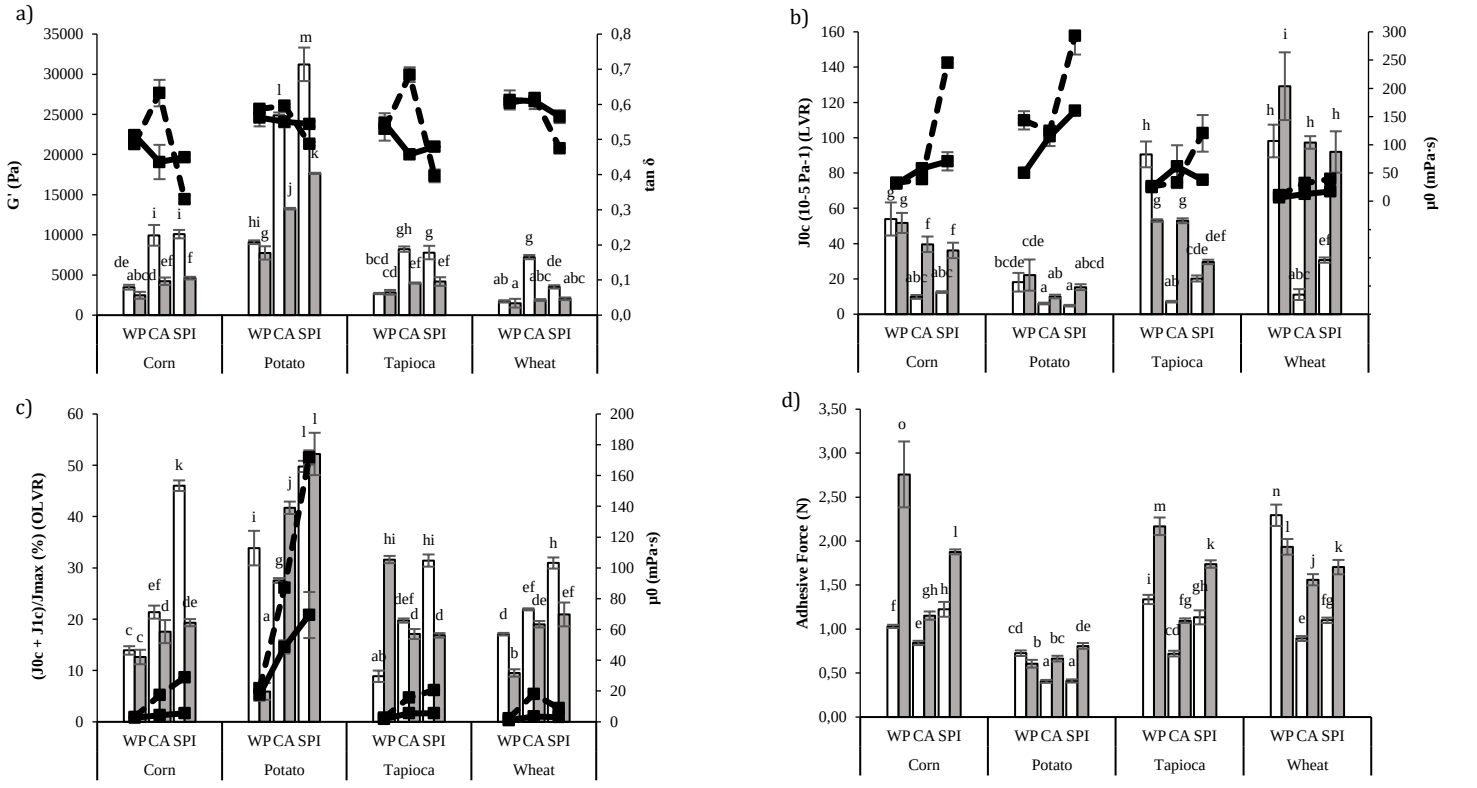

Figure 2. Rheological properties of bread doughs depending on the starch source, type of protein and acid addition. Elastic modulus and loss tangent from oscillatory tests (a), Instantaneous elastic compliance and steady viscosity from creep tests in the linear viscoelastic region (LVR) (b) Percentage of total elastic compliance with respect to maximum compliance from creep tests measured outside the lineal viscoelastic region (OLVR) (c) and adhesive force obtained from stickiness tests (d) of bread doughs. WP: doughs without protein, CA: doughs with 5% calcium caseinate, SPI: doughs with 5% soy protein isolate. Void bars (principal axes) and discontinue lines (secondary axes) correspond to doughs without acid addition, Filled bars and continuous lines correspond to acidified doughs. Error bars represent the mean standard deviation. Different letters within each graph mean statistically significant differences between means ($p<0.05$).

Similar tendency was observed in corn and tapioca doughs (Fig.2a). In both cases, tan δ decreased in unacidified doughs as result of protein addition, denoting an increase in the predominance of dough elasticity. In acidified doughs, both proteins CA and SPI, led to different effects. Acidification of CA-enriched doughs led to a marked increase in the loss tangent, which indicates an increment in the viscous to elastic moduli ratio, while in the case of SPI-added doughs a decrease was observed. The similarities between corn and tapioca starches could be due to their similar particle size and shape, completely different from potato (very big size) and wheat (bi-modal size distribution with small and big granules) starches. These structural differences and therefore, their functional properties, could change the behaviour of the continuous phase of the dough which results in changes of viscoelasticity. According to Singh, Singh, Kaur, Sodhi, & Gill (2003), the presence of a high phosphate monoester content and the absence of lipids and phospholipids in the potato starch may also be responsible for the high G' and G'' of their doughs. The presence of phospholipids and the more rigid granules of corn starch could explain the lower consistency of doughs.

3.3. Creep-recovery tests

Creep-recovery tests were carried out both at 1 Pa, within the linear viscoelastic region (LVR), and at 50 Pa, outside the linear viscoelastic region (OLVR). The results within the LVR are easier to correlate with the molecular structure of the sample components. However, during the baking process (mixing, moulding, fermentation, baking) the doughs are subjected to stress outside the LVR. Therefore, OLVR tests are useful for predicting the deformations that the doughs will experience during processing.

Table 3. Single effects on the rheological properties from creep recovery tests inside (LVR) and outside (OLVR) the linear viscoelastic region of gluten-free bread doughs made with starches from different sources, without or with protein (5 g calcium caseinate or soy protein isolate per 100 g of starch+protein) with or without acid addition (acetic+lactic acid 0.1+0.4 g/100 g of starch+protein)

Variable	Unit	Mean	Level	Starch		Protein		Acid	
Creep recovery test in LVR									
J_{0c}	10^{-5} Pa^{-1}	41	1	34	b	65	b	30	a
			2	13	a	29	a	52	b
			3	42	c	30	a		
			4	76	d				
SE				2		2		1	
J_{1c}	10^{-5} Pa^{-1}	201	1	138	a	309	b	163	a
			2	100	a	163	a	240	b
			3	194	b	131	a		
			4	373	c				
SE				19		16		11	
λ_c	s	30	1	28	a	32	a	31	a
			2	35	a	29	a	30	a
			3	33	a	30	a		
			4	26	a				
SE				5		4		3	
μ_0	10^{+3} Pa·s	74	1	79	c	41	a	95	b
			2	148	d	59	a	54	a
			3	51	b	123	b		
			4	20	a				
SE				10		8		7	
J_{0r}	10^{-5} Pa^{-1}	76	1	58	b	122	c	58	a
			2	27	a	57	b	94	b
			3	70	c	51	a		
			4	150	d				
SE				2		2		1	
J_{1r}	10^{-5} Pa^{-1}	226	1	125	a	354	c	166	a
			2	104	a	191	b	285	b
			3	191	b	132	a		
			4	482	c				
SE				13		12		9	
λ_r	s	94	1	94	a	109	b	93	a
			2	94	a	92	a	97	a
			3	93	a	83	a		
			4	98	a				
SE				6		5		4	
Recovery	%	43.2	1	39.0	a	41.2	a	42.1	a
			2	52.0	b	42.7	ab	44.2	a
			3	40.9	a	45.8	b		
			4	40.9	a				
SE				1.7		1.6		1.2	
Creep recovery test OLVR									
J_{0c}	10^{-5} Pa^{-1}	10	1	15	c	8	b	8	a
			2	10	b	5	a	11	b
			3	6	a	15	c		

			4	8	ab				
SE				3		1		1	
J_{1c}	10^{-5} Pa^{-1}	229	1	232	c	309	c	172	a
			2	79	a	177	a	285	b
			3	178	b	200	b		
			4	427	d				
SE				7		6		5	
λ_c		11	1	11	c	9	a	12	b
			2	12	d	11	b	9	a
			3	9	a	12	c		
			4	10	b				
SE				0.3		0.2		0.2	
μ_0	10^{+3} Pa·s	24	1	10	b	7	a	33	b
			2	69	c	25	b	14	a
			3	9	b	39	c		
			4	6	a				
SE				1		1		1	
J_{0r}	10^{-5} Pa^{-1}	39	1	33	a	38	b	36	a
			2	30	a	35	a	42	b
			3	40	b	45	c		
			4	54	c				
SE				2		1		1	
J_{1r}	10^{-5} Pa^{-1}	131	1	114	b	155	b	118	a
			2	83	a	121	a	143	b
			3	130	c	115	a		
			4	196	d				
SE				4		4		3	
λ_r		34	1	30	a	29	a	38	b
			2	39	c	40	c	30	a
			3	33	b	32	b		
			4	33	b				
SE				0.6		0.5		0.4	
Recovery	%	24.6	1	16.9	a	15.9	a	31.0	b
			2	50.0	c	30.7	b	20.3	a
			3	18.9	b	30.1	b		
			4	16.6	a				
SE				0.5		0.4		0.3	

Starch level: 1: corn, 2: potato, 3: tapioca, 4: wheat; Protein level: 1: without protein, 2: Calcium caseinate, 3: Soya protein isolate; Acid level: 1: without acid addition, 2: with acid addition, Within each parameter, different letters in the corresponding column mean statistically differences between means at $p<0.05$. J_0 and J_1 are the instantaneous and retarded elastic compliances, λ_1 is the retardation time and μ_0 is the steady state viscosity. Recovery (%): $100*J_{steady}/J_{maxc}$. where J_{max} is the maximum creep compliance obtained at the end of the creep step and J_{steady} is the steady-state compliance in recovery step. SE: Pooled standard error

The single effects of starch source, protein type and acid addition on Burgers model parameters are summarized in Table 3. Fig.2b shows 3rd order (starch source x protein type x acidification) effects on the instantaneous elastic compliance (J_{0c}) and the steady viscosity (μ_0) obtained from the creep phase in

the LVR. The studied bread doughs showed the typical viscoelastic creep–recovery curves combining viscous and elastic components both in the LVR and OLVR. In the LVR, a strong correlation ($p<0.001$) was found for all creep compliance parameters and the equivalents for the recovery phase ($r>0.95$). Besides, it was observed that factors providing an increase in viscosity at the steady state, μ_0, decreased elastic and retarded elastic components (J_0 and J_1 respectively) in both creep and recovery phases. Creep-recovery tests made in the LVR revealed that doughs with the lowest elastic and viscoelastic compliances and the highest steady viscosity had also the highest G_1' and G_1'' values. Potato starch led to doughs with the highest μ_0 (148 kPa·s on average) and the smallest J_0 and J_1 compliances ($13 \cdot 10^{-5}$ Pa^{-1} and $100 \cdot 10^{-5}$ Pa^{-1}) denoting their highest resistance to deformation. Conversely, wheat starch doughs showed the highest deformations versus the application of a constant stress, with J_0 and J_1 averaged values of $76 \cdot 10^{-5}$ Pa^{-1} and $373 \cdot 10^{-5}$ Pa^{-1} (Table 3), and the lowest μ_0 (20 kPa·s). The addition of proteins, regardless of the starch source, always decreased all compliance values and increased the steady viscosity (Table 3, Fig.2b). In general, dough acidification had the opposite effect. This means the studied proteins reinforced dough structure while the acid addition, in general, led to the opposite effect. The effect of acidification depended significantly ($p<0.05$) on the starch source and the presence and type of the added protein (Fig.2b). Acidification was more effective on protein-enriched doughs than in only starch-based matrices. It increased the instantaneous elastic compliance, J_0, of all protein-enriched doughs with respect to the non-acidified ones. The maximum increases, 780% and 650%, were obtained for CA-wheat and CA-tapioca doughs. The pH reduction will shift to neutral or positive the sign of the charge of the ionic radicals of proteins which will affect its intramolecular interactions and alter its interactions with starch and consequently the dough consistency and its viscoelasticity (Villanueva, Ronda, Moschakis, Lazaridou, & Biliaderis, 2018). The obtained results support the ability of acidification to modulate and compensate the effect of protein addition on dough viscoelasticity.

Fig. 2c depicts $(J_{0c}+J_{1c})/J_{max}$, which represents the elastic (instantaneous+retarded) to total (elastic+viscous) compliance ratio, and the steady viscosity (μ_0) of formulated bread doughs, both obtained in the creep phase from OLVR tests. The highest $(J_{0c}+J_{1c})/J_{max}$ values were obtained for potato starch doughs that were always above the remaining doughs except the non-

acidified SPI-corn dough. This means a higher elastic deformation with respect to the total (elastic+viscous) deformation which is of relevance given it is the elastic deformation the only that can be recovered after the release of the applied stress. The measurements OLVR demonstrate steady viscosity increased markedly with proteins, particularly with SPI and corn and potato starch doughs. However, acidification reduced the steady viscosity counteracting the protein effect. The recovery capacity of bread doughs after the applied stress decreased with the intensity of applied stress (Table 3). The recovery (%) values obtained in the LVR tests were always higher than in the OLVR tests except in the case of potato starch dough that was unchanged. The OLVR tests, which seem to better simulate dough processing conditions (Ronda et al., 2017), also showed a greater capacity for discrimination between the analysed doughs.

3.4. Dough stickiness

Table 4 summarizes the single effects of starch, protein and acid blend on the adhesive force of formulated doughs. The 3rd order effects are presented in Fig.2d. The adhesive force correlated negatively with the modules G_1' and G_1'' ($p<0.001$; $r=-0.67$ and $r=-0.70$) indicating that the greater the consistency of the dough the less sticky it is. The stickiness evolution versus elastic or viscous moduli (data not shown) was not linear but potential. This means, stickiness decreased faster (from 2.8 to 1N) with increases of G_1' within the range 1000–7000Pa and decreased slower (from 1 to 0.5N) for G_1' values within the range 7000-30000Pa. The lowest stickiness values were obtained for doughs made from potato starch (0.4N corresponded to CA-fortified/non-acidified dough) and the highest for those made from wheat and corn (the maximum value, 2.8N, corresponded to protein-free corn starch/acidified dough). Stickiness should not overpass the 1N value to discard dough handling problems (Armero & Collar, 1997). Consequently, many of the tested doughs (see Fig.2d) could affect the handling and shaping/flattening purposes to get continuous strands or thin sheets of the doughs. Protein fortification always decreased dough stickiness except when SPI was added to corn starch, where the opposite effect was observed. In general, CA addition decreased more the dough adhesive force than SPI. The effect of acidification on dough stickiness was markedly dependent on the protein presence. The acidification of protein-supplemented samples significantly increased the adhesive force regardless the starch and the type of protein. However, the acidification of protein-free matrices decreased the dough

stickiness in the case of potato and wheat starch (-16% for both starches) and increased it for corn starch (+167%) and tapioca starch (+62%) doughs. Armero & Collar (1997) reported that the addition of sourdough to wheat dough, which led to a concomitant decrease in pH, resulted in more adhesive doughs. The lactic acid concentration was the acidity parameter best correlated with stickiness.

Table 4. Single effects on the stickiness and visco-metric properties of gluten-free bread doughs made with starches from different sources, without or with protein (5 g calcium caseinate or soy protein isolate per 100 g of starch+protein) with or without acid addition (acetic+lactic acid 0.1+0.4 g/100 g of starch+protein)

Variable	Unit	Mean	Level	Starch		Protein		Acid	
Stickiness									
Adhesive Force	N	1.26	1	1.48	c	1.61	c	1.01	a
			2	0.60	a	0.92	a	1.51	b
			3	1.37	b	1.25	b		
			4	1.58	d				
SE				0.02		0.01		0.01	
Pasting properties									
PV	mPa·s	926	1	1520	d	441	a	1062	b
			2	546	a	1292	c	791	a
			3	748	b	1046	b		
			4	891	c				
SE				16		14		11	
TV	mPa·s	324	1	461	c	101	a	391	b
			2	441	c	507	c	256	a
			3	260	b	363	b		
			4	130	a				
SE				8		7		6	
BD	mPa·s	603	1	1057	d	340	a	606	a
			2	105	a	784	b	534	a
			3	488	b	683	b		
			4	761	c				
SE				11		9		7	
SB	mPa·s	180	1	323	b	73	a	231	b
			2	113	a	298	c	144	a
			3	145	a	186	b		
			4	138	a				
SE				6		5		4	
FV	mPa·s	503	1	787	d	174	a	640	b
			2	554	c	787	c	400	a
			3	405	b	549	b		
			4	267	a				
SE				11		9		8	
PT	ºC	71.25	1	73.91	b	71.97	b	69.21	a
			2	67.45	a	69.82	a	73.29	b
			3	76.29	b	72.96	b		

			4	67.28	a				
SE				0.28		0.24		0.20	
P-time	min	4.49	1	4.38	a	3.99	a	4.65	a
			2	5.36	b	4.75	b	4.32	a
			3	3.89	a	4.72	b		
			4	4.34	a				
SE				0.03		0.03		0.02	

Starch level: 1: corn, 2: potato, 3: tapioca, 4: wheat; Protein level: 1: without protein, 2: Calcium caseinate. 3: Soya protein isolate; Acid level: 1: without acid addition, 2: with acid addition. Within each parameter, different letters in the corresponding column mean statistically differences between means at $p<0.05$. PV: peak viscosity, TV: trough viscosity, BD: breakdown, SB: setback, FV: final viscosity, PT: pasting temperature, P-time: peak time. SE: Pooled standard error

3.5. Pasting properties

During the heating and holding stages of the RVA run of a starch suspension, gelatinization, pasting and breakdown take place successively. When gelatinised starch cools, the molecules begin to reassociate into an ordered structure, and undergo retrogradation. Single effects of the design factors on the pasting and gelling viscometric parameters are presented in Table 4. Quantitative viscometric profiles of starch suspensions during pasting and gelling were systematically higher as compared to doughs formulated with or without proteins either unacidified or acidified (Fig. 3). Viscosity values were particularly high during the cooking stage, especially for potato starch suspensions (6000 mPa.s) versus wheat starch suspensions (2600 mPa.s). The presence of high phosphate monoester content and the absence of lipids and phospholipids in potato starch, associated to the great values for the dynamic moduli (Singh et al., 2003) as well as the high degree of reticulation of starch structure may explain the developed great viscosity during pasting. Besides the diluting effect on starch, the presence of non-starch components in the bread dough, and particularly HPMC, protein and lipids for smaller starch granules in starch blends can restrict swelling and gelatinization during cooking, in good agreement with the lower viscometric pattern observed in blended matrices (bread doughs) compared to native starches (Fig.3). Table 4 shows major effects on cooking and cooling parameters were provided by corn (starch), casein (proteins) and no acidification (acid). PT values of blends followed the order: Potato (67ºC) = Tapioca (68ºC) < Corn (74ºC) = Wheat (76ºC) (Table 4). A higher temperature of gelatinization reflects a greater internal stability of starch granule, normally associated with a greater presence of semi-crystalline areas and a higher content of amylose (Hirashima, Takahashi, & Nishinari, 2012). Corn

starch doughs exhibited the highest PV (1520mPa·s), value about two-times those of wheat (891mPa.s) and tapioca (748 mPa·s) starch blends and three-times those from potato (546 mPa·s) starch mixtures. In addition, the highest BD, FV and SB were obtained for corn starch doughs. SB value from RVA determination of starch was attributed to amylose leaching during heating (Naguleswaran, Vasanthan, Hoover, & Liu, 2010), therefore the highest SB of corn starch could be due to the greater amount of amylose leached from swelled granules. Potato, tapioca and wheat starch doughs led to similar SB values indicating similar amylose retrogradation extent on cooling. The incorporation of protein led to significant ($p<0.05$) increases in PV, TV, BD, SB and FV, greater for CA than SPI. Quantitative differences may be attributed to variable ability to retain water and interact with starch molecules at granule surface, and to their gel forming capacity as reported for whey proteins (Ribotta & Rosell, 2010).

Acidification decreased the pasting profile and delayed the PT. Majzoobi, Kaveh, & Farahnaky (2016) reported that the proton released from acetic acid dissociated in water can destabilize and depolymerize glycosidic bonds of the starch molecules and therefore, smaller molecules are formed as a result of starch degradation. These molecules are generally more soluble in water and have lower water absorption capacity.

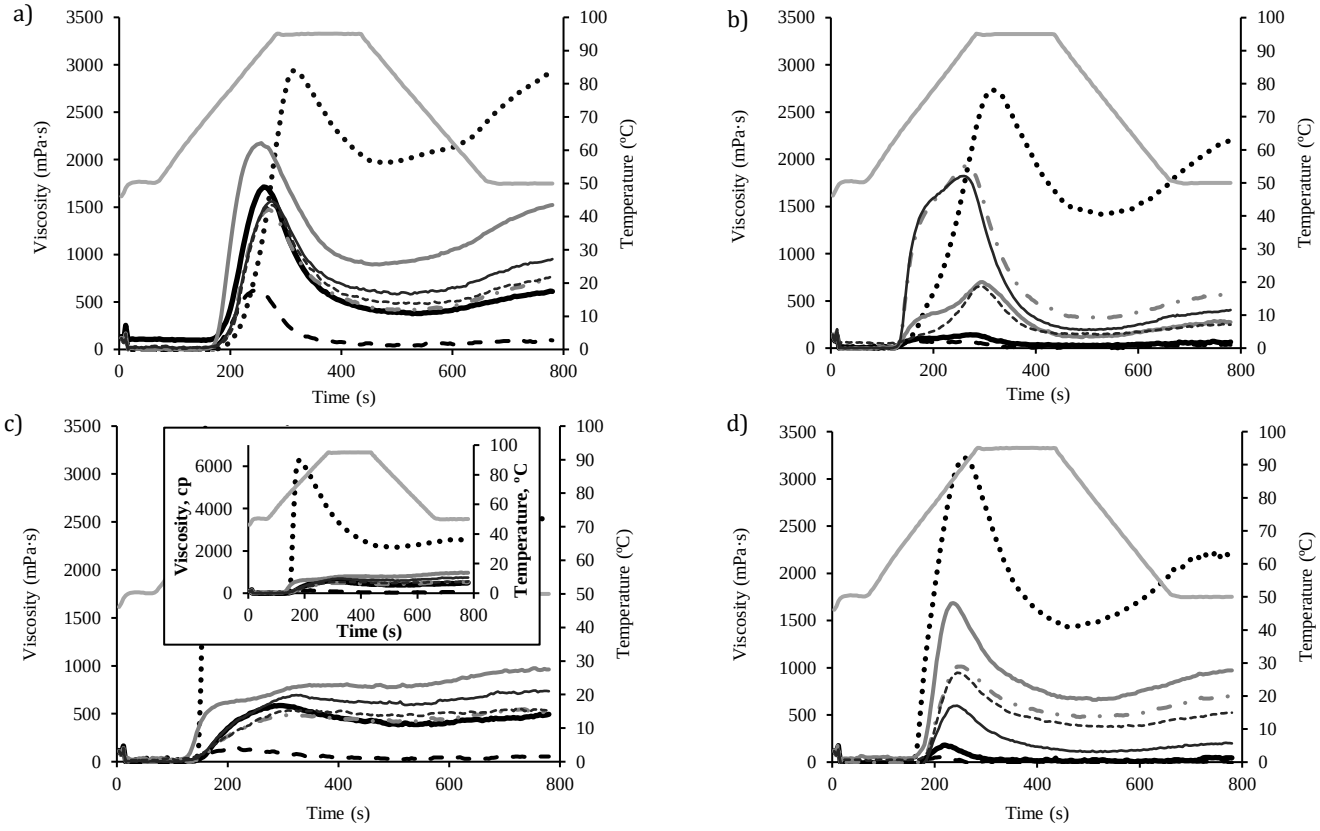

Figure 3. Effect of acidification and protein fortification on viscometric profiles of bread doughs made from corn (a), wheat (b), potato (c) and tapioca (d) starches. Doughs without protein are represented by ——, with 5% calcium caseinate by ——, and with 5% soy protein isolate by ——. Doughs with acid addition are represented by – – –, with 5% calcium caseinate acidified by – · –, and with 5% soy protein isolate acidified by -------. The ••••••• lines represent the viscometric profiles of aqueous starch dispersions with a dry matter content identical to that of the dough dispersion. The temperature profile is represented by —— in the second axis.

4. Conclusions

Acidification and protein supplementation modified the rheological and pasting properties of GF bread doughs. Those effects varied according to both the starch source and type of protein used as raw materials and the presence/absence of acid. In general, potato starch doughs revealed the most significant results. The incorporation of protein strengthened the dough, being structuring especially significant in the case of CA addition to potato, tapioca and wheat starch doughs, showing higher τmax values. However, the effect of protein on viscoelastic moduli depended on the type of protein and starch source. The acidification resulted in a weakening of the dough matrices structure. Creep-recovery test made in and outside the LVR revealed that the addition of protein decreased notably the values of maximum compliance compared to control doughs without protein, showing higher values with the addition of CA than SPI. In general, acid incorporation increased the values of compliance for all starches (enriched or not with proteins) in and outside the LVR, which indicates a greater capacity of deformation of the doughs to a given stress. Protein presence increased the pasting profiles, but with differences between the two proteins studied. The results of the present study can contribute to generating new knowledge and therefore the development and increase of the GF baked products quality to broaden the food product choices for GF products consumers. Additional studies are still required for extensive evaluation of the effect of acidification on these matrices and its applicability on the breadmaking process.

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CAPÍTULO II

CHAPTER II

EFECTO DE LA ACIDIFICACIÓN SOBRE LA VISCOELASTICIDAD DE MASAS DE ALMIDÓN DE ARROZ SIN GLUTEN ENRIQUECIDAS CON PROTEINAS EXÓGENAS

Ronda, F., Villanueva, M., Collar, C. 2014. Influence of acidification on dough viscoelasticity of gluten-free rice starch-based dough matrices enriched with exogenous protein. LWT – Food Science and Technology, 99, 12-20.

Influence of acidification on dough viscoelasticity of gluten-free rice starch-based dough matrices enriched with exogenous proteins

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Abstract

The impact of acid incorporation (acetic+lactic, 0.5%) into rice starch-based doughs enriched with different proteins (egg albumin, calcium caseinate, pea protein and soy protein isolates) at different doses (0, 5 and 10%) has been investigated on dough viscoelastic and pasting profiles. Oscillatory (stress and frequency sweeps) and creep-recovery tests were used to characterise the fundamental viscoelastic behaviour of the doughs, and thermomechanical assays were performed to assess dough viscometric performance. Supplementation of gluten-free doughs with proteins from vegetal sources led to more structured dough matrices (higher viscoelastic moduli and steady viscosities, and lower tan δ, instantaneous and retarded elastic compliances) effect being magnified with protein dose. Acid addition decreased these effects. Incorporation of proteins from animal source resulted in different viscoelastic behaviours according to the protein type, dosage and acidification, especially for casein. Acidification conferred lower dough deformation and notably higher steady viscosity and viscoelastic moduli for 5 %-casein-added dough. Protein-acid interaction favoured higher viscosity profiles, particularly for doughs with proteins of vegetable origin and lower dosage. Dough acidification decreased the pasting temperatures and the amylose retrogradation. Acidification of protein-enriched rice-starch doughs allowed manipulation of its viscometric

and rheological properties which is of relevant importance in gluten-free bread development.

Keywords: Acetic acid; Gluten-Free Doughs; Lactic acid; Proteins; Rheology

Abbreviations:

a: Exponent from fitting power law to G' data

b: Exponent from fitting power law to G'' data

BD: Breakdown viscosity

c: Exponent from fitting power law to $tan\ \delta$ data

FV: Final Viscosity

$G_1^{'}$: Elastic modulus at a frequency of 1 Hz obtained from fitting power law to G' data

$G_1^{''}$:Viscous modulus at a frequency of 1 Hz obtained from fitting power law to G'' data

J_{0c}: Instantaneous compliance obtained from creep test

J_{0r}: Instantaneous compliance obtained from recovery phase

J_{1c}: Retarded compliance obtained from creep test

J_{1r}: Retarded compliance obtained from the recovery phase.

LVR: Linear Viscoelastic Region

λ_{1c}: Retardation time in the creep phase

λ_{1r}: Retardation time in the recovery phase

μ_0: Steady state viscosity

PV: Peak Viscosity

PT: Pasting Temperature

SB: Setback

$(tan\ \delta)_1$: Loss tangent at a frequency of 1 Hz obtained from fitting power low to $tan\ \delta$ data

TV: Trough Viscosity

ω: Oscillation Frequency

1. Introduction

Gluten-free (GF) products are a growing sector in the food industry, and the related research constitutes a prioritised and challenging topic in cereal-based goods area. The development of new GF products is emerging not only because daily dietary requirements for essential nutrients of celiac disease patients are not fully covered at present by existing products (Mandala & Kapsokefalou, 2011). The target group of GF products is currently expanding to adhere join, in addition to celiac patients (1-3% of the population), people looking for nonallergenic ingredients, leading to a new market that needs a variety of products. Also, GF products can function as prototypes/templates for the development of other products addressed to specific vulnerable groups of population with special nutritional needs (e.g., diabetics). GF product approaches include: (1) reformulations (e.g., high-fiber gluten-free versions of traditional antecedents), (2) new forms of existing products (e.g., frozen and part-baked), (3) repackaging of existing products, and (4) innovative products (e.g., use of novel cereals) (Kelly, Moore, Elke, & Arendt, 2008). Concerning the first approach, complex formulations that appear promising in terms of technological improvement and nutritional quality have been developed so far, with variable success/failure regarding sensory appreciation and technological constraints. The formulations mainly involve the incorporation of starches of different origin, other non-gluten proteins such as dairy proteins, gums, and their combinations (Mariotti, Lucisano, Pagani, & Ng, 2009). These ingredients can mimic the viscoelastic properties of gluten and may result in improved structure, mouthfeel, acceptability, and shelf life of these products (Gallagher, Gonnley, & Arendt, 2004).

Rice flour is considered one of the most suitable cereal flour for preparing gluten-free products associated to its several significant properties such as natural, hypoallergenic, colorless, and bland taste. It has also very low level of protein, sodium, fat, fiber and high amount of easily digested carbohydrates.

Since most of the rice contain relatively small amount of prolamin (2.5–3.5%) (Gujral & Rosell, 2004), it is necessary to use some sort of gum, emulsifier, enzymes or dairy products together with rice flour for achieving desired viscoelastic mixture (Demirkesen, Mert, Sumnu, & Sahin, 2010). Gum type additives, such as hydroxy propyl methyl cellulose (HPMC) (Sivaramakrishnan, Senge, & Chattopadhyay, 2004) and the enzyme glucose oxidase (Gujral & Rosell, 2004) resulted in successful formation of rice bread showing the optimum volume expansion and a general improvement of bread quality, respectively (Nikolić, Dodić, Mitrović, & Lazić, 2011). Proteins from different sources can be added to increase both nutritional and functional values of GF products. Protein incorporation leads to the formation of a continuous protein phase (Moore, Tilman, Dockery, & Arendt, 2004), and are added to GF applications (Crockett, Ie, & Vodovotz, 2011) to increase elastic modulus by cross linking, to improve perceived quality by enhancing Maillard browning and flavour, to improve structure with gelation and to aid in foaming (Moore, Dal Bello, & Arendt, 2008). These result in bread with increased loaf volume, improved crumb regularity and improved sensory characteristics (Moore et al., 2008). The use of dairy powder in gluten-free baked product formulations has resulted in improved volume as well as better appearance and sensory aspects of the loaves (Gallagher et al., 2004). Soy protein isolate and dried egg white solids were investigated due to their foam-stabilizing activity and use in GF applications (Marco & Rosell, 2008; Moore et al., 2004). According to Stathopoulos (2008), the most used ingredients in gluten-free baked product formulations are caseinates, skim milk powder, dry milk, whey protein concentrate and milk protein isolate. It follows that the selection of the proteins used in a gluten-free formulation is a critical issue (Mandala & Kapsokefalou, 2011). Soybean protein isolates increases the nutritional value of rice cassava bread and increases elastic modulus, resulting in enhanced gas retention and loaf volume, and improves water binding in the bread loaves. Other authors stated that the addition of soybean protein isolate to an HPMC-treated rice cassava bread reduced dough stability by suppressing HPMC functionality, altering water distribution within the dough, weakening HPMC interactions with the starch matrix and reducing foam stability (Crockett et al., 2011). Green pea protein has been used in less extent than the soybean protein in GF breads evidencing also an increase in the elastic modulus (Marco & Rosell, 2008). Acetic and lactic acids confer suitable properties to final breads in terms of odour and taste either

when produced by the exogenous microflora or added to breadmaking matrices, increasing in addition protease and amylase activities that lead to a retarded staling during storage (Moore et al., 2008).

The combined effect of acid addition and protein supplementation in GF matrices has not been described so far despite inter and intra-molecular interactions established between exogenous proteins and starch molecules that are the main responsible for dough structurization, certainly depend on dough pH. In addition, despite several rheological techniques, including oscillation, stress relaxation, creep and creep-recovery measurements have been used extensively for assessing fundamental mechanical properties of gluten, the use of dynamic rheometry in studies of GF-dough rheological behavior has only been applied over the last decade (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007; Ronda, Pérez-Quirce, Angioloni, & Collar, 2013). Fundamental and empirical rheological properties of doughs inform about interactions among ingredients and the creation of structure at macromolecular and macroscopic levels, respectively. In addition, quality attributes of breads such as volume and texture can be correlated with dough rheological properties (Sahin, 2008; Pérez-Quirce, Collar, & Ronda, 2014).

This paper is intended to know the impact of acid incorporation (acetic:lactic, 0.1:0.4 g/100 g starch+protein basis) into GF rice starch-based dough matrices enriched with different proteins (egg albumin, calcium caseinate, pea protein and soy protein isolates) at different doses on dough viscoelastic, and pasting profiles, prior to assess comparatively the structure promoting ability in GF matrices of exogenous proteins in absence/presence of acid.

2. Material and methods

2.1. Materials

Rice starch (9.9 % moisture, 0.2 % ash and 0.5 % protein) from Ferrer Alimentación S.A. (Barcelona, Spain), and salt, sugar (Azucarera Ebro, Spain) and sunflower oil (branded Coosur Premium) purchased from the local market, were used to make gluten-free doughs. Hydroxypropylmethylcellulose (HPMC, Methocel K4M Food Grade) was provided by Dow Chemical (Midland, EEUU). Proteins used in gluten-free formulations were: soybean isolate Supro 500-E IP from Proveedora hispano-holandesa S.A. (Barcelona, Spain), calcium caseinate from Armor proteines (Saint-Brice-en-Coglès, France), egg albumin in dry

powder from Eurovo (Valladolid, Spain) and pea protein isolate branded Pisane C9, from Cosucra (Warcoing, Belgium). Acetic acid and lactic acid (analytical grade; Panreac, Barcelona) were used as a source of hydrogen ions.

2.2. Methods

Dough preparation

A straight dough process was performed using the following formula on a 100 g rice starch (or rice starch+protein) basis: 6 g oil, 5 g sucrose, 1.5 g salt, 2 g HPMC and 80 g water**.** All proteins were added at 0, 5 and 10 g/100 g levels. Doughs were supplemented with (0.1 + 0.4) g/100 g of acetic and lactic acid, respectively, when acid-treatment was applied. The experimental design is shown in Table 1.

Table 1. Experimental design

Formula	Protein				Acetic/Lactic acid
	CA	EA	SPI	PPI	
1	0	10	0	0	0.1/0.4
2	0	5	0	0	0
3	0	0	10	0	0
4	0	0	5	0	0
5	0	10	0	0	0
6	0	5	0	0	0.1/0.4
7	0	0	0	0	0
8	0	0	5	0	0.1/0.4
9	0	0	0	5	0
10	0	0	0	10	0.1/0.4
11	0	0	0	0	0.1/0.4
12	10	0	0	0	0.1/0.4
13	5	0	0	0	0
14	5	0	0	0	0.1/0.4
15	0	0	0	5	0.1/0.4
16	0	0	10	0	0.1/0.4
17	0	0	0	10	0
18	10	0	0	0	0

CA: Calcium Caseinate; EA: Egg Albumin;
SPI: Soya Protein Isolate; PPI: Pea Protein Isolate
Amounts are in % w/w, starch +protein basis

GF dough-making was achieved by blending first solid ingredients and oil in a kitchen-aid professional mixer (KPM5). Then water was added and hand mixed. Finally the dough was mixed with dough hook at a speed 4 for 8 min. Acid blend, when added, was diluted in a small part of water (7 % of total) and adjusted to the dough before the mixer was powered on.

2.3. Dough measurements

Oscillatory and creep recovery tests

Oscillatory and creep-recovery tests were carried out with a RheoStress 1 rheometer (Thermo Haake, Karlsruhe, Germany) with parallel plate geometry (60 mm diameter) of serrated surface and with 3 mm gap. The excess of batter was removed and vaseline oil was applied to cover the exposed sample surfaces. Before the measurement, the batter was rested for 10 min to allow relaxation. Frequency sweeps were carried out from 20 to 0.1 Hz in the linear viscoelastic region (LVR) previously established for each batter by means of stress sweeps from 0.1 to 1000 Pa at 1 Hz. The frequency sweeps of all batters were carried out at stress values between 2 Pa and 10 Pa. Temperature was 25 ºC. Frequency sweep data were fitted to the power law model as in previous works (Ronda et al., 2013):

$$G'(\omega) = G_1' \cdot \omega^a \; ; \; G''(\omega) = G_1'' \cdot \omega^b \; ;$$

$$\tan\ \delta(\omega) = \frac{G''(\omega)}{G'(\omega)} = \left(\frac{G''}{G'}\right)_1 \cdot \omega^{(b-a)} = (\tan\ \delta)_1 \cdot \omega^c$$

The coefficients G_1', G_1'', and $(\tan \delta)_1$, represent the elastic and viscous moduli and the loss tangent at a frequency of 1 Hz. The a, b and c exponents quantify the dependence degree of dynamic moduli and the loss tangent with the oscillation frequency, ω. Creep tests were performed by imposing a sudden step shear stress in the LVR for 150 s. In the recovery phase the stress was suddenly removed and the sample was allowed for 300 s to recover the elastic (instantaneous and retarded) part of the deformation. Each test was performed in triplicate. The data from creep tests were modelled to the 4-parameter Burgers model (Lazaridou et al., 2007) given by:

$$J_c(t) = J_{0c} + J_{1c}\left(1 - \exp\left(\frac{-t}{\lambda_{1c}}\right)\right) + \frac{t}{\mu_0}$$

In the equation, J_c (t) is the creep compliance (strain divided by stress), J_{0c} is the instantaneous compliance, J_{1c} is the retarded elastic compliance or viscoelastic compliances, λ_{1c} is the retardation time and μ_0 gives information about the steady state viscosity. Similar equations were used for the recovery compliance $J_r(t)$. As there is no viscous flow in the recovery phase, equations consist only of parameters describing the elastic response after removal of the shear stress. The data from creep tests were modelled to the 3-parameter Burgers model given by:

$$J_r(t) = J_{\max} - J_{0r} - J_{1r}\left(1 - \exp\left(\frac{-t}{\lambda_{1r}}\right)\right)$$

J_{max} is the maximum creep compliance obtained at the end of the creep step.

Thermoviscous test: Viscometric profile

Viscometric profiles (gelatinization, pasting, and setback properties) of formulated starch rice doughs were obtained with a Rapid Visco Analyser (RVA-4, Newport Scientific, Warriewood, Australia) using ICC Standard 162. Freeze-dried hydrated samples (3.5 g, 14 % moisture basis) were transferred into canisters and ≈25 ± 0.1 mL of distilled water were added and processed following standard method. The pasting temperature (PT), peak time (when peak viscosity occurred) (VT), peak viscosity (PV), holding strength or trough viscosity (TV), breakdown (BD), final viscosity (FV) and setback (final viscosity minus peak viscosity) (SB) were calculated from the pasting curve (Collar, 2003) using Thermocline v. 2.2 software. For each viscometric measurement, 3 samples were used.

2.4. Statistical analysis

Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) was used for multivariate non-linear regression and Pearson correlation matrix. STATISTICA package (Tulsa, OK, EEUU) v.6, allowed performance of MANOVA analysis, and LSD (Least Significant Difference) test was used to evaluate significant differences ($p<0.05$) between samples.

3. Results and discussion

Table 2 and 3 show the single and 2nd order interactive effects of protein and acid addition on pH and rheological and pasting properties of GF doughs. Protein

presence increased the dough pH between 7 % and 12 % with respect to the control dough, depending on the dose. The lower increase was obtained with albumin. The acidification of protein-enriched doughs resulted in pH values 15 % – 34 % higher than the acid-added control dough.

3.1. Fundamental rheology

Dynamic oscillatory rheology

Protein–enriched rice starch-based doughs were submitted to both stress and frequency sweeps in the linear visco-elastic region (LVR), which oscillatory rheological behaviour for selected samples is illustrated in Figures 1.a. and 1.b., respectively. Stress sweep tests allowed to know the maximum stress (τ_{max}) that GF matrices can tolerate in the LVR -from 6 to 108 Pa- providing structure preservation. Lower τ_{max} values corresponded to control samples without protein addition and to albumin-enriched samples regardless either acid or protein level addition, whereas higher τ_{max} were obtained for no acid/10 g/100 g pea protein or 10 g/100 g soya protein enrichment and for acid/10 g/100 g casein incorporation. Except for the egg albumin, the presence of protein encompassed a significant ($p<0.01$) increase in τ_{max} values as a result of dough structurization. Increased dosage from 5 to 10 g/100 g promoted τ_{max} for doughs enriched with vegetal proteins (+63 % soya, +89 % pea), whereas only acidified matrices containing casein underwent a relevant structure promotion with protein dose (+160 %). For vegetal proteins, dough acidification led to a weakening effect regardless the dose, and consequently to a decrease in $\tau_{max,}$ more prominent in pea enriched samples (-54 %) than in soya samples (-37 %). Samples supplemented with casein proteins observed a strengthening effect in acid medium when added at 10 g/100 g (+37 %), but underwent weakening impact with acid addition when added at 5 g/100 g to the doughs (-47 %).

Frequency sweep tests of unacidified and acidified 5 g/100 g casein added dough matrices are illustrated in Figure 1.b. Viscoelastic behaviour of dough samples corresponded with no exception to solid-like samples with storage modulus values (G'_1) higher (from 2568 to 70665 Pa) than loss modulus values (G''_1) (from 477 to 10465 Pa), slight frequency dependence, and values for tan δ (G''/G') under 1, in good accordance with earlier results found for rice doughs enriched with protein isolates (Gujral & Rosell, 2004). In this work, protein addition affected dough viscoelasticity, the extent of the changes being

dependent on the type and the dose of protein and on the absence/presence of acid (Table 2), and on the interactive effects of protein x acid (Table 3).

Table 2. Single effects of design factors at different levels on the dynamic oscillatory, creep-recovery, visco-metric parameters and pH of protein-enriched rice starch-based gluten-free doughs.

Parameter	Unit	Overall Mean	Level	Egg albumin	Calcium caseinate	Isolated pea protein	Isolated soya protein	Acid
Dynamic Oscillatory Rheometry								
G'_1	Pa	21858	0	14382b	14382a	14382a	14382a	ns
			1	3945a	19330a	17987a	23138a	
			2	3042a	28690b	37600b	56313b	
G''_1	Pa	4152	0	2738b	2738a	2738a	2738a	ns
			1	888a	5443b	3519b	4104a	
			2	603a	9146c	6254c	7952b	
tan δ		0.2070	0	0.19a	0.19a	0.19b	0.19b	ns
			1	0.23b	0.31b	0.20b	0.18ab	
			2	0.20a	0.32b	0.17a	0.15a	
Creep recovery test								
J_{oc}	10^{-4} Pa^{-1}	1.19	0	0.99a	0.99b	0.99c	0.99c	ns
			1	2.70b	0.81b	0.64b	0.55b	
			2	3.84c	0.53a	0.34a	0.30a	
J_{1c}	10^{-4} Pa^{-1}	1.14	0	0.54a	ns	0.54c	0.54b	ns
			1	2.64b		0.41b	0.33a	
			2	3.44c		0.24a	0.25a	
λ_{1c}	s	20	0	17a	17a	17a	17a	22.37b
			1	21b	18a	18b	29b	18.57a
			2	17a	21b	20c	24ab	
μ_0	10^6 Pa·s	3.69	0	4.33b	4.33b	4.33a	ns	ns
			1	0.63a	3.02ab	3.34a		
			2	0.40a	0.86a	5.50b		
J_{or}	10^{-4} Pa^{-1}	1.45	0	1.14a	ns	1.14c	1.14c	ns
			1	3.23b		0.71b	0.59b	
			2	4.67c		0.39a	0.36a	
J_{1r}	10^{-4} Pa^{-1}	1.16	0	0.90a	ns	0.90c	0.90b	ns
			1	2.26b		0.42b	0.35a	
			2	3.17c		0.27a	0.25a	
λ_{1r}	s	77	0	121b	121b	121b	121b	ns
			1	65a	97ab	60a	65a	
			2	63a	69a	72a	83a	

Table 2. *Continued*

Parameter	Unit	Overall Mean	Level	Egg albumin	Calcium caseinate	Isolated pea protein	Isolated soya protein	Acid
Viscometric profile								
PV	mPa.s	2340	0	ns	2860c	2860c	2860b	ns
			1		2222b	2426b	2518a	
			2		1665a	2035a	2294a	
TV	mPa.s	1804	0	2429b	2429c	2429c	2429c	ns
			1	2225ab	1821b	1679b	1955b	
			2	1986a	1377a	1216a	1545a	
FV	mPa.s	2683	0	ns	3228c	2429c	3228b	ns
			1		2655b	1679b	2822ab	
			2		2164a	1216a	2349a	
PT	ºC	81.54	0	ns	79.31a	79.31a	79.31a	82.87b
			1		83.94b	77.78a	82.03b	80.21a
			2		87.30c	79.63a	83.69b	
pH of the medium								
pH		5.20	0	4.54a	4.54a	4.54a	4.54a	5.73b
			1	5.08b	5.22b	5.23b	5.16b	4.73a
			2	5.27c	5.47c	5.53c	5.43ab	

Levels: 0, absence; 1, 5% protein addition (starch + protein basis) or acetic/lactic acid addition (0.1/0.4, w/w, starch + protein basis); 2, 10% protein addition (starch + protein basis). ns: non significant effects $p>0.05$. Within each parameter, different letters in the corresponding column mean statistically differences between means at $p<0.05$. Abbreviations used for the measured parameters are presented in the materials and methods section.

Interactions between starch and proteins depend upon the molecular structure of protein, the starch: state of the granules and the amylose/amylopectin ratio, the composition of protein and starch, as well as the phase transition temperatures of starch gelatinization and protein denaturation. There is also an electrostatic association between the two polymers. Anionic polysaccharide and protein are incompatible at pH values above the protein's isoelectric point (point of minimum solubility, pH ~ 5.1) and completely compatible below it due to the net opposite charges they carry (Rao, 2007). Factors affecting protein-polysaccharide compatibility and the characteristics of their complexes include the molecular characteristics of the two molecules (e.g., molecular weight, net charge, and chain flexibility), the pH, ionic strength, temperature, the protein/polysaccharide ratio, rate of acidification, and rate of shear during acidification (Rao, 2007). Vegetal proteins significantly increased ($p<0.01$) both the elastic and viscous components in doughs (Table 2), increments being larger in soya protein samples (+143 % G', +94 % G'') than in pea protein matrices

(+109 % G', +78 % G'') by increasing the dose from 5 to 10 g/100 g, starch-protein basis. Acid addition modulated dough viscoelasticity in soya protein matrices at higher dose, so that a weakening effect denoted by a significant drop in G' (-61 %) and G'' (-40 %) with a concomitant increase in tan δ (+52 %) was observed (Table 3). Animal proteins significantly modified mechanical spectra of protein-enriched matrices depending on the type of protein, when compared to both unacidified and acidified control doughs. Casein addition observed a dependence on the frequency for both dynamic moduli (Figure 1.b), a higher consistency than the control and albumin enriched samples, but a lower predominance of G'_1 over G''_1, (higher tan δ values) compatible with a more viscous nature (Table 2). The acidification of casein supplemented samples increased G' (+52 %) when added at 5 g/100 g and decreased G'' depending on the dose of addition (-34 % at 5 g/100 g, -25 % at 10 g/100 g) (Table 3). Doughs enriched with albumin exhibited a different behaviour with lower mechanical spectra profiles than unsupplemented protein-samples, regardless the dose of addition and the absence/presence of acid (Table 2 and Table 3). Slight dependence of the moduli on angular frequency (a and b values ranged 0.11-0.28) and values of phase shift tangent (tan δ) varying in the range $0.1 < \tan \delta < 0.4$ are both characteristic features for the systems which so called weak gels (elastic behaviour). This is in agreement with earlier observations regarding viscoelastic properties of GF dough (Witczak, Korus, Ziobro, & Juszczak, 2010). Significant variation in dough viscoelastic moduli was also observed by Nunes, Ryan, and Arendt (2009) who supplemented GF bakery products with milk and whey proteins. In the case of albumin a significant decrease of G' and G'' was accompanied with a slight, but statistically significant increase of phase shift tangent when added at 5 g/100 g. All other protein preparations caused significant increase of moduli G' and G'' (Table 2). Although the addition of pea protein resulted in a significant growth of G' and G'', it caused only a slight shift of phase shift tangent in the range of low frequencies, in accordance with previous reports (Ziobro, Witczak, Juszczak, & Korusa, 2013).

Table 3. Selected second order interactive effects (protein x acid) on the dynamic oscillatory, creep-recovery, visco-metric parameters and pH of protein-enriched rice starch-based gluten-free doughs

Parameter	Unit	Overall Mean	Level protein	Level Acid	Albumin X Acid	Caseinate X Acid	Pea protein X Acid	Soya protein X Acid
Dynamic Oscillatory Rheometry								
G'_1	Pa	21858	0	0	ns	15763a	ns	15763a
			0	1		11620a		11620a
			1	0		15360a		27920b
			1	1		23300b		20748ab
			2	0		30480c		70665c
			2	1		26900bc		27610b
G''_1	Pa	4152	0	0	ns	2852a	ns	2852a
			0	1		2511a		2511a
			1	0		6568c		4903c
			1	1		4317b		3705b
			2	0		10465e		9184d
			2	1		7826d		5487c
tan δ		0.2070	0	0	ns	0.18a	ns	0.18b
			0	1		0.22b		0.22c
			1	0		0.43e		0.18b
			1	1		0.19a		0.18b
			2	0		0.34d		0.13a
			2	1		0.29c		0.20bc
Creep recovery test								
J_{oc}	10^{-4} Pa^{-1}	1.19	0	0	0.88a	0.88c	0.88e	0.88e
			0	1	1.10b	1.10e	1.10f	1.10f
			1	0	2.45c	1.05d	0.57c	0.47c
			1	1	2.96d	0.57b	0.71d	0.62d
			2	0	4.52f	0.54ab	0.31a	0.19a
			2	1	3.16e	0.51a	0.36b	0.42b
J_{1c}	10^{-4} Pa^{-1}	1.14	0	0	0.44a	0.44b	0.44d	0.44d
			0	1	0.65b	0.65c	0.65e	0.65e
			1	0	2.69d	2.38f	0.40c	0.29b
			1	1	2.60cd	0.37a	0.43d	0.38c
			2	0	4.33e	1.27e	0.23a	0.12a
			2	1	2.55c	0.85d	0.26b	0.39c
λ_{1c}	s	20	0	0	16a	ns	ns	16a
			0	1	17ab			17ab
			1	0	22c			40e
			1	1	19b			18b
			2	0	19b			25d
			2	1	16a			22c
μ_0	10^6 Pa·s	3.69	0	0	5.79f	5.79f	5.79f	5.79d
			0	1	2.87e	2.87d	2.87a	2.87a
			1	0	0.67d	0.56a	3.08b	9.09e
			1	1	0.60c	5.47e	3.61c	3.68b
			2	0	0.33a	0.62b	5.52e	13.6f
			2	1	0.47b	1.10c	5.48d	3.85c
J_{or}	10^{-4} Pa^{-1}	1.45	0	0	0.97a	0.97d	0.97e	0.97e
			0	1	1.30b	1.30e	1.30f	1.30f
			1	0	2.96c	1.74f	0.67c	0.49b

			1	1	3.50d	0.67a	0.75d	0.69d
			2	0	5.39f	0.84c	0.37a	0.20a
			2	1	3.94e	0.74b	0.41b	0.52c
J_{1r}	10^{-4} Pa^{-1}	1.16	0	0	1.02b	1.02d	1.02d	1.02e
			0	1	0.78a	0.78b	0.78c	0.78d
			1	0	1.83c	2.66f	0.41b	0.31b
			1	1	2.68d	0.66a	0.43b	0.39c
			2	0	3.51f	1.37d	0.28a	0.13a
			2	1	2.83e	0.92c	0.25a	0.37c
λ_{1r}	s	77	0	0	153f	153f	153f	153e
			0	1	888e	88d	88e	88c
			1	0	50a	69b	66c	63a
			1	1	80d	124e	53a	67b
			2	0	52b	62a	83d	100d
			2	1	75c	75c	60b	67b
Viscometric profile								
PV	mPa.s	2340	0	0	3091e	3091f	3091d	ns
			0	1	2628c	2629e	2629c	
			1	0	2384b	1990c	2239b	
			1	1	2852d	2454d	2612c	
			2	0	2086a	1536a	1869a	
			2	1	2770cd	1794b	2200b	
TV	mPa.s	1804	0	0	2771d	2771e	2771f	2771d
			0	1	2087b	2087d	2087e	2087c
			1	0	2158bc	1615c	1455c	2008bc
			1	1	2291c	2028d	1903d	1902b
			2	0	1876a	1275a	1151a	1581a
			2	1	2095b	1479b	1281b	1509a
FV	mPa.s	2683	0	0	3783d	3783e	3783e	3783d
			0	1	2672a	2672d	2672d	2672b
			1	0	3401c	2558c	2521c	2986c
			1	1	3128b	2752d	2593cd	2658b
			2	0	3077b	2068a	2227b	2518b
			2	1	2955b	2259b	1952a	2181a
PT	ºC	81.54	0	0	80.23b	80.23b	80.23c	80.23ab
			0	1	78.38a	78.38a	78.38b	78.38a
			1	0	81.05b	86.03d	81.30c	83.52c
			1	1	78.10a	81.85c	74.27a	80.53b
			2	0	83.57c	88.10e	79.00b	83.05c
			2	1	77.63a	86.50d	80.27c	84.33c
pH of the medium								
pH		5.20	0	0	5.21d	5.21d	5.21c	5.21d
			0	1	3.88a	3.88a	3.88a	3.88a
			1	0	5.56e	5.71e	5.73d	5.68e
			1	1	4.46b	4.73b	4.72b	4.64b
			2	0	5.73f	5.84ef	5.85de	5.82ef
			2	1	4.80c	5.10cd	5.20c	5.03c

Levels: 0, absence; 1, 5% protein addition (starch + protein basis) or acetic/lactic acid addition (0.1/0.4, w/w, starch + protein basis); 2, 10% protein addition (starch + protein basis). ns: non significant effects $p>0.05$. Within each parameter, different letters in the corresponding column mean statistically differences between means at $p<0.05$. Abbreviations used for the measured parameters are presented in the materials and methods section.

In oscillatory studies, Crockett et al. (2011) observed an increase of storage modulus accompanied by the drop in phase shift tangent of the dough supplemented with soy protein isolate, which was potentially due to protein aggregation within the medium. The application of casein significantly modified rheological image of dough structure, shifting its properties toward values typical for strong gels, probably caused by its special arrangement, in which regularly occurring amino acid sequence favoured the formation of tight polypeptide-strands stabilized by covalent and hydrogen bonds, as described for collagen (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). Current results in agreement with previous studies (Crockett et al., 2011; Ziobro et al., 2013) are compatible with the creation of a robust crosslinked structure by added proteins, especially supported in the case of soya protein by glicinin and a high water retention ability (Crockett et al., 2011). In studies using acid in rice flour based doughs, chemical acidification encompassed a dough softening effect highly dependent on both the final dough pH and the type of acid (Blanco, Ronda, Pérez, & Pando, 2011). Some authors have reported an increase in wheat flour dough stiffness (viscosity or complex shear modulus) with decreasing pH in the range 6-5.6 to 4 (Jekle & Becker, 2012) probably as result of the change in the conformation of the proteins. The decreased pH would lead to the change in the overall net charge from neutral (near the isoelectronic point) to positive. A neutral charge causes less repulsion forces and less space for water molecules between the proteins. This repulsion forces increase with increasing charge and more water molecules can be attached to the protein strands whereby less mobile water is available in the dough system (Jekle & Becker, 2012).

Creep-recovery tests

Creep-recovery tests were also conducted on formulated GF doughs. Stress applied in the LVR ranged from 2 Pa to 10 Pa, and were maintained for 150 s, sufficient for the sample to reach the steady-state flow. Creep-recovery curves of GF doughs exhibited a typical viscoelastic behaviour combining both viscous fluid and elastic components (Figure 1.c), similar to the corresponding curves obtained previously for rice flour (Sivaramakrishnan et al., 2004) and other gluten-free doughs (Lazaridou et al., 2007; Ronda et al., 2013).

Creep parameters for all GF dough formulations are summarized in Table 2. Major impact on creep-recovery parameters was associated to vegetal proteins

and albumin incorporation. Increased vegetal protein incorporation led to significantly lower instantaneous (J_0) and retarded (J_1) elastic compliance in both creep and recovery phases associated to a lower dough deformation submitted to a constant stress, and a higher recovery when stress is removed, respectively. Maximum depletion in compliance values was observed for soya protein enriched matrices at 10 g/100 g of addition: -70 % (J_{0c}), -54 % (J_{1c}), -70 % (J_{0r}), -72 % (J_{1r}). For animal protein supplemented doughs, albumin incorporation notably promoted J values compared to control doughs, increases being magnified with protein dosage; whereas casein inclusion in dough formulation only affected J0c when added at 10 g/100 g, encompassing a 40 % decrease in values (Table 2).

Addition of protein from both animal and vegetal source encompassed higher retardation times in the creep phase (λ_{1c}) and lower retardation times in the recovery phase (λ_{1r}), indicating a slower and quicker retarded elastic response, respectively (Table 2). pH decrease as a result of acidification significantly affected major creep-recovery parameters (Table 3). In unacidified doughs, J_{1c} values were higher in presence of animal proteins but similar or even 50-60 % lower in presence of vegetal proteins, in accordance with a higher deformation at a constant stress with time for animal proteins encompassing a lower dough consistency. Dough acidification led to a decrease in J_{1c} when albumin or casein was incorporated while for vegetal protein addition, the opposite effect was observed. Protein addition to unacidified matrices significantly increased values of λ_{1c} except for doughs supplemented with 5 g/100 g pea protein.

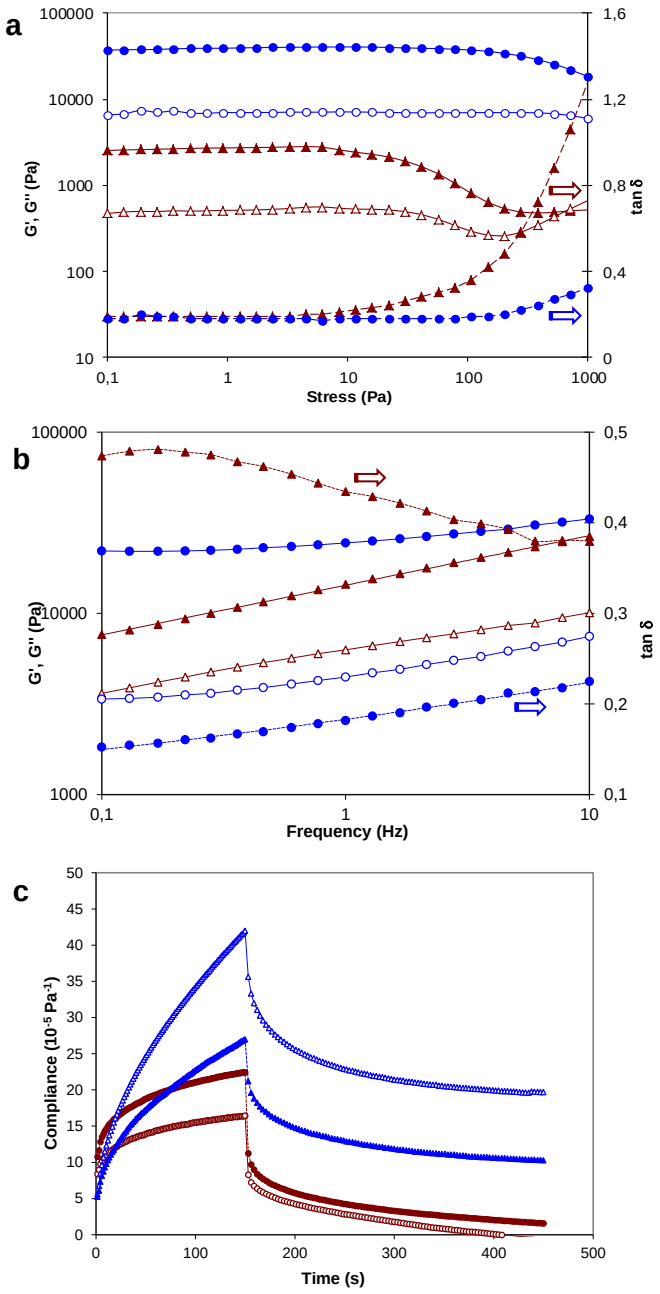

Figure 1. Curves of dough stress sweep with 10 g/100 g albumin (triangle) and pea (circle) (a), dough frequency sweep with 5 g/100 g casein without acid (triangle) and with acid (circle) (b) and creep-recovery (c) tests of control (circle) and casein 10 g/100 g (triangle), both without acid (continuous lines) and with acid (discontinuous lines) dough. Elastic modulus, G', is represented by solid points and the viscous modulus, G'', by void points. The loss tangent is represented by discontinuous lines in the secondary (right) scale.

Acidification induced longer λ_{1c} with respect to control doughs only in doughs formulated with casein, pea protein or soya protein added at 10 g/100 g (Table 3). Viscosity at steady state (μ_0) marked increased with soya protein addition although decreased with the remaining proteins. It decreased notably with dough acidification in soya protein presence (-67 % for 5 g/100 g and -72 % for 10 g/100 g) and slightly, but significantly, in presence of 10 g/100 g pea protein and 5 g/100 g albumin. Rice starch control dough also showed a decreased viscosity at lower pH. Doughs with 10 g/100 g albumin, 5 or 10 casein or 5 g/100 g pea protein observed the opposite trend. In acidified doughs, vegetal protein incorporation led to increased values for μ_0 while animal proteins, except casein at 5 g/100 g dose, encompassed a significant decrease (Table 3). As it was established for cake batters (Sahi and Alava, 2003) there is probably an optimum consistency for gluten-free doughs, more similar to batters than to wheat doughs, to achieve breads of high volume. A proper consistency, with high enough G' and G'' moduli and viscosity, μ_0, helps to hold the carbon dioxide produced during fermentation. Too strength doughs, with too low J_0 and J_1 compliances, can restrict dough expansion and lead to less developed breads (Pérez-Quirce et al., 2014).

3.2. Visco-metric profile

Impact of protein addition and acidification (Table 2) and interactive effects of protein x acid (Table 3) on the RVA primary parameters evidenced significant changes on the pasting and gelling behaviour of protein-enriched rice starch-based matrices. Major single effects on cooking and cooling parameters were provided by casein and vegetal proteins, especially by pea protein (Table 2). Pasting occurs when the starch granules absorb sufficient water and swell after gelatinization. The initial increase in viscosity with temperature during heating could be attributed to the increase in the leachates from the starch granules and the formation of a homogeneous mass resulting from the remaining fragile starch granules (Atwell, Hood, Lineback, Marston, & Zobel, 1988). A sharp decrease in peak viscosity was observed with the addition of casein and vegetal proteins with a concomitant general increase in pasting temperature, with changes being magnified with increased dose of protein (Table 2). The importance of protein in the initialization of pasting (Meadows, 2002) as well as in peak and final viscosity (Fitzgerald, Martin, Ward, Park, & Shead, 2003) has been strongly evidenced in rice. In addition, protein-starch linkages established

in presence of proteins stabilise starch structure, and hence delayed the gelatinization process (Crockett et al., 2011). Lower values for pasting viscosities are an indication of a reduction in starch available for gelatinization. This reduction is likely due to a general reduction in the starch content of the pastes because of replacement with proteins that can additionally retain water from the starch granules. The reduction of available water in the system would reduce initial starch granule swelling and, hence, add to the explanation of lower peak viscosities of the pastes. In addition to the retention of the integrity of the starch granules, it is suggested that a reduction in pasting characteristics may be associated with a reduced enthalpy of starch gelatinization as observed in dietary enriched biscuits (Brennan & Samyue, 2004). Acidification decreased pasting temperature in protein-free and protein-enriched doughs with the exception of both soya and pea proteins added at 10 g/100 g. Effects of acid incorporation on peak viscosity revealed a decrease in protein-free doughs and an increase in protein enriched doughs with the exception of soya protein, where no significant effects were observed. The viscosity of the paste that had been gelatinized in acetic/lactic acid solution was decreased by shearing thinning effect caused by stirring in the RVA test. Takahashi (1974) mentioned that the part where the molecular associative strength was weak in starch granule collapsed and dispersed when gelatinized starch paste was sheared by mechanical power. In the presence of acetic/lactic acid, the structure of the starch became more fragile by stirring, resulting in the decrease of viscosity and the increase of breakdown. It was considered that the residual proteins prevented the increase in viscosity and the collapse of starch granules during heating. Proteins mainly exist among the starch granules as protein bodies. Proteins around starch granules might indirectly disturb the gelatinization of starch (Ohishi, Kasai, Shimada, & Hatae, 2007).

Upon subsequent cooling, a gel is formed that consists of an amylose matrix in which amylopectin enriched granules are embedded (Miles, Morris, Orford, & Ring, 1985). Effects of protein supplementation and acidification on the parameters characterizing the gelling process were particularly significant for the final viscosity on cooling (Table 2, Table 3). This parameter sharply decreased in presence of increasing amounts of either vegetal or animal protein except for albumin. Dough acidification promoted the decrease in final viscosity values for unsupplemented and supplemented protein matrices particularly for soya protein, except for casein-enriched samples that underwent an increase

(Table 3). In earlier reports, final viscosity of the rice paste with acetic acid was lower than that with distilled water. It was suggested that cooked rice with acetic acid might exhibit less tendency to retrogradation when rice was soaked in acetic acid solution; proteins were eluted from rice grains and degraded by aspartic proteinase and carboxypeptidase (Ohishi et al., 2007). The different nature of added proteins may be responsible for the different behaviour. General results are in accordance with those reported by others for protein isolates (Ribotta & Rosell, 2010) and acetic acid incorporation (Ohishi et al., 2007).

3.3. Correlations between fundamental and empirical rheological parameters

Multivariate data handling of rheological variables supplied useful information on the significantly correlated viscoelastic and viscometric characteristics of GF dough samples. Using Pearson correlation analysis, a range of correlation coefficients (r) (from 0.46 to 0.95) was obtained for the relationships between fundamental and empirical properties of protein-free and protein-supplemented rice starch-based matrices with/without acid addition (Table 4). A significant interdependence ($0.51<r<0.98$) within both rheometer and mimetic measurements was found. This is especially true for parameters retrieved from the same fundamental (oscillatory measurements and creep-recovery features) and mimetic (pasting and gelling) tests. Storage and loss moduli, indicators of dough strengthened structure and solid-like behavior, strongly correlated ($p<0.001$, $r=0.81$). The loss tangent tan δ indicating solid-like or liquid like nature, is highly connected to the "a" exponent ($p<0.001$, $r=0.98$), indicating a correspondence between less structured doughs with high viscous nature expliciting elastic component G' more dependent on the frequency. As expected, a strong correlation was found between creep compliance parameters and the recovery phase counterparts ($p<0.001$), since the creep-recovery tests were carried out in the LVR (data not shown). In addition it was observed that factors increasing viscosity at the steady state (μ_0) decreased compliance values J_0 ($r=-0.56$) and J_1 ($r=-0.66$), in good accordance with previous observations (Lazaridou et al., 2007; Ronda et al., 2013), and increased G'_1. The larger the maximum stress τ_{max} providing structure integrity, the greater are the dynamic moduli, the poorer are the instantaneous

and retarded compliance, and the lower is the visco-metric profile of the corresponding doughs.

Table 4. Correlations between dough functional properties

	a	G''_1	b	tan δ	c	τ_{max}	J_{0c}	J_{1c}	μ_o	PV	TV	BD	FV	SB	TP
G'_1	-	0.81***	-	-	-	0.75***	-0.70**	-0.66**	0.80***	-	-0.56*	0.52*	-0.55*	-	-
a		-	-	0.98***	-0,84***	-	-	-	-	-	-	-	-	-	-
G''_1			-	-	-	0.90***	-0.73***	-0.55*	-	-0.65**	-0.71***	-	-0.71***	-	0.59*
b				-	0,68**	-	0.66**	0.66**	-	-	-	-	-	0.65**	-
tan δ					-0,81***	-	-	-	-	-	-	-	-	-	-
c						-	-	-	-	-	-	-	-	0.47*	-
τ_{max}							-0.67**	-0.51*	-	-0.67**	-0.74***	-	-0.68**	-	0.57*
J_{0c}								0.95***	-0.56*	-	-	-	0.54*	-	-
J_{1c}									-0.66**	-	-	-0.52*	-	-	-
μ_o										-	-	0.56*	-	-	-
PV											0.85***	-	0.73***	-	-0.66**
TV												-	0.92***	-	-
BD													-	-	-0.46*
FV														0.50*	-
SB															-

Protein: is referred to the dose of protein (0. 5. 10 %) independently of the type of protein; Acid: varied between 0 (without acid addition) and 1 (with addition); *p<0.05; ** p<0.01; *** p<0.001; ns: not significant. Abbreviations used for the correlated parameters are presented in the materials and methods section.

4. Conclusions

A gluten-free formulation based on rice starch can be obtained with a suitable combination of different proteins (egg albumin, calcium caseinate, pea protein and soy protein isolates) and acid. Supplementation of GF doughs with proteins from vegetal sources led to more structured dough matrices (higher viscoelastic moduli and steady viscosities, and lower tan δ, instantaneous and retarded elastic compliances) effect being magnified with protein dose. Acid addition produced weakening of the structure dough matrices. Acidification of soya-added doughs decreased G' and G'' (20–60 % depending on the dose) and the steady viscosity (60-70 %) and increased the loss tangent (up to 50 %) and the elastic compliances, J_{0c} (30 – 120 %) and J_{1c} (30 % - 230 %). The effect of acidification on pea protein-enriched doughs was similar although the changes

in viscoelastic moduli and loss tangent did not result significant. Incorporation of proteins from animal source resulted in different viscoelastic behaviours according to the protein type, dosage and acidification, especially for casein. Acidification conferred lower dough deformation and notably higher steady viscosity, G' and G'' for dough with 5 g/100 g casein. Protein-acid interaction favoured higher viscosity profiles, particularly for doughs with proteins of vegetable origin and lower dosage. Dough acidification decreased the pasting temperatures and the amylose retrogradation. It can be concluded that acidification of protein-enriched rice-starch doughs allows manipulation of dough rheological properties which is of relevant importance in GF bread development.

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CAPÍTULO III

CHAPTER III

EFECTO DE LA ACIDIFICICACIÓN DE MASAS DE ALMIDÓN DE ARROZ ENRIQUECIDAS CON PROTEÍNAS EXÓGENAS SOBRE LA PANIFICACIÓN SIN GLUTEN

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Acidification of protein-enriched rice starch doughs: effects on breadmaking

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Abstract

The impact of acid incorporation (acetic+lactic, 0.5%) into rice starch-based doughs enriched with different proteins (egg albumin, calcium caseinate, pea and soy protein isolates) at different doses (0, 5 and 10%) was investigated on dough proofing and thermal properties, and bread quality evaluated from physical and sensory measurements. Proteins from vegetable sources led to breads with lower specific volume and harder crumb, effects being magnified with protein dose and reduced with acid addition. Incorporation of proteins from animal source resulted in different behaviours according to the protein type, dosage and acidification. Protein addition increased the dough pH and total titratable acidity, and reduced the impact of acid addition on dough acidity. Albumin-added doughs had significantly higher temperature of gelatinization than most of other supplemented doughs, while vegetable proteins led to significantly lower gelatinization enthalpy than the control dough. Acid addition affected dough proofing and significantly improved the volume and texture of protein-enriched breads without detriment of either odour or taste.

Keywords: Acetic acid; Gluten-Free Doughs; Lactic acid; Proteins; Gluten-free bread

1. Introduction

Legume- and cereal-based foods such as bread are rich in complex carbohydrates and other essential nutrients, representing staple foods in many countries worldwide. Rising demands for gluten free (GF) products parallels the apparent or real increase in coeliac disease, and other intolerances to gluten consumption (Lazaridou, Duta, Papageorgiou, Belc and Biliaderis, 2007). The target group of GF products is currently expanding to adhere people looking for nonallergenic ingredients, and specific vulnerable groups of population with special nutritional needs leading to a new market that welcomes a variety of products (Kelly, Moore & Arendt, 2008). One of the main challenges of the bakery industry is still to improve the quality of GF breads currently available in the market.

Starch plays an important role in the structure and mechanical properties of bread. The role is even greater in the case of GF bakery products, where the visco-elastic wheat protein network is replaced by blends of different structuring agents (Gallagher, 2009; Ziobro, Korus, Witczak & Juszczak, 2012). Rice starch is one of the more preferred starches for GF bread production, associated to its characteristics of natural, hypoallergenic, colorless, and bland taste (Kittisuban, Ritthiruangdej and Suphantharika, 2014) and very low level of protein, sodium, fat, fiber and high amount of easily digested carbohydrates (Gujral & Rosell, 2004).. Proteins from different sources (soybean, pea, egg albumen and casein) can be added to GF basic formulations resulting in nutritional benefits and, in some cases, improved volume and appearance of GF breads (Marco and Rosell, 2008b; Gallagher, Kunkel, Gormley and Arendt, 2003a; Ziobro, Witczak, Juszczak and Korus, 2013). Proteins are added to increase elastic modulus by cross linking, to improve perceived quality by enhancing Maillard browning and flavour, to improve structure with gelation and to aid in foaming (Ronda, Villanueva and Collar, 2014b). Protein addition results in breads with increased loaf volume, improved crumb regularity and enhanced sensory characteristics (Crocket, Ie and Vodovotz, 2011). Starch-protein interactions can affect starch gelatinization and further retrogradation, bread quality and shelf life.

Acidification through lactic acid and acetic acid addition confers suitable properties to final breads. Enhanced fresh bread odour and taste and increased protease and amylase activities leading to retarded staling are observed in

acidified matrices (Moore, Dal Bello and Arendt, 2008). This has been attributed to an increase in net protein charge, which leads to promoted solvent interaction and easier protein unfolding, but that could eventually prevent strong network formation (Jayaram, Cuyvers, Verstrepen, Delcour and Courtin, 2014). Despite dough acidification by acetic acid and lactic acid addition is known to impact the properties of dough (Ohishi, Kasai, Shimada and Hatae, 2007; Jeckle and Becker, 2012; Ronda et al., 2014b;) and, therefore, also to affect the quality of the final baked product (Jayaram et al., 2014), the impact of acidification in protein enriched GF breads has not been reported so far.

It is assumed that inter and intra-molecular interactions established between exogenous proteins and starch molecules, responsible for dough structurization and bread characteristics, are certainly dependent on dough pH. This study aims to determine the combined effect of acid addition and protein supplementation on dough proofing, thermal properties and quality attributes of GF breads.

2. Material and methods

2.1. Materials

Rice starch (9.9 % moisture, 0.2 % ash and 0.5 % protein) from Ferrer Alimentación S.A. (Barcelona, Spain), and salt, sugar and sunflower oil purchased from the local market, were used to make GF doughs. Hydroxypropylmethylcellulose (HPMC, Methocel K4M Food Grade) was provided by Dow Chemical (Midland,USA). Proteins used in GF formulations were: soybean protein isolate (SPI) Supro 500-E IP from Proveedora hispano-holandesa S.A. (Barcelona, Spain), calcium caseinate (CA) from Armor proteins (Saint-Brice-en-Coglès, France), egg albumin (EA) in dry powder from Eurovo (Valladolid, Spain) and pea protein isolate (PPI) branded Pisane C9, from Cosucra (Warcoing, Belgium). Acetic acid and lactic acid (analytical grade; Panreac, Barcelona) were used as a source of hydrogen ions.

2.2. Methods

Dough preparation and breadmaking

A straight dough process was performed using the following formula on a 100 g rice starch (or rice starch+protein) basis: 6 % oil, 5 % sucrose, 1.5 % salt, 2 % HPMC, 3% dried yeast and 80 % water**.** All proteins were added at 0 %, 5 % and 10 % w/w (starch+protein basis) levels. Doughs were supplemented with 0.1 %

+ 0.4 % (w/w starch+protein basis) of acetic and lactic acid, respectively, when acid-treatment was applied. The studied factors in the experimental design where: i) Protein type (four levels): EA, CA, SPI, PPI; ii) Protein dose (three levels): 0, 5% and 10% and ii) Acid (two levels): presence/absence. Eighteen elaborations were carried out in a randomized way by means of the programme Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) (see Table 1).

Table 1. Experimental design

Formula	Protein				Acetic/Lactic acid
	CA	EA	SPI	PPI	
1	0	10	0	0	0.1/0.4
2	0	5	0	0	0
3	0	0	10	0	0
4	0	0	5	0	0
5	0	10	0	0	0
6	0	5	0	0	0.1/0.4
7	0	0	0	0	0
8	0	0	5	0	0.1/0.4
9	0	0	0	5	0
10	0	0	0	10	0.1/0.4
11	0	0	0	0	0.1/0.4
12	10	0	0	0	0.1/0.4
13	5	0	0	0	0
14	5	0	0	0	0.1/0.4
15	0	0	0	5	0.1/0.4
16	0	0	10	0	0.1/0.4
17	0	0	0	10	0
18	10	0	0	0	0

CA: Calcium Caseinate; EA: Egg Albumin;
SPI: Soya Protein Isolate; PPI: Pea Protein Isolate
Amounts are in % w/w, starch +protein basis

GF dough-making was achieved by blending first solid ingredients and oil in a kitchen-aid professional mixer (KPM5). Then water was added and hand mixed. Finally the dough was mixed with dough hook at a speed 4 for 8 min. Acid blend (acetic+lactic acid in the ratio 1:4 w/w), when added, was previously diluted in a small part of water (7 % of total) and adjusted to the dough before the mixer was powered on. The dough, 200 g, was placed into an alumnium pan of 14cm x 9 cm x 4cm and was proofed at 27ºC and (85 ± 5) % relative moisture for 50

min. Subsequently, baking was carried out in a Salva oven (Lezo, Spain) at 190ºC for 40 min. After baking, six breads for each formula were left for one hour at room temperature before analysis. To study the effect on staling, breads were stored for 2 days in a refrigerator at 4 ºC (± 2 ºC) in polyethylene bags. This temperature was chosen to accelerate the bread staling and to measure greater storage effects.

Dough measurements

Gas production of formulated GF doughs was continuously measured in the rheofermentometer (Chopin Rheofermentometer F3, Chopin Technologies, Villeneuve-La-Garenne Cedex, France). In contrast to the traditional method, the weight of dough was reduced to 200 g and the four weights of 0.5 kg were removed adapted to dough softness. Fermentation was carried out at 37 ºC for 4 h. The parameters registered included: H_m, height of dough at maximum development time (mm); h, height of dough at the end of the test (mm); $(H_m-h)/H_m$ that is inversely related to dough stability; T_1 time corresponding to H_m (min); T_2: Time of stabilisation, where the dough height is above 90% H_m (min); H'_m, maximum height of CO_2 production (mm); T'_1 , time of the maximum gas formation (min); V_T, total volume of CO_2 (mL) produced during 4 h of fermentation; V_r, total volume of the CO_2 (mL) retained by the dough; R_C, the CO_2 retention coefficient V_r/V_T, which measures the amount of CO_2 liberated and retained from the dough and therefore is related to the porosity of the dough; T_X, the time (min) when the porosity of the dough develops.

pH measurements of dough samples were made with pH-meter (Oakton-Eutech Instruments PH6) at regular intervals of 5 min up to 60 min during proofing.

Total titratable acidity (TTA) was measured on ten grams of dough blended with 100 mL acetone/water (5/95, v/v) under constant stirring. The titration was carried out against 0.1N NaOH until a final pH of 8.5. The results were expressed as milliequivalents of lactic acid/g of dough. This measurement was carried in triplicate on un-yeasted doughs.

Thermal characteristics of doughs were determined using a differential scanning calorimeter (DSC-822e, Mettler Toledo, SAE). Freeze-dried hydrated doughs (≈ 6 mg dry matter) were weighed into aluminium pans of 40 μL (ME-29990, Mettler Toledo, SAE) and distilled water was added using a micropipette to make 70% moisture content. The samples were scanned from 20 to 110°C at

5 °C/min using an empty pan as reference. Starch retrogradation was evaluated in the samples previously gelatinized in the DSC oven stored in the pans at 4 ºC (2 ºC) for 2 days. These samples were scanned from 0 to 110 °C at a heating rate of 5 °C/min. The enthalpy (H), left temperature (T_{left}), peak temperature (T_p) and the difference T_{right}- T_{left} (ΔT), as a measurement of the width of the endotherm peaks, were measured in both scans, at 0 and 2 days of storage. Reported values are the mean of duplicate measurements.

Evaluation of bread quality

The volume, height and width of bread were determined from four replicates using a Volscan profiler 300 (Stable Microsystems, Surrey, UK) analyser. The breads were weighed immediately after removal from the pan once cooled.

Crumb texture was determined in quadruplicate with a TA-XT2 texture analyser (Stable Microsystems, Surrey, UK) provided with the software "Texture Expert". An Aluminium 20 mm diameter cylindrical probe was used in a compression test (TPA) to penetrate to 50% depth, at 1 mm/s speed test. Hardness (N) was the force at the maximum deformation. Analysis were carried out at (20 ± 2) ºC for two bread slices of 20 mm thickness taken from the centre of the loaf. Two loafs were measured. The texture analysis was carried out on fresh and two-day stored breads to evaluate bread staling.

Colour was measured with a Minolta spectrophotometer CN-508i (Minolta, Co.LTD, Japan). Results were obtained in the *CIE L*a*b* and *CIE L*C*h* coordinates using the D65 standard illuminant, and the 2º standard observer. Colour determinations were made 5x5 times: bread crumb and crust colours were checked at five different points on each bread and every point was measured five times. The colour of proteins was also measured.

Sensory analysis was performed by a panel of ten trained judges (two males and eight females aged 25–53) from the baking laboratory. Eight training sessions were carried out; five of them in the specific attributes tested in breads. An intensity non-structured scale from 1 to 10 was used. The attributes tested were cell regularity (1 = very irregular size cells; 10 = very regular size cells), acid taste (1: very little; 10: very much), acid odour (1: very little; 10: very much), taste intensity (1: very little; 10: very much), odour intensity (1: very little; 10: very much) and aftertaste persistency (1: very little; 10: very much).

Statistical analysis

STATISTICA package (Tulsa, OK, EEUU) v.6, allowed performance of MANOVA analysis, and LSD (Least Significant Difference) test was used to evaluate significant differences ($p < 0.05$) between samples. Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) was used for Pearson correlation study.

3. Results and discussion

3.1. Dough properties

pH and total titratable acidity of doughs

Table 2 compiles values for dough properties according to the presence, type and dose of the different proteins. All doughs were made with and without acid addition in order to study the effect of acid presence and its possible interaction with the type or dose of protein. Protein presence increased significantly ($p<0.01$) dough pH between 7 % (5% EA) and 12 % (10% CA, PPI or SPI) with respect to control dough. The lowest increase was obtained with EA. The dose of protein also affected significantly ($p<0.01$) dough pH. Acidification of protein-enriched doughs resulted in pH values 15 % – 34 % higher than the acid-added control dough. Proteins exerted a buffering effect on doughs, also confirmed by the higher TTA of the protein-enriched doughs in spite of its higher pH. TTA also increased significantly ($p<0.01$) as the protein dose increased. The highest effect on TTA was exerted by CA. As expected, acid addition increased dough TTA. In general, acidification of protein-enriched doughs led to significantly higher TTA than the acid-added control dough. However the increase was not very relevant except for CA-added doughs. The decrease of pH during proofing, ΔpH, in unacidified doughs, with or without proteins, was 0.21-0.36. However, acidified doughs had a stable pH that varied less than 0.1 units in spite of acid generation during proofing, showing the highest buffering capacity around pH=4.5.

Fermentative properties of doughs

Acid addition to control dough delayed significantly both gas production and maximum dough development along proofing (Fig. 1). Low pH of acid-added control doughs, 3.9, could explain the effect as a result of yeast inhibition (Blanco, Ronda, Pérez and Pando, 2011). The undissociated forms of acetic acid can pass across the membrane into the cell by simple diffusion (Stratford, 1999; Piper, Calderon, Hatzixanthis and Mallapour, 2001). Once inside the cytoplasm, the undissociated form of the acid dissociates, liberating protons. The lower internal pH prevents normal yeast growth (Krebs, Wiggins, Stubbs, Sols and

Bedoya, 1983) and leads to other physiological alterations, affecting fermentative activity, yeast cell viability (Peres, Tininis, Souza, Walker and Laluce, 2005) and effective dough development (Blanco et al., 2011). Acidification of protein-enriched doughs showed a much more smoothing effect on the time of maximum gas production except for EA-added doughs that increased by 20% and 50% with respect to unacidified doughs for 5 and 10% dosages, and for 5% SPI- and 5% PPI-added doughs that showed a delay in gas production of 27% and 168%, respectively. A significant negative correlation was obtained between dough pH and time of the maximum gas production, T_1' ($p<0.001$; $r = -0.80$) in agreement with the effect of acetic acid on yeast activity explained previously.

The maximum height of gas production (H'_m) declined with the presence of protein in doughs. However, except for 10% PPI, where no effect was observed, the acidification of the protein-enriched doughs improved the H'_m value, opposite to the effect observed when the control dough was acidified. In general, little or no permeability was observed in rice starch doughs. More than 90% of the gas produced during fermentation was retained in all doughs. The lowest retention coefficient was achieved for 10% PPI- or SPI-added doughs. Again, vegetable and animal proteins led to different behaviour. The effect of protein and acid addition on dough development depended significantly ($p<0.001$) on the type and dose of protein. The maximum height of dough development, H_m, was dependent not only on the volume of gas produced or retained by dough during proofing, but also on the capacity of expanding under the action of the gas produced. Positive correlations were obtained between H_m and V_T and V_r ($p<0.01$ $r = 0.87$ and 0.95 respectively).

Table 2. Dough properties: Total Titrable Acicity (TTA), initial pH (pH_0), change of dough pH during proofing (ΔpH) and thermal properties: ΔH_{gel}: Enthalpy associated to gelatinization; ΔH_{ret}: Melting enthalpy of the recrystalized amylopectin after storage of the gelatinized sample at 4ºC for 2 days. ΔT: T_{peak}-T_{onset} of the peak; $T_{peak\ ret}$: amylopectin recrystallization peak temperature.

	TTA (meq/g)	pH_0	ΔpH	ΔH_{gel} (J/g starch)	T_{peak} (ºC)	T_{onset} (ºC)	ΔT (ºC)	ΔH_{ret} (J/g starch)	$T_{peak\ ret}$ (º C)
Control	0.0048 a	5.21 f	0.36 f	11,5 efgh	70.86 cde	63.98 efgh	6.88 abc	0.52 a	48.7 a
Control-A	0.0281 g	3.88 a	0.04 a	11,4 defg	70.82 cde	63.97 efg	6.85 abc	1.35 a	49.4 a
Egg albumen									
5%	0.0070 b	5.56 g	0.29 ef	11,4 cdefgh	71.38 de	64.44 efgh	6.94 abcd	0.79 a	55.0 abc
5%-A	0.0304 i	4.46 b	0.01 a	11,8 gh	71.14 cde	64.23 efgh	6.91 abcd	1.32 a	54.3 abc
10%	0.0081 cd	5.73 hij	0.21 bcde	11,8 fgh	71.39 de	64.51 fgh	6.88 abcd	0.82 a	53.9 abc
10%-A	0.0279 g	4.80 d	0.08 ab	12,2 h	71.56 e	64.76 h	6.80 abc	0.74 a	56.2 abc
Calcium caseinate									
5%	0.0097 e	5.71 hi	0.29 ef	11,3 cdefg	69.65 a	62.34 ab	7.31 cd	0.58 a	53.3 abc
5%-A	0.0321 j	4.73 cd	0.09 a	10,7 abcd	69.60 a	62.28 ab	7.32 cd	0.54 a	52,5 abc
10%	0.0134 f	5.84 ij	0.24 def	11,0 abcdefg	69.65 a	62.37 ab	7.28 cd	1.21 a	54.5 abc
10%-A	0.0436 k	5.10 ef	0.12 abcd	10,7 abc	69.58 a	62.92 bc	6.67 a	1.08 a	58.7 c
Pea Protein Isolate									
5%	0.0075 bc	5.73 hij	0.30 ef	10,8 abcd	70.41 bc	63.68 de	6.73 ab	1.17 a	56.5 abc
5%-A	0.0298 hi	4.72 cd	0.07 a	10,6 ab	71.19 de	64.58 gh	6.61 a	0.96 a	58.5 bc
10%	0.0085 d	5.85 j	0.21 bcde	10,9 abcde	69.89 ab	62.45 ab	7.44 d	1.21 a	54.1 abc
10%-A	0.0289 h	5.20 f	0.10 abc	10,9 abcde	70.46 bc	63.25 cd	7.22 bcd	0.80 a	53.8 abc
Soy Protein Isolate									
5%	0.0075 bc	5.68 gh	0.27 ef	10,6 abc	69.64 a	62.41 ab	7.23 cd	0.73 a	51.3 bc
5%-A	0.0296 hi	4.64 c	0.03 a	11,0 bcdefg	70.59 bcd	63.88 def	6.93 abc	0.92 a	53.1 abc
10%	0.0097 e	5.82 hij	0.23 cdef	10,3 a	69.52 a	62.09 a	7.43 c	0.72 a	53.5 abc
10%-A	0.0314 j	5.03 e	0.13 abcd	11,0 abcdef	69.51 a	62.18 a	7.33 cd	0.54 a	52.1 bc
SD	0.0003	0.04	0.03	0.10	0.09	0.09	0.05	0.12	6,3

-A: Acid addition. Each data is the average of duplicates. The standard deviation (SD) was established from MANOVA

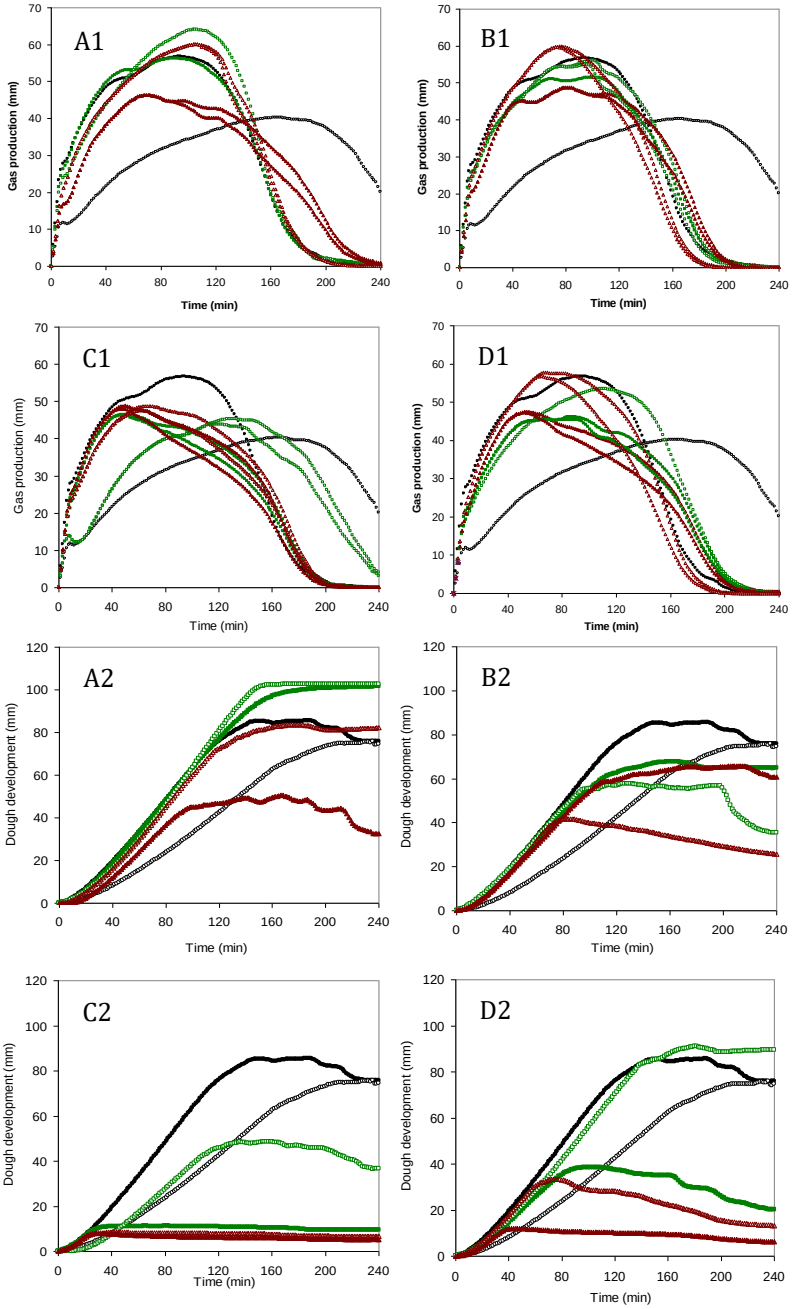

Figure 1. Gas production (1) and dough development (2) obtained from rheofermentomer of doughs with egg albumin (A) calcium caseinate (B) isolated pea protein (C) isolated soya protein (D); Control doughs are included as references in all graphics. Control (without protein) (●○); 5% protein (■ □); 10% protein (▲△); without acid (solid points) or with acid (void points). The same symbol is used for gas produced and retained.

Thermal properties of doughs

Dough thermal properties, studied by DSC, are summarized in Table 2. A significant effect of protein presence on starch gelatinization enthalpy (ΔH_{gel}) was observed. The enthalpy, expressed on starch basis, is independent on the sample starch content, that obviously was lower in protein-enriched doughs. The average ΔH_{gel} of the control dough, 11.4 J/g starch, was similar to that of doughs enriched with animal proteins but superior to that of doughs with vegetable ones with an average value of 10.8 J/g starch, for both SPI and PPI. The increase in protein dose did not exert a significant decrease on this transition enthalpy. Noisuwan, Bronlund, Wilkinson and Hemar (2008), who studied the effect of milk proteins on thermal properties of rice starch, found a decrease in ΔH_{gel} when the concentration of sodium caseinate passed from 5% to 10% in the mixture casein/rice starch. Similar tendency was observed in the current study although the difference was very small and not significant. In this case, the different counter-ion, calcium instead of sodium, and the application of mechanical energy during mixing for dough making of protein/starch, could account for the observed differences. The acidification of the dough did not affect ΔH_{gel} significantly as was reported by others (Ohishi et al., 2007).

The gelatinization temperature was significantly affected by the type and dose of protein and by the interaction (protein type*dose). Acid also affected rice starch gelatinization temperature in presence of vegetable proteins. When gluten was added to wheat starch, the gelatinization temperature of the starch increased. However, the influence of proteins other than gluten on DSC parameters were not very deeply determined (Eliasson & Larsson, 1993). Both CA and SPI decreased T_{onset} and T_{peak} by ~1.4ºC and ~1.1ºC respectively with respect to the control dough. Doughs with EA, showed a higher gelatinization temperature than the remaining protein-enriched doughs, although values were not significantly different than that of control dough. With vegetable proteins, the higher the dose the lower the temperature of gelatinization, although only in PPI presence the effect was significant. No effect of casein dose was observed on T_{onset} and T_{peak} as reported Noisuwan et al., (2008). The acidification of

the dough did not affect T_{onset} and T_{peak} of the control dough and doughs enriched with animal proteins; other authors found endothermic peak temperatures of rice starch lower in presence of acetic acid (Hibi, 2002; Ohishi et al., 2007). However, the acetic acid concentration added to rice starch was 6 – 10 times the amount of acetic+lactic used in the current study, where the addition was only the acid concentration usually provided by sourdoughs. The limited acid concentration was probably unable to promote starch hydration and further enhancement of starch gelatinization, as stated by Ohishi et al., (2007). Conversely, T_{onset} and T_{peak} increased significantly ~1.5 ºC and ~1.0ºC, respectively with the acidification of 5%/10% PPI- or 5% SPI-enriched doughs. The nature of proteins and their physico-chemical properties (size, shape, composition, structure, net charge and charge distribution, ability to interact or to repel other components, etc.) affect the gellatinization of studied doughs. The higher temperatures obtained with EA might be due to its lower water absorption (Damodaran, 2008).

The second scan from 0ºC to 110ºC applied to gelatinized samples stored in the DSC pans at (4±2)ºC for 2 days (retrogradation scan) led to two visible peaks. The first one, very wide, at a peak temperature that ranged 49-50ºC was related to the melting of the recrystallized amylopectin, and the second one at temperatures of 97-102ºC probably related to the amylose-lipid complex dissociation (Eliasson, 1994). This transition, not observed in the gelatinization scan, took place at an usual temperature for this phenomenon when the water content of the starch suspension is high enough (> 60-70%) as it was the case. Eliasson (1994) reported that the increased values during the second scan, also observed by other authors, are probably due to better conditions for complex formation after the first heating. In the case of starch it has relation to the leaking of amylose from granules that occurs at temperatures above gelatinization temperature range. The average enthalpy quantified in tested samples for amylose-lipid complex dissociation was 1.2 J/g starch ranging between 0.8 y 1.8 J/g starch, without significant differences based on acid or protein presence. Similar enthalpy for this endotherm was previously reported (1.3 J/g starch) for wheat starch in excess water where the lipid

compound of the complex was almost exclusively attributed to lysolecithin (Eliasson & Larsson, 1993). The melting enthalpy of the recrystallized amylopectin (ΔH_{ret}) in two days ranged 0.5 – 1.3 J/g starch. The large width of these small peaks was responsible for the low accuracy of the enthalpy and peak temperature values making it difficult obtaining significant differences. It seems that, in protein presence the retrograded amylopectin melted at higher temperatures meaning higher stability of crystal structure. It is still pending to measure retrogradation after long storage periods to assess the effect of proteins on retrogradation extension.

3.2. Bread quality properties

Bakeloss

The loss of weight during baking varied between 15% (no acid 10% EA) and 21% (acidified 5% EA) (Table 3). Protein addition affected loss of weight, effects being dependent on the protein type and dose, and on the absence/presence of acid. In general, except for CA, the presence of protein led to a decrease in the loss of weight during baking that was more marked at higher protein concentration, with a reduction of 21% when 10% of EA, PPI and SPI were added. The well established water binding capacity of proteins would explain this effect (Zayas, 1997). Acidification effect depended on protein presence. Acidification of control dough reduced 10% the losses during baking, while significant increases in presence of protein -7% and 15% for 5 and 10% of SPI and 7% for 5% EA- were observed. The increase of protons concentration in dough may stabilise some high electronic density functional groups, decreasing interactions with water molecules by hydrogen bridges and, hence water binding capacity. This must be dependent on dough pH and protein nature. The correlation study of the loss of weight with dough rheological properties previously reported (Ronda et al., 2014b) denoted a strongly correlation when the study was confined only to vegetable proteins. The loss of weight showed a general significant positive correlation with Hm and Hm' ($p<0.01$; $r=0.69$ and $p<0.05$; $r=0.63$ respectively) and with the specific volume ($p<0.001$; $r=0.86$). The same

factor (the presence of protein) that decreased the dough development during proofing and led to lower bread volumes, improved the water binding capacity causing a decrease in baking loss.

Table 3. Textural and morphogeometrical properties of bread

	Baking Loss (%)		Specific Volume (mL/g)		Heigth/ Width		Firmness (N)		ΔFirmness (2days) (N)	
Control	19.42	f	4.44	g	0.81	c	1.02	abc	8.9	abcd
Control-A	17.19	bc	2.90	c	0.65	bc	1.73	d	7.4	abc
Egg albumin										
5%	19.43	ef	4.39	g	0.79	bc	1.21	abcd	10.1	abcd
5%-A	20.89	g	4.59	g	0.79	bc	1.34	bcd	8.7	abcd
10%	15.16	a	2.19	b	0.40	a	2.97	e	16.8	e
10%-A	15.48	a	3.38	d	0.70	bc	1.61	cd	13.0	de
Calcium caseinate										
5%	19.00	ef	3.77	def	0.73	bc	0.98	ab	10.3	bcd
5%-A	18.88	ef	4.34	g	0.83	c	0.69	a	6.2	a
10%	18.64	def	3.95	ef	0.77	bc	0.94	ab	9.9	abcd
10%-A	18.44	de	3.96	ef	0.81	c	0.79	ab	11.3	cd
Pea Protein Isolate										
5%	16.68	b	1.69	a	0.40	a	4.72	f	81.3	g
5%-A	16.67	b	3.74	de	0.91	c	1.00	ab	7.3	ab
10%	15.28	a	1.36	a	0.36	a	11.36	h	90.3	h
10%-A	15.60	a	1.48	a	0.37	a	7.20	g	93.1	h
Soya Protein Isolate										
5%	17.94	cd	3.62	de	0.75	bc	1.04	abc	9.4	abcd
5%-A	19.28	f	4.00	f	0.77	c	1.07	ab	7.9	abc
10%	15.42	a	1.60	a	0.56	ab	11.75	h	31.1	f
10%-A	17.69	cd	3.54	d	0.71	bc	0.90	ab	6.7	ab
SD	0.30		0.12		0.09		0.22		1.5	

Values with a common letter in the same column are not significantly different ($p>0.05$); SD: Standard deviation obtained from variance analysis

Specific volume and height/width

Loaf-specific volume, which varied from 1.36 mL/g (no acid 10% PPI) to 4.59 mL/g (acidified 5% EA), and the loaf height/width, which varied

from 0.36 to 0.91, exhibited similar trends (Table 3). Both properties were strongly correlated ($p<0,001$, $r= 0.82$) as could be expected in pan breads. A slight lack of symmetry in breads could explain a Pearson coefficient different than 1. The presence of protein affected bread specific volume differently depending on the type and dose and on the absence/presence of acid. In acid-free doughs, both CA and PPI decreased the specific volume by 13% and 65% respectively, irrespective of the dose. This is similar to the findings of Gallagher, Gormley and Arendt (2003b), including dairy powders in gluten free breads, reduced the loaf volume by about 6% independently of the type and dose of dairy product. Conversely, the effect of the addition of EA or SPI was strongly dependent on the dose. The addition of 5% SPI only reduced 18% the volume while the highest dose led to a decrease of 64%. In gluten-free breads, a negative effect of SPI (Ribotta, Ausar, Morcillo, Pérez, Bertramo and León, 2004; Marco and Rosell, 2008a; Crockett et al., 2011) and PPI and other proteins, except EA, (Ziobro et al., 2013) on final bread quality has been reported. 5% EA was the only protein addition with no negative effects on bread volume as observed by others (Ziobro et al., 2013). These breads were significantly bigger than the rest of supplemented breads. This effect could be attributed to EA foaming capacity (Zayas, 1997). EA exhibits relatively low molar masses, and contains mostly acidic amino acids. Their ability to bind carbon dioxide could have an important influence on dough structure during baking (Ziobro et al., 2013). The EA addition at 10% level reduced the volume dramatically.The acid addition exerted a variable effect depending on the type and dose of protein. The acidification of the control dough reduced the bread specific volume in ~34%. However, when applied to protein-supplemented doughs always increased the bread volume, except with 5% EA, 10% CA and 10% PPI, where no significant effect was obtained. Specific volume showed a significant correlation with H_m ($p<0.001$; $r = 0.80$). The different expansion of doughs during proofing and baking could be due to several factors: i) the lower gas production as consequence of a lower substrates concentration for yeast as took place in our case in protein presence (Witczak, Korus, Ziobro and Juszczak, 2010; Ziobro et al., 2013); ii) the

higher dough consistency that can restrict dough expansion under the action of the stress produced by the gas formed during proofing and expanded during baking (Perez-Quirce, Collar and Ronda, 2014) until the bread crumb structure is formed, which is mainly dependent on starch gelatinization and protein denaturation (Eliasson & Larson, 1993). A proper consistency of doughs is necessary. A too low dough consistency, as those with 10% EA (Ronda et al., 2014b) could explain the low specific volume of breads by its inability to retain the produced and expanded gas.

Colour

The crust colour, an important parameter for consumer acceptance (Ziobro et al., 2013), is mainly determined by Maillard reaction. It depends directly on the available water, the concentration of carbonyl groups from reducing sugars, the amount of amine groups mainly proceeding from the proteins added (Purlis, 2010), and the pH (Pyler, 2000). It varied significantly with the type and dose of protein, and the presence/absence of acid (Figure 2). It was also influenced by 2nd order interactions of all the factors studied. The crust lightness, L_c^*, ranged 66 – 45 (Fig. 2a). As could be expected the protein presence always decreased it leading to darker crusts as consequence of higher Maillard reaction extent. The lightest crust corresponded to the control bread and the darkest one to that with 10% EA. The acid addition led to lighter bread crusts, with L* values 6% (5% SPI) to 25% (10% SPI) higher than the unacidified doughs. The hue of the crust (Fig 2b) ranged from 69 (control bread) to 50 (10% EA) degrees and followed a parallel evolution to the lightness. Protein concentration decreased the hue significantly, leading to more reddish crust. At 5% level the hue reduction ranged 6% - 16% for SPI and EA while it decreased to 16% -28% for the same proteins at 10% level. Acid addition also increased the crust hue. So that, acid blend counteracted the protein effect. The crust of acidified 5% protein-added breads had similar hue to that of the control bread whereas acidified 10% protein-added breads still showed a smaller crust hue than the control.

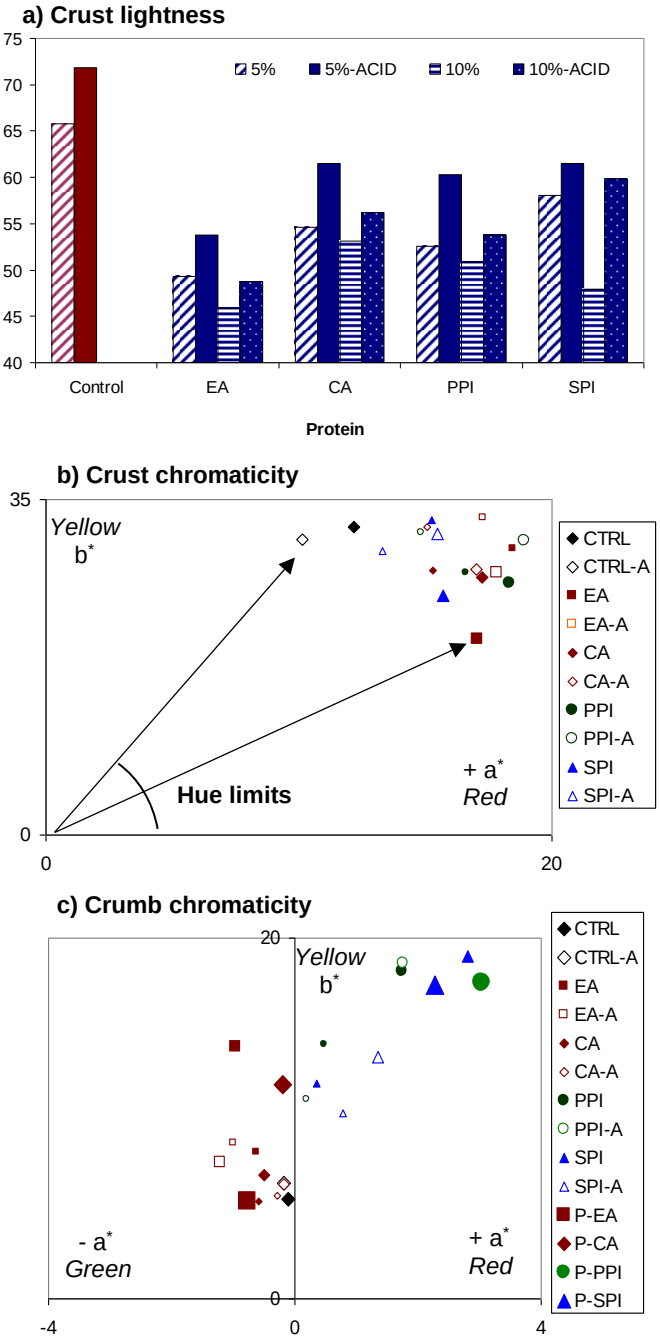

Figure 2. Crust lightness (a) crust chromaticity (b) and crumb chromaticity (c) depending on protein presence type and dose and acid blend addition. EA: Egg albumin; CA: Calcium caseinate; PPI: Isolated pea protein; SPI: Isolated soya protein; A- With acid addition; P-EA, P-CA; P-PPI; P-SPI are referred to the colour of pure protein ingredients

The crust chroma (Fig. 2b) ranged from 26 to 37, for no acid 10% EA- and acid/5% EA-enriched bread respectively. The latter showed the most vivid colour. Proteins, except EA and SPI, at 5% level decreased the crust chroma between 6% and 8%, while breads with 10% SPI or EA showed a crust chroma 22% lower than the control bread. Colours of the proteins used for the enrichment of breads were measured in order to explain the trends observed in crumbs. The L^*C^*h coordinates were: CA (95.8; 5.5; 98.0), EA (96.3; 11.9; 91), PPI (87.3; 17.8; 80.2) and SPI (85.7; 17.5; 82.6). Consequently, both, EA and CA were practically white, with a very slight pure yellow hue with a very little tendency to red in the case of EA and to green in the case of CA. PPI and SPI were hardly darker than the two former, as indicated by smaller L* coordinate. The hue of these vegetable proteins was also mainly yellow although they had a small reddish component (Fig.2c). PPI and SPI colours are represented in the first quadrant of the chromatic a*-b* diagram while CA and EA are depicted in the second quadrant, very near the y-axis. The crumb of breads enriched with EA or CA showed a*-b* coordinates that fell in the same quadrant than the pure proteins (Fig. 2c), showing similar hues. The crumb lightness varied from 66 to 80%. The highest lightness was from 10% EA- and 5% CA-enriched breads, with an increase of 14% and 9% with respect to the control crumb lightness. 10% SPI-added breads had the lowest lightness, 7% lower than the control.

Texture

Fresh bread crumb firmness varied from 0.7 N (5% CA with acid) to 11.8 N (no acid 10% SPI) (Table 3). In this work, protein addition affected bread hardness, the extent of the changes being dependent on the type and the dose of protein and on acid addition. At 5% level only PPI increased the crumb firmness significantly with respect to the control bread (360%). Increased dosage from 5 to 10% promoted dramatically the crumb hardening of breads enriched with vegetable proteins, by two fold and eleven fold the value of the control for PPI and SPI, respectively, while only breads containing EA animal protein underwent a significant hardening promotion (+145%). Current results in agreement with previous studies (Ahlborn, Pike, Hendrix, Hess and Huber, 2005; Marco

and Rosell, 2008a; Crockett et al., 2011; Ziobro et al., 2013) are compatible with the creation of a robust cross linked structure by added proteins, especially supported in the case of SPI by glicinin and a high water retention ability (Crockett et al., 2011). CA did not promote any crumb hardening with respect to the control bread regardless either the protein level or acid addition. Acid blend addition encompassed a great decrease in vegetable-supplemented bread firmness, (-92% for 10% SPI and -79% and -37% for 5% and 10% PPI respectively), as a result of the higher amount of air entrapped in the crumb of more developed breads. Acidification of 10% EA-added breads also reduced significantly ($p<0.05$) crumb firmness (- 46%). However, the acidification of the control bread led to a 70% firmness increase. A neutral charge causes less repulsion forces and less space for water molecules between the proteins. This repulsion forces increase with increasing charge and more water molecules can be attached to the protein strands whereby less mobile water is available in the dough system (Jekle & Becker, 2012). As expected, a strong negative correlation was obtained between the crumb firmness and the bread specific volume ($p<0.001$; $r=-0.85$).

The effect of acid on crumb firmness could also be related to a reduction of the extent of amylose retrogradation, as it was previously reported (Ronda et al., 2014b). Amylopectin retrogradation is related to bread staling while amylose recrystallization can be partially responsible for the initial firmness of fresh breads (Zobel & Kulp, 1996).

Bread staling

Bread staling was assessed by means of crumb firmness evolution after two days of storage at (4 ± 2)ºC. (Table 3). It has been reported that amylopectin recrystallization occurs faster in GF bread, than in traditional bakery products, where the structure is determined by the presence of gluten (Eliasson & Larsson, 1993). Ziobro et al., (2012) observed that bread staling was accelerated by vegetable protein supplementation. The increase of hardness in two days of storage of no acid PPI-, both at 5% or 10% level, and 10% SPI-enriched bread crumbs was markedly higher than that of the control bread while animal proteins

hardly affected bread staling. Dough acidification counteracted the protein effect on bread ageing except for 10% PPI, which effect was kept despite the addition of the acid blend. The hardening of the crumb is a complex phenomenon in which multiple mechanisms operate. All involve starch recrystallization and water migration (Ronda, Caballero, Quilez and Roos, 2011; Ronda, Quilez, Pando and Roos, 2014a). Factors affecting crumb bread staling have been extensively investigated (Zobel and Kulp, 1996; Chinachoti and Vodovotz, 2001; Osella, Sánchez, Carrara, de la Torre & Buera, 2005; Ziobro et al., 2012). In the current study, differences among 2-day-retrograded amylopectin extent were not detectable. The averaged melting enthalpy of the recrystallized amylopectin was 0.8 J/g starch. Probably, molecular mobility and water restriction in the real system played an important role in bread staling that could not be detected in the DSC pan-baked doughs where starch was in an excess of water in order to no restricting starch gelatinization.

Sensory evaluation

The results of sensory analysis of rice bread samples are given in Table 4. The presence of protein notably improved cell regularity of bread. The lowest scores were obtained for the control breads, with larger and inhomogeneous cells and the highest for 10% EA, CA or SPI and 5% PPI. Acidification did not show a significant effect on cell regularity of breads enriched with protein. Only the acidification of SPI-added breads promoted a score reduction (-42% and -24% for 5% and 10% respectively) with respect to the un-acidified breads. The use of acetic and lactic acid tried to simulate the midly acid taste of conventional white bread that comes from water-soluble organics acids formed by yeast and bacterial fermentation (Pyler, 2000). What stood out from the sensory analysis was protein addition neutralised the acid odour and taste of acidified breads. When the acid blend was added to the control bread the odour and taste scores increased markedly (57% and 175% respectively). However, any significant effect was observed in protein-enriched breads, except in breads supplemented with 10% SPI whose acidification was detected by panellist. Acidified 10% CA-enriched breads had the highest acid taste score among protein-supplemented breads,

95% higher than no acid control bread. The highest taste intensity and persistency scores were obtained by 10% PPI-enriched breads either with or without acid addition.

Table 4. Sensory evaluation of breads

	Cell Regularity		Odor Intensity		Acid Odour		Taste Intensity		Acid Taste		Taste Persistency	
Control	2,07	ab	6,79	a	2,43	bcd	5,30	ab	1,95	abcd	3,82	ab
Control-A	1,21	a	6,89	a	5,63	e	5,66	abc	5,38	f	3,64	ab
Egg Albumin												
Albumin 5%	3,61	cd	6,46	a	2,08	abc	5,41	ab	1,40	ab	3,71	ab
5%-A	4,47	de	7,13	a	2,64	abcd	5,48	abc	1,13	abc	3,13	ab
10%	6,81	gh	6,03	a	1,51	ab	4,96	a	1,43	abc	3,30	ab
10%-A	6,81	gh	5,32	a	0,98	ab	6,00	abcd	2,10	abcde	3,94	abcd
Calcium caseinate												
Casein 5%	5,11	ef	6,53	a	1,36	ab	5,90	abcd	1,48	abc	3,23	ab
5%-A	4,84	ef	6,70	a	3,48	cd	5,93	abcd	2,51	bcde	3,63	ab
10%	7,71	h	6,43	a	2,26	abcd	5,97	abcd	2,79	cde	2,63	a
10%-A	7,52	h	6,74	a	3,76	d	6,06	abcd	3,80	e	3,36	ab
Pea Protein Isolate												
Pea 5%	7,51	gh	5,83	a	1,62	abc	6,42	bcd	1,38	abc	4,80	bcd
5%-A	6,82	gh	6,65	a	1,54	ab	6,80	cd	1,29	ab	4,49	bcd
10%	6,04	fg	6,19	a	1,00	ab	7,04	cd	1,34	abc	5,86	cd
10%-A	7,19	gh	6,70	a	1,49	ab	6,84	d	2,62	cde	5,70	d
Soya Protein Isolate												
Soya 5%	4,96	ef	6,72	a	3,28	cd	6,05	bcd	1,54	abc	3,55	ab
5%-A	2,87	bc	6,60	a	2,54	abcd	6,36	bcd	2,18	abcde	3,84	abc
10%	7,09	gh	6,30	a	0,97	a	5,81	abc	1,29	a	4,07	b
10%-A	5,35	ef	6,74	a	2,86	bcd	6,11	abcd	3,09	de	3,97	ab
SD	0.5		0.7		0.8		0.6		0.7		0.75	

Value with a common letter in the same column are not significantly different ($p>0.05$);
SD: Standard deviation obtained from variance analysis

In general, bakery products based on starch with proteins of different sources are characterized by typical odour and taste of such ingredients (Ziobro et al., 2013). EA or PPI odour and taste intensity and persistency could adversely affect overall acceptability as happened to other authors (Crockett et al., 2011). Although the use of proteins significantly improves the nutritional value of gluten-free breads because of its enrichment in lysine and methionine (Marco & Rosell, 2008a), the addition of these proteins usually reduced its sensory acceptability (Ziobro et al., 2013). Acid addition improved significantly the volume and instrumental texture of protein-enriched breads without detriment of its odour and taste.

4. Conclusions

Gluten-free formulations based on rice starch in association with different proteins (egg albumin, calcium caseinate, pea protein and soy protein isolates) and acid addition led to dough matrices with variable acidification, fermentative, and thermal profiles, and subsequent different fresh and stored bread quality. Proteins exhibited a dosage dependent buffering capacity on doughs, particularly for caseinate, and counteracted, in general, the delay and decline of gas production and the inhibition of dough development in acidified doughs. Supplementation of GF doughs with vegetable proteins, that modified starch gelatinization transitions, led to lower volume and harder breads, effects being magnified with protein dose and reduced with acid addition. Incorporation of animal proteins gave different functional impact depending on the protein type, dosage and acidification. Albumin-added doughs provided significantly higher temperature and enthalpy of gelatinization than most of the other supplemented breads. It can be concluded that acid addition improved significantly the colour, volume and texture (instrumentally measured) of protein-enriched breads without impairment of sensory scores.

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CAPÍTULO IV

CHAPTER IV

IMPACTO DE LA ACIDIFICACIÓN Y ENRIQUECIMIENTO PROTEICO SOBRE LAS PROPIEDADES TÉRMICAS DE ALMIDONES DE ARROZ, PATATA Y TAPIOCA Y LAS PROPIEDADES REOLÓGICAS DE SUS GELES

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Impact of acidification and protein fortification on thermal properties of rice, potato and tapioca starches and rheological behaviour of their gels

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Abstract

The impact of acidification and non-gluten protein fortification (egg-albumin and soy-protein isolate) on thermal transitions of rice, potato and tapioca starches as well as the viscoelastic properties of their gels prepared at two casting temperatures, 90ºC and 120ºC, was investigated. The thermal and rheological behaviour of starches depended on their botanical origin and were significantly influenced by the presence and type of protein added as well as by the pH of the aqueous dispersion. Acidification to pH 4.5 increased the gelatinization temperature of rice starch in the presence of albumin or soy proteins, while reduced it in the case of tapioca starch, regardless of the presence of proteins. Acidification of rice starch dispersions decreased significantly the apparent gelatinization enthalpy; this effect was even greater in the presence of proteins. The addition of proteins brought about a structuring effect on tapioca gels leading to higher viscoelastic moduli and lower tan δ values. In general, acidification led to weaker gel structures, with more

pronounced effect for potato starch, most likely related to its higher phosphate content (charge screening). Much weaker gels were obtained at 120ºC compared to those processed at lower temperatures; however, protein incorporation reinforced gel structure, an effect that was not observed in gels formed at 90º, as also revealed by microstructure analysis using confocal scanning laser microscopy. In conclusion, protein addition and pH adjustments of aqueous starch dispersions can provide an effective means to modulate the functional and textural properties of gel-like starch-based gluten-free formulations.

Keywords: Acidification; starch; Thermal properties; Rheological properties of starch gels; Non-gluten proteins; Confocal scanning laser microscopy

1. Introduction

Gluten-free (GF) products are a growing processed foods sector and the related research activity constitutes a prioritized and challenging topic in the area of cereal-based products. The development of new GF food items is emerging not only because the daily dietary requirements for essential nutrients of celiac disease patients are not fully met at present by existing products, but also because of industry needs for product diversification (Mandala & Kapsokefalou, 2011). Moreover, the target group of GF products is currently expanding to include, in addition to clinically diagnosed celiac patients (1-3% of the population), people looking for non-allergenic ingredients, thus leading to a new market product category where a variety of items are being offered to the consumer.

Starch is a macro-constituent in many raw materials and processed foods with particular importance from both a nutritional and a functional point of view; in many formulated products its role is pivotal as texture modifier (Walkenström and Hermansson, 1998), contributing to many quality aspects of processed foods (Chen et al., 2015). Rice, potato and tapioca are among the most commonly used starches for texture

development in many gluten-free products. In almost all applications, starchy food matrices are heated and sheared during manufacturing. When the starch granules are heated in the presence of water several changes at a molecular and structural level occur and the ordered (partially crystalline) native granules swell and form a viscous dispersion (Biliaderis, 2009; Carlstedt et al., 2015). The temperature and energy required for these phase transitions are usually probed by differential scanning calorimetry (DSC) that monitors differences in structural organization and phase transition behavior among starches subjected to thermal treatments in aqueous media (Biliaderis, 1983, 2009; Roos, 1995; Singh et al., 2003). During thermal processing-manufacturing and consumption of starchy foods, hydrocolloid gels are formed and they are subjected to large deformations that may cause either irreversible deformation or structural failure due to fracture (Tabilo-Munizaga and Barbosa-Cànovas, 2005). Therefore, the study of rheological properties of starch gels is essential for proper handling and modification of sensory attributes of starch-based foods.

Rice starch is one of the most common ingredients used in GF products (Matos & Rosell, 2015), although it does not form self-supporting gel networks, even at concentrations above 20%, that are easily disintegrated (Abebe & Ronda 2014). Starches from tubers and roots, such as potato and tapioca, with notable higher amylose content, are also useful hydrocolloid alternatives in gel-like gluten-free formulations (Burrel, 2003).

Several methods to modify texture of starchy foods have been explored. Among them, acidification of rice-based food systems has been tested by means of addition of acetic or lactic acid (Blanco et al., 2011; Kasai et al., 2001) and their blends (Ronda et al., 2014; Villanueva et al., 2015), or their production from lactic acid bacteria fermentation in the form of sourdough (Moore, Del Bello & Arendt, 2008), which seems to be a promising alternative. Acetic acid increased the transparency, glossiness and stickiness and decreased the hardness of cooked rice (Kasai, Tanihata, Ohishi, Shimada & Hatae, 2001). Acidification also had an effect on complex systems such as rice based gluten-free doughs (Jekle &

Becker, 2012; Ohishi et al., 2007; Ronda et al., 2014), imparting the quality of the final baked products (Blanco et al., 2011; Jayaram et al., 2014; Villanueva et al., 2015). Moreover, acetic and lactic acids confer acceptable sensorial properties to GF breads in terms of odour and taste, either when produced by a starter culture or added as ingredients in bread formulations, leading to staling retardation (Moore, Del Bello & Arendt, 2008). This could be attributed to an increase in net protein charge, which enhances protein-solvent interactions and protein unfolding, but eventually prevents formation of a strong network structure, as noted for gluten-based doughs (Jayaram et al., 2014). The higher solubility and degradation of rice flour proteins in acetic acid than in distilled water might also accelerate the absorption, swelling and gelatinization of starch of rice flour (Ohishi et al., 2003).

Incorporation of exogenous proteins is another alternative to improve the final properties (physical, textural and nutritional) of starchy products; i.e. besides polysaccharides, proteins are known as structure- and texture-macromolecular modifiers in foods (Da Silva and Rao, 2007). Such enrichment is even more important for gluten-free formulations that usually have a poor protein/starch balance. Soy protein isolate (SPI) and dried egg albumin (EA) have been investigated in GF applications due to their nutritional quality and foam-stabilizing activity (Crockett et al., 2011; Marco & Rosell, 2008a, 2008b; Ronda et al., 2014; Villanueva et al., 2015). These globular proteins have the ability to heat-set in a gel state where a number of intra- and intermolecular disulfide bonds as well as hydrophobic interactions between nonpolar amino acid groups are involved (Chang et al., 2016). The structure of these gels is dependent on both pH and ionic strength (Brownsey et al., 1989). However, the impact of acidification on protein-enriched starch systems has been little studied. We have previously reported a structure weakening effect by acidification on protein-enriched rice starch bread doughs (Ronda et al., 2014) that led to improved texture of protein-enriched gluten free breads (Villanueva et al., 2015). However, the impact of proteins and the modulating effect of pH on the thermal properties of starches from different sources as well as the rheological behavior of their gels have not

been extensively studied so far. Also, pending and important for the development of autoclaved gel-like foods, is the evaluation of the effect of processing temperatures on formation of mixed starch-protein gels and their rheological properties.

Considering all these factors, the aim of the present work was to study the effect of decreasing the pH of starch dispersions to 4.5 on the thermal properties of rice, potato and tapioca starches and their mixtures with egg white and soybean protein isolate (at 10% by weight level) and on the rheological properties of their gels prepared at 90º (common cooking temperatures) and 120ºC (sterilization temperatures). Evaluation of the microstructure of the starch dispersions and gels was also carried out using confocal laser scanning microscopy (CLSM) for elucidation of their structural features. The knowledge gained from this work may help to modulate the formulation and improve texture of new gel-like gluten-free food products.

2. Material and methods

2.1. Materials

Rice starch (9.9 % w/w moisture, 0.5 % protein and <10% amylose content referred to total starch) and potato starch (19.1 % w/w moisture, 0.45 % w/w protein and 22% amylose referred to total starch) were supplied by Ferrer Alimentación S.A. (Barcelona, Spain), whereas tapioca starch (13 % moisture, 0.1% w/w protein, and 17% amylose content referred to total starch) was supplied by Cargill (Brenntag) S.L. Soy bean protein isolate was Supro 500-E IP (purity ~82%) from Proveedora hispano-holandesa S.A. (Barcelona, Spain) and egg albumin in dry powder form (purity ~92%) was from Eurovo (Valladolid, Spain). A sodium acetate (100 mM, pH 4.5) buffer was used to acidify the starch-protein dispersions; glacial acetic acid or 1.0 M NaOH (analytical grade), supplied by Panreac (Barcelona, Spain) were used to adjust the pH at the desired level. Double-distilled water was used to prepare all the solutions and aqueous hydrocolloid dispersions.

2.2. Methods

Starch dispersions and gel preparation

Starch gels were made by heating 20g of starch or starch+protein blend in 100 g of suspension in a hermetically sealed cylindrical stainless steel container (25 mm inner diameter and 60 mm height) at 90 or 120ºC. The protein content in the solid starch-protein mixtures was either 0 or 10 g/100 g of mixture which means 0 or 2 g of proteins in 100g of aqueous dispersion. When the buffer was added, the double distilled water was replaced by the 100 mM acetate buffer (pH=4.5). It was checked and found that in this case, the resultant pH in the dispersions was always 4.5. Gels were made by immersion of the stainless steel container containing the starch dispersion in a preheated oil bath (at 120ºC) or in a water bath (maintained at 90ºC) and kept there for 7 min to accomplish gelation of the starch/protein dispersions. During the heating period the device was shaken manually and once the time elapsed, the device was rapidly cooled by immersion in a water bath at 18 ± 3 ºC for 5 min, without loss of water or any mechanical damage (lack of shear) of the preformed upon heating gel network.

Thermal properties

Differential scanning calorimetry (DSC) measurements were carried out with a PL DSC-Gold calorimeter (Polymer Labs. Ltd, Epsom, UK). Samples of about 4.5-6.5 mg dry matter of starch or starch-protein mixtures were dispersed in water or in the acidified medium (20% w/w dispersions) and sealed hermetically into 120 µL DSC medium pressure stainless steel pans (ME-29990, Mettler, Toledo, SAE). The dispersions were made either with distilled water or with a 100 mM acetate buffer (pH=4.5). Samples were scanned from 10 to 125 °C at 5 °C/min using an empty pan as reference. The onset, endset, and peak temperatures, T_o, T_e, T_p (ºC), or temperature values at which the transition starts, ends or gives the maximum signal, respectively, and the apparent enthalpy, ΔH (J/g of starch), of starch gelatinization and amylose-lipid dissociation endotherms were evaluated from the thermograms. Reported values are the means of at least duplicate measurements for each system.

Rheological measurements

The gel cylinders were cut into slices and placed between the parallel plates (serrated upper plate with 25 mm diameter) of a Physica MCR 300 rheometer (Physica Messtechnic GmbH, Stuttgart, Germany). Rheological tests were carried out with 1 mm gap. Before the measurement, the gel was rested for 10 min to allow sample relaxation. Strain sweeps were carried out from 0.01 to 1000% strain at 1 Hz frequency. From strain/stress sweep tests of gels the maximum stress, τ_{max}, was also calculated as the stress at which the elastic modulus of the gels dropped in the LVR by 10%. Frequency sweeps were carried out from 0.1 to 100 Hz in the linear viscoelastic region (LVR); the stress value chosen for the frequency sweep tests of all gels was 0.5 Pa, which was in the LVR. A constant temperature of 25ºC was chosen and maintained by the controlled Peltier system of the rheometer (TEZ 150P/MCR). Data analysis was carried out by the supporting software US200 V2.21 of the rheometer. Frequency sweep data were fitted to the power law model as in previous works (Ronda et al., 2011; 2014). The recorded viscoelastic parameters, G_1' and G_1'', and $(\tan \delta)_1$, represent the elastic and viscous moduli and the loss tangent, respectively, at a frequency of 1Hz. The a and b exponents quantify the dependence of the dynamic moduli on the oscillation frequency (power law model). Each test was carried out at least in duplicate.

Confocal Laser Scanning Microscopy

A Leica TCS SP5 confocal laser scanning microscope (CLSM), mounted on a Leica DMI 6000B inverted microscope base, was operated in the fluorescence mode with a 60x oil-immersion objective of numerical aperture 1.40. The starch and protein phases were stained with Nile Blue. Fluorescence from the sample was excited with the 633 nm of a red HeNe laser line. The signal from the samples was collected and eight scans were averaged for the creation of each image. For granular starches or the mixed starch-protein aqueous dispersions, samples of ~ 5ml were transferred into a small beaker. A 10µl aliquot of Nile Blue solution (0.01% w/v) was added and the dispersions were thoroughly mixed. In the case of gels, the starch or starch/protein dispersions were initially

stained with the dye and afterwards the gels were formed as described in section 2.2.1.

Statistical analysis

Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) allowed performance of ANOVA analysis of the data; the LSD (Least Significant Difference) test was used to evaluate significant differences ($p < 0.05$) between samples.

3. Results and discussion

3.1. Thermal properties

The thermal properties of different aqueous starch dispersions (at 20 % w/w total solids) as influenced by pH and the presence of proteins are shown in Figure 1 and Table 1. Potato and tapioca starch exhibited one endothermic peak on the DSC thermograms, while rice starch and its mixtures with proteins showed two peaks; i.e. the main endotherm related to starch gelatinization and a much smaller peak at higher temperature due to the amylose-lipid complex dissociation (Biliaderis 2009; Eliasson, 1994). The temperature values of the latter (second) peak (rather broad transition) appeared slightly lower in the buffered dispersions than in distilled water (83.8ºC, 95.2ºC and 106.7ºC versus 84.8ºC, 96.1ºC and 108.1ºC for T_o, T_p, T_e, respectively); the dissociation enthalpies of the amylose-lipid complex in both types of rice starch dispersions (2.1 versus 1.9 J/g starch) were not significantly different. For the gelatinization peak, T_p, the highest temperature corresponded to tapioca (72.6 ºC) and the lowest to potato starch (64.5 ºC), and an intermediate value for rice starch (68.6ºC) (Table 1). Similar findings were reported by other authors (da Cruz Francisco et al., 1996; Singh et al., 2003). Potato starch exhibited the highest apparent gelatinization enthalpy values (13.3 J/g starch), followed by tapioca starch (10.6 J/g starch), whereas the lowest values corresponded to rice starch suspensions (6.9 J/g starch). The differences observed may be attributed to differences in several factors such as size, shape and distribution of starch granules, amylose/amylopectin ratio (that followed the same

order, potato>tapioca>rice, with gelatinization enthalpy values), macromolecular assemblies of starch polymers within the granule and the presence of internal monoacyl lipids, in the case of rice starch, which upon heating form complexes with amylose, an exothermic process that reduces the 'apparent' gelatinization enthalpy of the starch crystallites (Biliaderis, 2009; Schirmer et al., 2013). The enthalpy value reflects the composite thermal energy effect resulting from melting (endothermic) of starch crystallites as well as the amount of heat associated with formation (exothermic) or disruption (endothermic) of short range order (e.g. amylose lipid complexes) in the heated starch matrix (Biliaderis, 2009).

The presence of proteins at the weight ratio used in the mixed systems (10% with respect to the solid starch-protein mixture) did not seem to significantly alter the T_{onset}, T_{peak}, T_{end} and ΔH of starch gelatinization endotherm (Table 1), although the analysis of variance did show significant interaction effects (protein x starch and starch x protein x pH) (Table 1); this means that the effect of proteins depended on starch type and pH of the dispersion. The apparent gelatinization enthalpy values of different starches significantly increased in the presence of soy protein in both pure aqueous (distilled water) and acidified dispersions of potato starch, as well as for egg albumin in the acidified dispersions of tapioca starch, while ΔH decreased when both proteins were added in the acidified dispersions of rice starch; no change in ΔH values was observed in the remaining mixtures of starches-proteins. It is worth noting that calorimetry of the protein samples alone (25 % w/w aqueous dispersions) did not show any endothermic peak in the DSC thermograms over the same temperature range (data no shown). This means the proteins used in this study were completely denatured as a consequence of their manufacturing process; otherwise some endothermic transitions would instead occur as found for native protein systems (e.g. a double endothermic transition in the case of SPI, corresponding to 7S and 11S globulins of oilseeds and legume seeds, respectively; Biliaderis, 1983).

Acidification of the starch dispersions did not have a significant single effect on the gelatinization thermal transition parameters (Table 1). An influence was only noted, depending on the starch source and the type of

starch-protein mixture used, through significant interactions effects (starch x pH and starch x protein x pH). At pH 4.5, the gelatinization enthalpy of the rice starch suspension was significantly decreased (~26 %) and this effect was more pronounced in the presence of proteins (-29% and -35% for EA and SPI, respectively). Similar effects of acidification were recently reported for rice starch by Colussi et al. (2015). However, no significant effects of the acidified medium on potato and tapioca starch gelatinization enthalpy were noted (Table 1). These two tuber starches have similarities between them and differ from rice starch (e.g. significantly higher amylose content and absence of lipids) that could explain their similar behaviour upon acidification versus the rice starch. On the other hand, differences between starches from potato and tapioca, such as the higher phosphorus content and the longer average chain length of amylopectin in the former (da Cruz Francisco et al., 1996), may explain differences between their gelatinization temperature and enthalpy values (Table 1); for tapioca starch higher T_o, T_p, and T_e values and lower ΔH were found compared to those from potato.

The change of the dispersion medium from distilled water to acetate buffer (pH 4.5) increased the gelatinization temperature, T_p, of rice starch by approximately 2ºC in the presence of EA or SPI proteins, while it did not exert any effect on pure rice starch dispersions.

Table 1. Starch gelatinization parameters of rice, potato and tapioca starches and their mixtures with egg albumin and soy protein isolate in aqueous (distilled water) and pH 4.5 (acetate buffer 100mM) dispersions (20 % w/w).

Starch	Protein	Acetate Buffer	T_o (ºC)	T_p (ºC)	T_e (ºC)	ΔH (J/g starch)
Rice	0	0	60.3 abcd	68.6 c	77.2 bcd	6.9 b
		1	59.9 abc	68.8 c	77.0 bcd	5.2 a
	EA	0	59.9 abcd	68.9 c	77.8 cd	7.0 b
		1	63.5 fgh	70.7 de	77.6 cd	5.0 a
	SPI	0	61.0 bcdef	68.4 c	78.1 d	7.8 bc
		1	62.1 cdefg	70.1 d	76.9 bcd	5.1 a
Potato	0	0	59.3 ab	64.5 a	75.9 bc	13.3 hij
		1	59.6 abc	64.9 ab	78.7 de	13.7 ij
	EA	0	59.3 ab	64.7 ab	75.2 b	14.3 jk
		1	57.9 a	64.2 a	72.4 a	14.6 jk
	SPI	0	60.1 abcd	64.5 ab	78.5 d	15.4 kl
		1	60.5 abcde	65.5 b	80.8 ef	16.6 l
Tapioca	0	0	63.1 efgh	72.6 f	81.2 f	10.6 def
		1	60.3 abcd	70.6 de	82.2 f	9.3 cd
	EA	0	64.0 gh	72.8 f	82.9 f	11.8 fgh
		1	62.6 defg	71.3 e	85.5 g	12.4 ghi
	SPI	0	65.3 h	73.0 f	83.1 f	11.2 efg
		1	61.0 bcdef	70.9 de	82.3 f	9.7 de
SE			0.90	0.36	0.77	0.56
Analysis of variance and significance (p-values)						
Factor 1 (starch)			***	***	***	***
Factor 2 (protein type)			ns	ns	ns	ns
Factor 3 (pH)			ns	ns	ns	ns
Factor 1x2			ns	ns	***	*
Factor 1x3			**	***	ns	**
Factor 2x3			ns	ns	ns	ns
Factor 1x2x3			***	***	***	***

Protein: 0: without protein; EA: egg albumen; SPI: soy protein isolate. Acetate buffer (pH=4.5): 0: without acetate buffer (aqueous medium); 1: Acetate buffer medium. Different letters in the corresponding column indicate statistically significant differences between means at $p<0.05$. SE: Pooled standard error obtained from ANOVA analysis. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant.

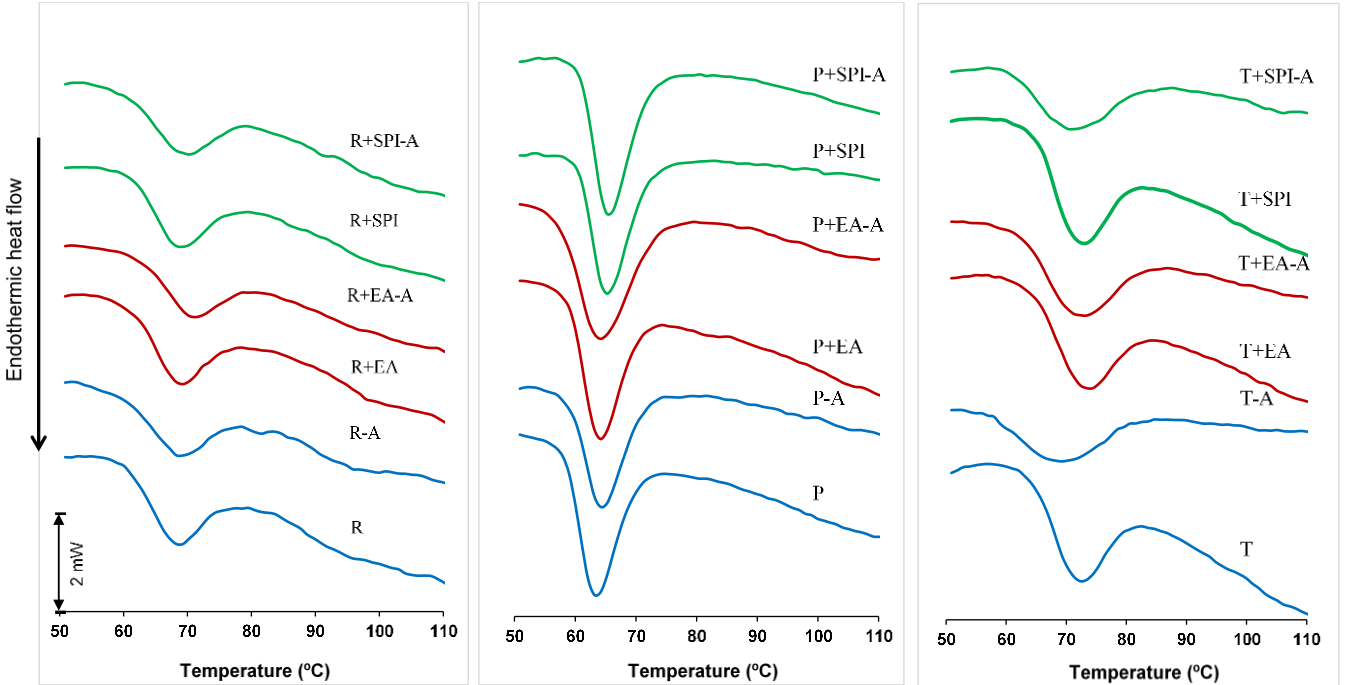


Figure 1. DSC thermograms obtained from aqueous (distilled water) or acidified (acetate buffer 0,1M pH 4.5) dispersions of different starches (rice, potato and tapioca) and their mixtures with proteins (egg albumen or soy protein isolate). R: rice starch; P: potato starch; and T: tapioca starch. EA: egg albumin; SPI: soy protein isolate. -A: Acidified dispersion.

Ohishi et al. (2007) found that addition of 0.2 M acetic acid resulted in a significantly lower gelatinization temperature (~ 1ºC) for rice starch obtained from a brown rice japonica variety. The authors attributed this shift to acceleration of water absorption by the starch granules (i.e. promotion of its hydration by adding acetic acid). Hibi (2002) also reported that the endothermic peak temperature of rice starch was lower in the presence of 0.33M acetic acid. In both previous cases the resultant pH of the heated starch dispersions was not reported by the authors, but it should be lower than that of the present study, where the rice starch dispersion was adjusted to pH 4.5; this might explain why we did not observe any effect on gelatinization temperature for the pure rice starch dispersions. In contrast, acidification of tapioca starch dispersions facilitated the gelatinization, as demonstrated by the lower onset and peak temperatures of the acidified starch samples, compared to their distilled water counterparts, regardless the presence or absence of proteins (Table 1). Acidification of tapioca starch also led to a wider gelatinization range, i.e. (Te-To) difference, implying a more complex phase transition behaviour (Table 1 and Figure 1).

3.2. Rheological properties

Strain sweeps

The responses of viscoelastic moduli of starch gels, prepared at 90ºC and 120ºC as a function of strain are shown in Figures 2 and 3, respectively. In rice starch gels it was possible to discriminate two different regions, the linear viscoelastic region in which G' and tan δ (data not shown) values were practically constant, and the non-linear domain in which G' started to decrease, and at the same time the tan δ began to increase with increasing strain until the curves of G' and G'' intersected (tan δ=1). However, for tapioca (at 90ºC and 120ºC) and potato starch (at 120ºC) gels, the loss tangent was maintained well below 1 over the entire strain range, revealing no cross-over of the G' and G'' curves. The maximum stress, τ_{max}, that gels can resist before structure disruption (end of LVR) is depicted in Table 2.

Figures 2 and 3 show that the elastic modulus (G') of pure rice gels (formed at both 90ºC and 120ºC) yielded at lower strain values than tapioca and potato starch gels, indicating a more susceptible structure which breaks easier under the applied stress than those of tuber starch gels. In fact, it was not possible to determine the maximum stresses, τ_{max}, of the latter gels over the entire strain range recorded. Rice starch gels formed at 90ºC raised their τ_{max} values with EA protein addition (Table 2). On the other hand, fortification with SPI and acidification (pH = 4.5) of starch gels prepared at 90ºC and 120ºC did not significantly affect this parameter. With increasing gel setting temperature there was decreased gel resistance to breakage; i.e. much lower τ_{max} (between 35% and 100% decreased values) for gels formed at 120ºC than their respective samples prepared at 90ºC. Previous works showed similar effects and reported a significant decrease of the rigidity and strength of gels when prepared at autoclaving temperatures (Christianson et al., 1986; Doublier et al., 1987). Due to the high solids content of the heated aqueous starch dispersions (20 % w/w) and the lack of shear forces during heat-setting of the gel network structures it is unlikely that extensive phase separation involving amylose leaching out of the swollen granules takes place. However, chain ordering (short scale crystallization) of the linear starch fraction in the high temperature casted gels, forming the continuous phase, could be more pronounced and it might contribute to the observed weakening of the network structure for these samples (Biliaderis, 2009). In the mixed gels, the included proteins most likely partition at the intergranular regions following swelling of the granules upon gel setting.

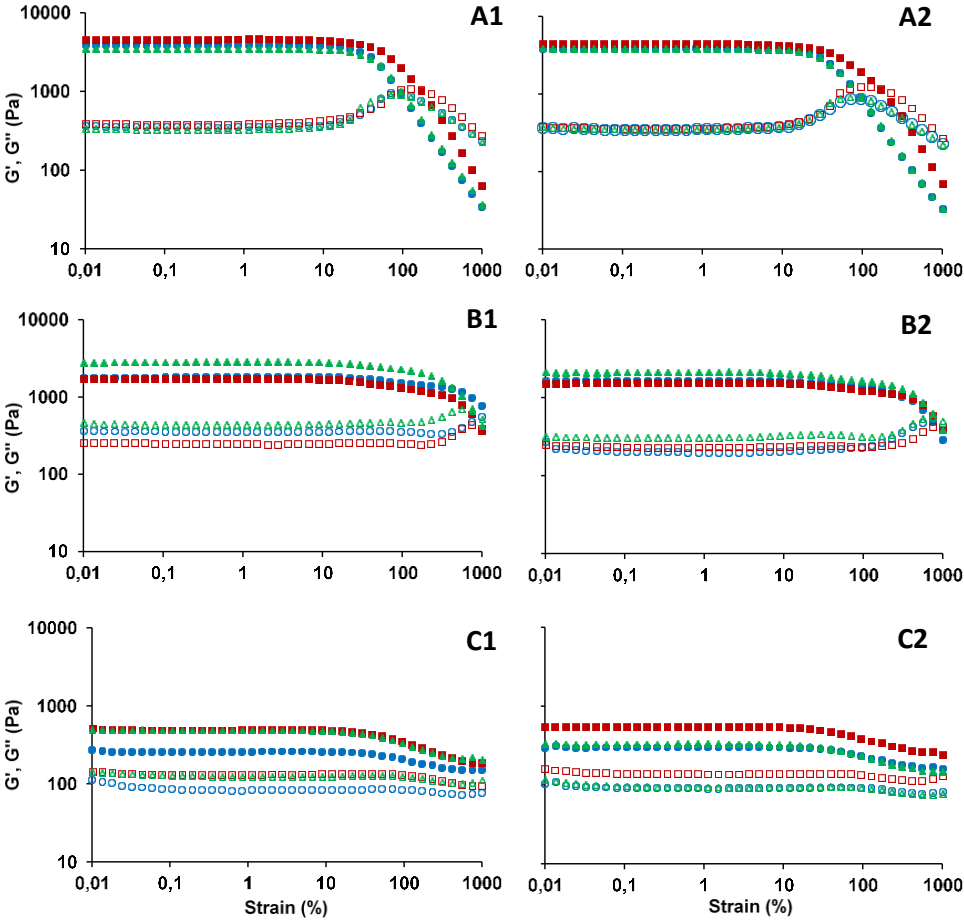


Figure 2. Strain sweeps of rice (A), potato (B) and tapioca (C) starch gels prepared at 90ºC using distilled water (A1, B1, C1) and acetate buffer (pH 4.5) (A2, B2, C2), without protein addition (circle symbols), with egg albumin (square symbols) or soy protein isolate (triangle symbols). Elastic modulus, G', is represented by solid symbols and viscous modulus, G'', by open symbols.

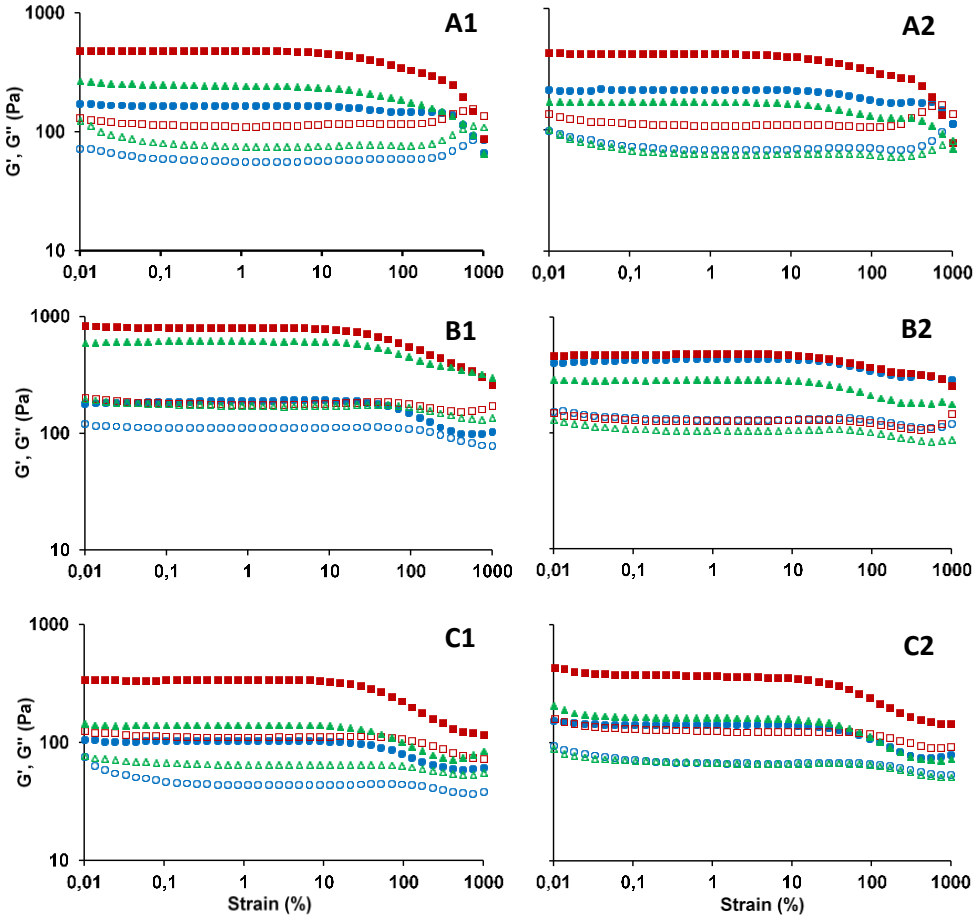


Figure 3. Strain sweeps of rice (A), potato (B) and tapioca (C) starch gels prepared at 120ºC using distilled water (A1, B1, C1) and acetate buffer (pH 4.5) (A2, B2, C2), without protein addition (circle symbols), with egg albumin (square symbols) or with soy protein isolate (triangle symbols). Elastic modulus, G' is represented by solid symbols and viscous modulus, G'', by open symbols.

Frequency sweeps

The viscoelastic moduli at 1 Hz, G1', G1'', and (tan δ)1 and the obtained exponent "a" and "b" values by fitting the power law model to the frequency sweep data (from 0.01 to 10 Hz) are summarized in Table 2. Frequency sweeps showed that G' exceeded G'' over the entire frequency range for all the gels tested, with the tan δ values being low enough (Table 2) to classify such rheological responses as being of "true" gels (Krystyjan et al., 2015; Ribotta and Rosell, 2010). The natural logarithms of G1' and G1'' were used for the ANOVA analysis, allowing more conclusive results on the impact of the studied factors (Ronda et al., 2011).

The elastic and viscous moduli were much higher in gels formed at 90ºC than their counterparts formed at 120ºC, whereas the loss tangent increased markedly showing a transition from a 'rigid' (tan δ1 ≈ 0.1) to a 'weak' (tan δ1 ≈ 0.4) type of gels with increase of casting temperature (Table 2). Christianson et al. (1986) found that rice, corn and wheat starch gels prepared at 121 ºC had a much higher fraction of starch molecules released from the gel (obtained in the hot filtrate), not solubilized but rather dispersed, than when the temperature was 80ºC or 94ºC. This could be attributed to the 'weakening' of the gel structure due to extended deformation and rupture of the swollen starch granules, allowing demixing of the two starch polymeric fractions (amylose and amylopectin) that could count for the lower consistency of gels prepared at 120ºC.

Viscoelasticity of gels prepared at "cooking" temperatures (90ºC)

Rice starch provided gels with the highest consistency (3182-5094Pa for G_1'), particularly when compared to tapioca starch; these results concur with the findings of Ronda et al. (2011), who observed the highest moduli for layer cake batters made from rice starch compared to potato, corn and wheat starches.

The rheological properties of gels prepared from the protein-starch blends also showed significant interaction effects (starch x protein) (Table 2), suggesting that the same protein exerted different effect depending on the starch source. Ribotta and Rosell (2010) observed

different effects of SPI on the viscoelastic properties of corn and tapioca starch gels, demonstrating an increase in G' and G'' and a decrease in tan δ of tapioca starch gels as a result of SPI addition that was not noted in the case of corn starch gels. The data of the present study pointed to a similar impact of SPI on tapioca and potato starch gels, and just an opposite effect on rice starch gels (Table 2). With the EA addition, there was a significant increase in the elastic and viscous moduli of rice starch gels, while it decreased potato gels consistency. Tapioca gels, which were the less structured and exhibited viscoelastic moduli five to ten times below those of the potato and rice starch gels, showed a significant increase of consistency when both proteins, SPI and EA, were added (with increases up to ~70% in G_1' and ~ 47% in G_1'' values).

Acidification of starch dispersions to pH 4.5 always weakened potato gels prepared at 90ºC, regardless the presence and type of protein added. However, only the SPI-enriched tapioca and rice gels exhibited a significant change in consistency, with a notable decrease in G_1' and G_1'' moduli, as a consequence of dispersion acidification. The effect of pH on viscoelasticity of SPI-added gels was dependent on starch source, which concurs with the significant interaction effect found (starch x protein x pH). Acidification of SPI-starch dispersion promotes a change in the collective charge of soybean protein (pI~4.5-5.0) leading to decreased strength of the protein-protein interactions and an increase in the surface of contact with the medium (Ribotta and Rosell, 2010).

Table 2. Rheological properties of gels of rice, potato and tapioca starches and their mixtures with egg albumin and soy protein isolate prepared from both aqueous (distilled water) and pH 4.5 buffered dispersions at two different temperatures; all viscoelastic parameters were measured at 25ºC.

Starch	Protein	Acetate Buffer	G_1' (Pa)		a		G_1'' (Pa)		b		tan δ_1		τ_{max} (Pa)	
			90 ºC	120 ºC	90 ºC	120 ºC	90 ºC	120 ºC	90 ºC	120 ºC	90 ºC	120 ºC	90ºC	120ºC
Rice	0	0 (6.39)	3955 i	212 b	0.057 a	0.187 c	373 hij	70 bc	0.306 h	0.362 cdef	0.094 bc	0.334 def	1196 a	886 ab
		1	4090 i	199 b	0.055 a	0.187 c	381 ijk	66 b	0.306 h	0.368 def	0.093 abc	0.333 de	1102 a	1264 b
	EA	0 (6.68)	4697 j	615 g	0.053 a	0.131 a	402 jk	132 fgh	0.192 a	0.281 a	0.085 ab	0.217 a	2159 b	1061 ab
		1	5094 j	502 fg	0.053 a	0.137 ab	421 k	124 efg	0.200 bc	0.305 ab	0.083 a	0.245 ab	2192 b	1232 b
	SPI	0 (7.07)	3182 h	282 c	0.061 a	0.180 c	314 g	88 d	0.274 f	0.336 bcd	0.099 c	0.312 cde	1239 a	600 a
		1	3668 i	186 b	0.058 a	0.186 c	354 hi	67 bc	0.296 g	0.365 def	0.095 bc	0.360 efg	1101 a	793 ab
Potato	0	0 (7.34)	2375 g	377 de	0.099 c	0.231 e	346 gh	152 i	0.205 c	0.345 cd	0.157 e	0.423 ij	nd	nd
		1	1862 d	600 g	0.078 b	0.135 ab	229 d	139 ghi	0.221 d	0.309 ab	0.124 d	0.236 ab	nd	nd
	EA	0 (6.73)	2109 fg	504 fg	0.083 bc	0.161 abc	271 f	142 hi	0.196 ab	0.305 ab	0.129 d	0.285 bcd	nd	nd
		1	1867 de	474 f	0.080 b	0.162 abc	238 de	139 fghi	0.200 bc	0.336 bcd	0.127 d	0.307 cde	nd	nd
	SPI	0 (7.20)	3014 h	317 cd	0.080 b	0.227 de	380 ij	120 ef	0.192 ab	0.359 cde	0.126 d	0.388 fghi	nd	nd
		1	2197 ef	561 fg	0.078 b	0.143 ab	257 ef	141 ghi	0.199 bc	0.311 ab	0.123 d	0.259 abc	nd	nd
Tapioca	0	0 (4.94)	336 a	125 a	0.157 e	0.275 f	99 a	56 a	0.314 i	0.396 fg	0.294 gh	0.446 jk	nd	nd
		1	320 a	134 a	0.169 e	0.251 ef	97 a	66 bc	0.321 i	0.413 g	0.303 h	0.488 k	nd	nd
	EA	0 (6.77)	570 bc	428 ef	0.141 d	0.167 bc	145 c	141 ghi	0.266 e	0.303 ab	0.253 f	0.325 de	nd	nd
		1	604 c	290 c	0.138 d	0.193 cd	150 c	110 e	0.275 f	0.358 cde	0.246 f	0.364 efgh	nd	nd
	SPI	0 (7.19)	514 b	183 b	0.138 d	0.233 e	132 b	77 cd	0.276 f	0.329 bc	0.256 f	0.418 hij	nd	nd
		1	348 a	184 b	0.166 e	0.233 e	99 a	74 bc	0.307 h	0.381 efg	0.285 g	0.405 ghij	nd	nd
SE			99	36	0.005	0.012	11	7	0.003	0.013	0.004	0.02	150	184
Analysis of variance and significance (p-values)														
Factor 1 (starch)			***	***	***	***	***	***	***	**	***	***	ns	ns
Factor 2 (protein type)			ns	***	ns	***	ns	***	***	***	ns	***	***	ns
Factor 3 (pH)			ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
Factor 1x2			***	***	*	ns	***	***	***	**	***	ns	nd	nd
Factor 1x3			**	***	*	***	***	ns	ns	**	*	***	nd	nd
Factor 2x3			ns	ns	ns	*	ns	ns	ns	ns	ns	*	ns	ns
Factor 1x2x3			***	***	***	***	***	***	***	***	***	***	nd	nd

Protein: 0: Without protein; EA: egg albumen; SPI: soy protein isolate. Buffered: 0: NO (aqueous suspension, into brackets the measured pH); 1: YES (pH 4.5 acetate buffered suspension); Different letters in the corresponding column indicate statistically significant differences between means at p<0.05. nd: Not determined. SE: Pooled standard error obtained from ANOVA analysis. Analysis of variance and significance: *** p<0.001. ** p<0.01. * p<0.05. ns: not significant.

The different effects of acidification on viscoelastic properties, depending on starch source, were also related to the phosphate groups present in some tuber starches; potato starch exhibits the highest content of phosphate ester residues (Hoover, 2001). The granular shape and size, the presence of lipids (phospholipids) and phosphate monoester residues on amylopectin, and the amylose content seem to be among the most important factors that significantly affect the rheological properties of different starch gels (Biliaderis, 2009; Singh et al., 2003). The effect of pH on the viscosity of potato starch gels could arise from formation of a counter-ion layer at the phosphate groups on the surface of the potato starch granules (Muhrbeck and Eliasson, 1987). These counter-ions diminish the electric charge of the starch molecules, thus blocking the extensive repulsion forces among the phosphate groups. This was not the case for tapioca starch gels, where the viscoelastic properties were not largely affected by the pH; apparently, tapioca starch has much lower phosphate content than potato starch (Hoover, 2001).

The loss tangent of rice starch gels was below 0.1 for all tested samples (Table 2), implying a well physically cross-linked network structure. The very low values of "a" exponent, near zero, for rice and potato starch gels mean that the elastic modulus, G', was hardly dependent on frequency, indicating a stable gel structure. With exception of rice starch gels, the presence of protein in all mixed starch/protein preparations decreased the tan δ regardless of the protein type. The addition of protein led to a network that shifted to a more elastic-like behaviour and a more structured gel (Ribotta et al., 2012). The tapioca starch gels had the highest values of loss tangent, which means the viscous modulus was higher with respect to the elastic one than in the remaining systems (Da Silva and Rao, 2007). The elastic moduli of tapioca gels also showed the highest dependence on frequency, as shown by the highest "a" exponent values, among the gels made by different starch sources.

Rheological properties of gels prepared at autoclaving temperatures (120ºC)

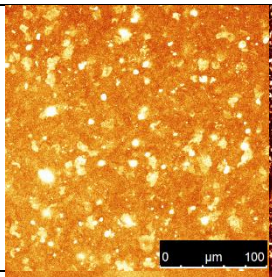
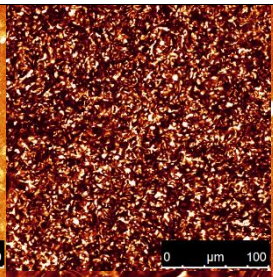
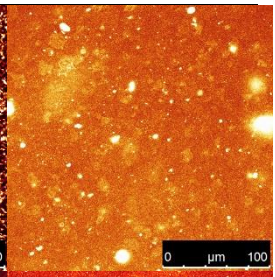
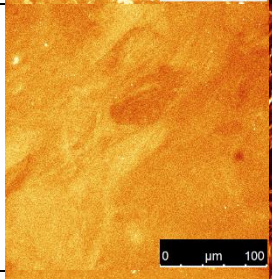
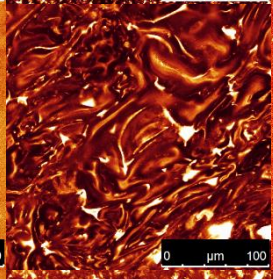
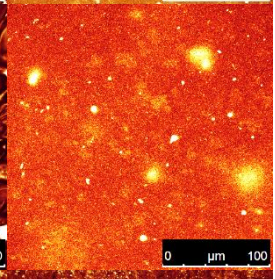
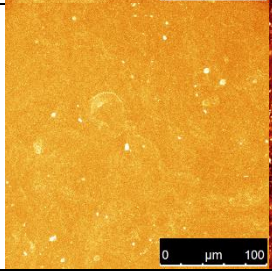
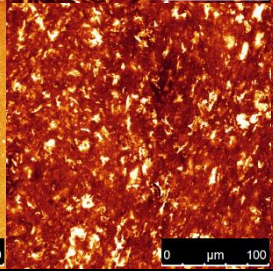
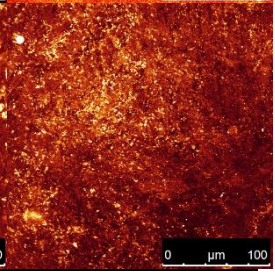
Table 2 also shows the values of viscoelastic parameters for gels casted at 120ºC. At this temperature, potato starch gave the highest consistency gels. Acidification of aqueous dispersions of potato starch increased the elastic moduli (59%) and decreased tan δ (44%), meaning a reinforcement of the gel network structure obtained at 120ºC, while no effect was observed for all other starch gels. The high phosphate content of potato starch might be the origin of the different effect of pH on gels prepared at such harsh conditions due to the formation of a counter-ion layer at the phosphate groups on the surface of the potato starch granules (Muhrbeck and Eliasson, 1987). The presence of protein in general reinforced all the gels prepared at 120ºC (Table 2). Rice and tapioca gels exhibited a significant increase in the elastic and viscous moduli when protein was added, with increments being larger when EA was incorporated (190 % and 88 % for rice and 234% and 151% for tapioca for G_1' and G_1'', respectively) than with SPI (33 % and 26 % for rice and 47% and 37% for tapioca). Incorporation of EA also affected the potato starch gels (distilled water as medium), increasing their elastic modulus, G_1' (34%), and decreasing the loss tangent (30%). Lowering the pH of protein-enriched starch suspensions had a different effect depending on the protein and the starch source, as revealed by the significant interaction effects (starch x protein x pH) (Table 2). Acidification of the SPI-potato starch dispersions strengthened the structure of the gel formed as shown by the increase of elastic modulus (77%) and the decreased tan δ value (33%). On the contrary, acidification of rice gels supplemented with SPI, resulted in reduction of both elastic and viscous moduli (34% and 24%, respectively) with a concomitant increase in tan δ (15 %). The opposite effect was reported in the previous section for rice and potato gels at 90ºC, implying that the temperature of gel setting also plays an important role on the viscoelastic properties of mixed starch/protein gel systems, especially when SPI is present in the composite gel matrix.

3.3. Microscopy of starch granule dispersions and gels

CLSM imaging has been employed to investigate the microstructure of starch dispersions and starch gels formed with or without addition of egg

albumin and soy protein isolate at 90ºC and 120ºC (Fig.4). CLSM did not reveal differences between aqueous and acidified granular starch dispersions (pictures are not shown). The micrographs showed the typical size and shape of starch granules for each species which have often been reported in the literature (Delcour and Hoseney, 2010; Schirmer et al., 2013; Van de Velde et al., 2002). Potato starch displayed mostly large oval or round granules, ranging from 15 to 100 μm, compared to the much smaller rice starch granules which varied in the range of 2-8 μm. Moreover, rice and tapioca starch consisted of a mixture of truncated and round granules with the largest granules being round and the smaller ones truncated, while the shape of tapioca starch granules was more uniform and their size varied between 5 and 40 μm.

Upon heating the starch or protein-starch dispersions, the type of starch and composition of the mixed dispersions had a large impact on the system's microstructure (Biliaderis, 2009). That is, heating a diluted starch or protein-starch dispersion in distilled water or in acid medium, at 90 or 120ºC for 7 min, resulted in pronounced deformation (swelling, rupture and collapse) of the granules with a subsequent amylose leaching out of the swollen granular structure, as shown in Fig.4. The microstructures of the different gels seem to be dependent on the type of starch used, the addition of protein, and the composite mixture of the biopolymers involved in the mixed gel networks. Specifically, upon heating, when the dispersions consisted of solely starch, discrete structural elements with a spherical-like shape were detected. The size and the number of these entities were dependent on the starch type and the temperature employed for gelation; i.e., the gels from rice starch had more pronounced structural features which became more globe-shaped and of smaller size at 120ºC. On the other hand, the potato starch gels displayed more uniform ('smooth') microstructure than the other two types of starch.

A) 90ºC	Without protein	With EA	With SPI
Rice			
Potato			
Tapioca			

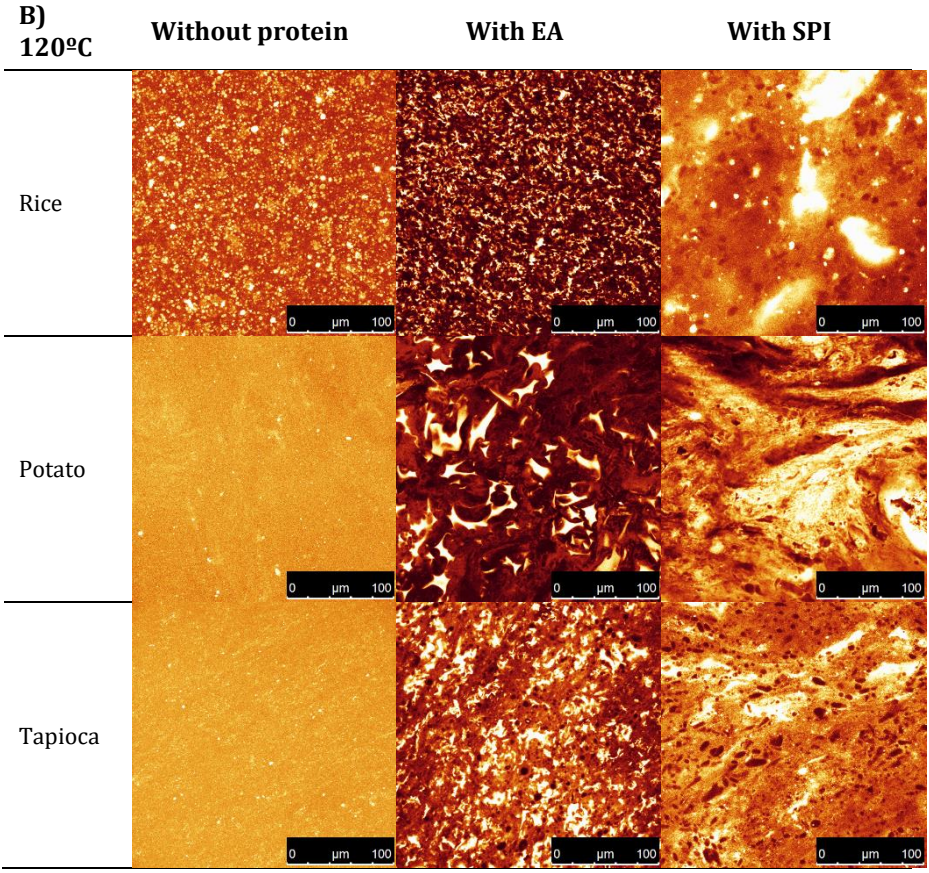


Figure 4. Confocal laser scanning micrographs of starch gels prepared at 90ºC (A) and 120ºC (B). EA: Egg albumin; SPI: Soy protein isolate.

The microscopic observations clearly showed that the incorporation of proteins in starch dispersions resulted in different microstructures. As can be seen in Fig.4, the final microstructure was not predominantly determined by one of the individual biopolymers used in this study. Although equal amounts of protein and starch were used in making the primary dispersions, the divergent microstructures formed indicate the strong impact of each specific combination of the two biopolymers involved in the final mixed gelling system.

4. Conclusions

The addition of egg albumin and soy protein isolate at 10% (by weight in the solid starch-protein mixtures) combined with a reduction of pH to 4.5 proved to be an efficient way to modify the thermal and rheological properties of rice, potato and tapioca starches and starch gels. Gels prepared at 120 °C were much weaker (lower consistency and more prone to break-down upon deformation) than those formed at 90 °C; the rice starch being the most sensitive to increasing gelification temperature and potato the most resistant. In general, proteins worked as structure enhancing agents at both temperatures; in gels prepared at 120ºC the strengthening effect was more pronounced. In contrast, acidification weakened the structure of these gels. It can be concluded that both pH and addition of exogenous proteins are useful approaches to modify the functional properties of gel-like starchy food products. The rheological observations made in this study could be helpful in attempting to modify gluten-free formulations, which are mostly based on non-wheat cereal or tuber starches to optimize the end-product quality attributes (texture, microstructure, thermal stability of autoclaved food products to extend their shelf life). Further studies are needed to clarify the role of acidification into heated starch-protein mixtures by employing a range of structure probing analytical techniques (calorimetry, various rheological tests, chromatography, different spectroscopies, etc.) to unravel the molecular interactions among the hydrocolloids involved and thereby fine tune end-product quality.

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SECCIÓN II

SECTION II

ESTRUCTURACIÓN MEDIANTE TRATAMIENTOS FÍSICOS DE LA HARINA: IMPACTO DEL TRATAMIENTO HIDROTÉRMICO ASISTIDO CON MICROONDAS SOBRE LA MODULACIÓN DE LAS PROPIEDADES TECNO-FUNCIONALES DE SISTEMAS MODELO Y SISTEMAS PANARIOS

CAPÍTULO V

CHAPTER V

CAPACIDAD DE ABSORCIÓN DE MICROONDAS DE LA HARINA DE ARROZ. IMPACTO DE LA RADIACIÓN SOBRE SU ESTRUCTURA Y SUS PROPIEDAES TÉRMICAS Y VISCOMÉTRICAS

Villanueva, M., Harasym, J., Muñoz, J.M., Ronda, F. 2018. Microwave absorption capacity of rice flour. Impact on the radiation on rice flour microstructure, thermal and viscometric properties. Journal of Food Engineering, 224, 156-164.

Microwave absorption capacity of rice flour. Impact of the radiation on rice flour microstructure, thermal and viscometric properties

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Abstract

The microwave radiation thermal treatment of rice flour was studied and its impact on physical and structural characteristic in relation to the initial moisture content (IMC) (20% and 30%) was evaluated. To explain the fundamentals of observed changes the microwave radiation absorption capacity of flour as well as temperature and moisture change during the treatment were evaluated. The flour particle morphological structure as well as crystallinity/amorphous region ratio changed after the treatment. The flour thermal properties also altered revealing IMC significant impact on the gelatinization temperature, that rised up to 3ºC, and the amylopectin retrogradation extent that increased up to a 7% in the most intense microwave-treated flours with respect to the native flour. Lower peak, setback and breakdown viscosities -that decreased with respect to the native flour up to 42%, 34% and 86% respectively- and higher pasting temperatures -that increased up to 10 ºC- were also

observed. An exceptional microwave irradiation efficiency resulting in rice flour physical changes in significantly shorter times, 4–8 min, than conventional heat-moisture treatment processes was concluded.

Keywords: Microstructure; Microwave treatment; microwave absorptivity; pasting properties; rice flour; thermal properties

1. Introduction

Rice flour, due to its low allergenicity is one of the most utilized raw materials in gluten-free production. However, the functional properties of native rice flour are insufficient for the creation of highly developed and stable dough structure. For improvement of rice flour functionality several different modification procedures were employed including hydrocolloids and fiber addition (Perez-Quirce et al, 2017; Ronda et al., 2015), enzyme application (Kim, 2016), protein enrichment (Villanueva et al., 2015; Phongtai et al., 2017), milling and particle size classification (Yano et al., 2017), high pressure processing (Cappa et al., 2016) and hydrothermal treatment in the excess of water (Bourekoua et al., 2016).

Microwave (MW) radiation can deliver energy with high efficiency depending mainly on dielectric properties of a treated sample. Therefore microwave treatment (MWT) provides a faster method than conventional heating to perform common hydrothermal treatment (HMT). MW are electromagnetic waves with frequencies between 1 and 300 GHz that polar and ionizable molecules (water and mineral salts, mainly) may absorb efficiently. The energy absorption takes place at a molecular level, producing a rapid increase in the temperature of all the sample volume, which significantly differentiates MW and convectional thermal heating. MWT impact studies on the physicochemical, structural and functional properties of cereals and legumes starches were carried out, but the effect on flours has been so far little studied.

Anderson and Guruya (2006) evaluated the effect of MWT of waxy and non-waxy rice starches at 20% water content revealing significant

changes in viscosity properties after microwave irradiation exposure. Ashraf et al. (2012) studied the effect of MWT on the functional properties of wheat and red bean flour and showed that MWT improved the water holding capacity, oil absorption, emulsifying and foaming ability and proteins solubility index. However, there is a lack of processing data e.g. the mixing characterizing and microwave radiation distribution as well as moisture evaluation during the treatment which is crucial for the proper evaluation of the microwaves' impact.

To develop effective microwave treatments, the dielectric properties of raw materials are critical as they measure the ability to store and absorb electromagnetic energy. The dielectric properties of interest are the dielectric constant ε´ and loss factor ε´´, the real part and imaginary part, respectively, of the relative complex permittivity (Guo et al., 2010):

$$\varepsilon = \varepsilon' + j\varepsilon'' \quad (j = \sqrt{-1}) \qquad \text{(Eq.1)}$$

The ε´ mainly reflects the ability of a material to store electromagnetic energy and ε´´ represents its ability to absorb it. Therefore the microwave attenuation of a sample is related to dielectric losses (ε´´) of studied flour while phase change is related to dielectric permittivity (ε´) of flour. The measurement of microwave dielectric properties of raw materials as a function of its water contents can be a useful method to estimate the effect of microwave radiation on other properties as well as to discriminate bound water from the total water content thanks to its different contribution to dielectric permittivity and loss. As the moisture is the functional variable of MWT, it is crucial to determine its specific contribution to flour heat absorption and physical modification during the microwave irradiation.

The microwave radiation physical modification of rice flour and its water content effect have not been studied up until now in spite of the fact that rice flour is a raw material more extensively used in gluten-free products formulation. The main objectives of this study were to analyze the microwave radiation absorption capacity of rice flour as a function of its water content and the microwave assisted thermal treatment impact on the physical characteristic, thermal and pasting properties of rice flour.

Moreover the procedure to obtain a uniform distribution of MW radiation within the sample to avoid flour burning/darkening was designed, and water content of the flour was controlled during the MWT to evaluate the relative importance of HMT and dry-heat-treatment (DHT).

2. Materials and Methods

2.1. Rice flour

Indica rice variety commercially available flour Herba NAT 300 (Herba Ricemills S.L.U., Tarragona, Spain) was used for all experiments. The initial water content was 13%, ash <0.9%, protein > 6.5%, fat < 1% and gluten < 10 ppm. The flour granulometry was as follows: 1% <250 μm, 250 μm >10-20%>210 μm, 210 μm > 35–45% > 150 μm, 150 μm > 20–35% >100 μm and 100 μm <10–20% (data provided by manufacturer).

2.2. Flour preparation

Initial rice flour water content was measured with Official Method AACC 44-19 (AACC, 2000) and the amount of water added for certain water content levels achievement was calculated. Flour water content levels were set at 2.5%, 5%, 10%, 13%, 15%, 25%, 30% and 39% ± 0.5%. The water content of 2.5% was obtained by rice flour lyophilization in FreeZone 1, Labconco (Kansas City, USA) lyophilizer. The calculated amount of water was sprayed onto the flour mixed in Teddy Bear mixer Mono Equipment (Swansea, UK) within 10 minutes. Water contents of 5 and 10% were obtained by mixing calculated amounts of 13% rice flour with lyophilized one. The prepared samples were stored for 24 hours at 4±2ºC for equilibration.

The water activity of flours was measured with Testo 650 Humidity Meter provided with a high precision relative humidity probe TESTO (Lenzkirch, Germany). Determination was made in duplicate at 25ºC.

2.3. Microwave absorption capacity of rice flours

Rice flour samples of different water contents were weighted and carefully homogenously distributed in Petri dishes. Absorption was measured with Keysight (Agilent) E5071C network analyzer with both

port connected via coaxial lines and attenuators to two coaxial waveguide (SMA to WR340) transitions. The two 40dB attenuators were placed between the coaxial lines and the waveguide transitions to minimize the impedance mismatch between a coaxial line joining the analyzer (50 ohms) and transition (the impedance depends on the measured sample) by reduction of stationary waves in those lines with the goal of improving the determination. The waveguides were aligned and faced each other at a constant distance and the 11 mm deep Petri dishes, with homogenously dispersed flour, were placed between them. The analyzer was configured to work between 2 and 3 GHz and to measure the scattering coefficient S_{12}. It was calibrated with an empty Petri dish for 0 dB and 0º of phase angle. Absorption was measured at 2.5 GHz because is the same frequency used by home microwave ovens and the only frequency that can be used without special permits. All samples were measured in duplicate.

In our system –moistened flour– water was responsible for microwave absorption while flour played the role of diluent. The application of the Lambert-Beer law required the normalization of the measurement to sample weight since the optical path could not be completely filled with the sample but also included air entrapped between the flour particles. The microwave absorption was expressed in terms of attenuation, calculated from the ratio of the power measured with the sample and the one obtained with an empty Petri dish (in dB) and in terms of phase change, calculated as phase shift of the received signal when placing the sample (in degrees), by using the following equations:

$$\alpha = 20 \cdot log \frac{S_{12}\,(filled)}{S_{12}(empty)} \qquad \text{(Eq.2)}$$

$$\Theta = arg \frac{S_{12}(filled)}{S_{12}(empty)} \qquad \text{(Eq.3)}$$

Where α is attenuation, Θ is the phase shift, S_{12} is the scattering coefficient when the sample container is filled with a sample (S_{12}(filled)) or empty (S_{12}(empty)).

In terms of power, the attenuation can be obtained from the formula:

$$\alpha = 10 \cdot log \frac{P\ (filled)}{P(empty)} \qquad \text{(Eq.4)}$$

Where *P(filled)* is the power detected for the sample filled Petri dish and *P(empty)* is the power detected for the empty Petri dish. The actual value of the complex permittivity is difficult to measure without waveguide sample holders, which require, at these frequencies, an inordinate amount of sample material.

2.4. Microwave treatment

The microwave treatment was provided with customized microwave oven (900 W) R342INW (Sharp, Sakai, Japan). Preliminary studies were undertaken for setting the microwave treatment conditions. Finally, 100 g of flour with two levels of initial water content (20% and 30%), were placed in a polyethylene container closed with a plastic film with a few (4-5) needle made small holes and continuously stirred by an external device at a speed of 60-70 rpm. The flour was exposed to microwave radiation for 2, 4, 6, 8, 12 and 16min in cycles of 20s of exposure and 40s of rest.

The flour temperature evolution during the MWT was measured with Testoterm thermometer strips of different scales, and 0.5ºC accuracy, from TESTO (Barcelona, Spain). Two strips of different scale were introduced with each sample into the polyethylene container and were continuously stirred and in contact with the sample during the established treatment time. Each measurement was made in duplicate.

2.5. Scanning electron microscopy (SEM)

A microscope model Quanta 200-F (FEI, Oregon, USA) was used to study the morphological changes in the flours. This microscope was equipped with an X-ray detector which allowed the analysis of samples of low conductivity without prior metallization. The samples were directly mounted on stubs and observed with an accelerating voltage of 1.5 keV.

2.6. X-Ray Diffraction

The diffraction assessment was proceeded using a Bruker-D8-Discover-A25 diffractometer (Bruker AXS, Rheinfelden, Germany) equipped with a copper tube operating at 40 kV and 40 mA, with CuKα radiation of 0.154-nm wavelength. Diffractograms of samples were obtained with scanning from 5° to 40°(2θ) at a rate of 1.2°/min, a step size of 0.02°, a divergence slit width variable of 5mm and a scatter slit width of 2.92° and a nickel filter 0.02 to exclude the Kβ radiation.

The crystallinity of samples was determined from diffractograms based on the relation between the global peaks area and the reduced peaks area assigned to the crystalline part of the sample, and expressed as a percentage. The "search–match" software DifracEVA with PDF2-2004 and COD database was used for this purpose.

2.7. Differential Scanning Calorimetry

Gelatinization and retrogradation transitions were assessed by DSC (DSC-822e, Mettler Toledo, SAE). Flour samples, ~6 mg, were weighed into aluminum pans (40µl) and distilled water was added to achieve the ratio 30:70 (flour:water). The samples were scanned from 0 to 115°C at 5°C/min using an empty pan as reference. The retrogradation of starch was evaluated in the samples previously gelatinized in the DSC pans after 7 days of storage at (4± 2) °C following the same protocol. The enthalpy (ΔH) values, J/g of solids, the onset and peak temperatures (To and Tp), and the temperature range (R_{gel}= 2·(Tp-To)) for the gelatinization peak, were established. Samples were run in duplicate.

2.8. Pasting Properties of Flours

Pasting properties were studied by using the Rapid-Visco-Analyzer (RVA-4, Newport Scientific Pvt. Ltd., Australia) using ICC Standard method 162.

Untreated flour was also measured as control. The pasting temperature (PT), peak time (VT), peak viscosity (PV), trough viscosity (TV), breakdown (BD), final viscosity (FV), and setback (SB) were calculated from the pasting curve using Thermocline v.2.2 software. The determination was carried out in triplicate.

2.9. Statistical analysis

The Statgraphics Centurion v.16 (Bitstream, Cambridge, MN, USA) software was used for MANOVA and ANOVA analyses. The Fisher's least significant difference (LSD) test was adopted to evaluate significant differences ($p<0.05$) among samples. Homogeneity of variance was checked for each studied variable.

3. Results and Discussion

3.1. Microwave absorption capacity of flour

The microwave radiation absorption capacity of rice flour, measured in terms of attenuation, and the phase shift of radiation when it passed through the samples as function of their water content are shown in Fig. 1A. The MW attenuation data are also represented, simultaneously with the water content of rice flour, versus flour water activity, a_w, in Fig.1B. The MW attenuation and phase shift increased with water content according to the equations:

$$\text{Attenuation(dB/g)} = 1.10\ (\pm 0.16)\cdot 10^{-5}\cdot W^2 + 9.00\ (\pm 1.1)\cdot 10^{-4}\cdot W - 1.2\ (\pm 1.4)\cdot 10^{-3}$$

$R^2 = 99.8$ (Eq.5)

$$\text{Phase-shift}(\Theta) = 0.0064(\pm 0.0017)\cdot W^2 + 0.37(\pm 0.12)\cdot W + 20.6\ (\pm 1.5)$$

$R^2 = 99.0$ (Eq.6)

Where W is the water content expressed in g/100 g solids. The standard error of each regression coefficient is given in parentheses. These results reveal that the greater the total amount of water contained in the flour the smaller is the signal reaching the receiver. This is due, on the one hand, to the fact that there is a greater absorption of radiation by the sample and on the other hand, that the proportion of incident radiation that is reflected by the sample is greater, since its dielectric permittivity is also greater. The combination of these two effects justifies the positive deviation from Beer's law that shows the absorption curve approaching a quadratic rather than a linear behavior. This fact cannot be confirmed, since it is possible that the increase of the attenuation is due to an increase in the reflected radiation and not in the absorbed one. The independent term of Eq.5 is not significantly different from zero which means attenuation is zero when the flour is completely dry. In consequence, it can be concluded that flour dry matter is unable to absorb the microwave radiation even though flour could experience a faster heating under MW-radiation when it loses water (Lewandowicz et al., 1997). This effect must be due to the dramatic reduction in flour specific heat with the reduction of its water content. Figure 1B shows a total parallelism in the evolution of water content and attenuation versus a_w. This indicates that the microwave absorption capacity of flour does not depend on water activity since the attenuation increased drastically even when a_w hardly varied around a_w=1, but depends on the total water content of the flour. These results also indicate that the attenuation and the phase shift measurements may be a fast, simple, and accurate method for determining in line the moisture content of rice flour as they allow a remote and non-destructive measurement of the flour, as concluded by Ince and Turner (1965), in biscuits investigations and Okabe et al. (1973), in rice and wheat grains study.

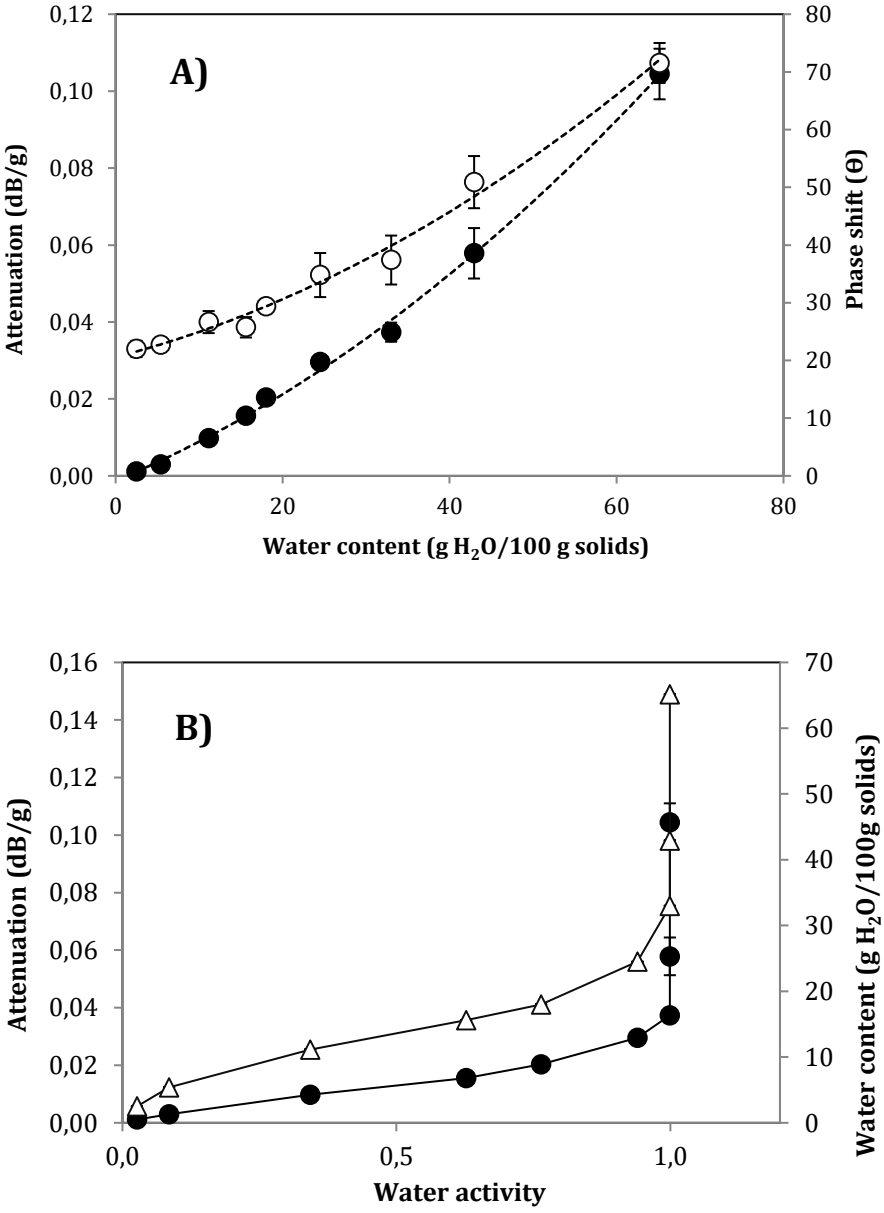

Figure 1. Attenuation of microwave radiation by rice flour in function of its water content and water activity. A) Attenuation (●) and phase shift (○) of rice flour in function of its water content. B) Attenuation (●) and water content (Δ) versus water activity.

3.2. Temperature evolution in flour during the microwave treatment

The evolution of the temperature and the water content of the flours during MWT are shown in Fig.2. As can be seen, it decreased in 8 min to 8% and 10% for the flours moistened to 20% and 30% respectively. These results suggest that MWT in a non-hermetic container, is in fact, the combination of two processes: HMT, in the first stage, and DHT in the last one. As can be seen in Fig.2 during the first 80s of treatment the energy absorbed by the samples was mainly involved in changing its temperature, while at 80s both flours attained a small plateau of constant temperature, where the heat absorbed by the sample was involved in boiling its small amount of free water. After that, the temperature increased again to attain a new plateau, after 4–5min of MW radiation, where the temperature was constant until the end of the treatment.

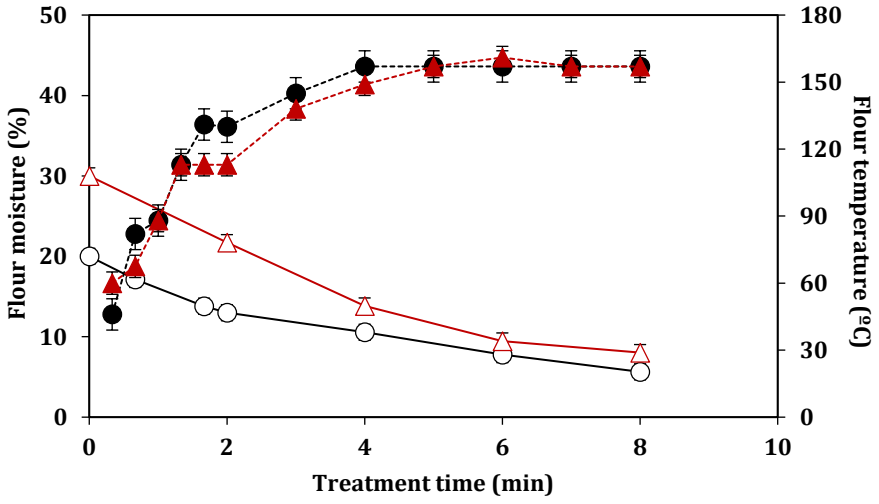

Figure 2. Evolution of moisture (○,Δ) and temperature (●,▲) of the flour as a function of microwave treatment of moistened rice flours at 20% (●,○) and 30% (▲,Δ) initial hydration levels.

The maximum temperature achieved by the samples was 150±10ºC. In the first 20s of treatment, the flours with 20% and 30% water content

reached 46ºC and 60ºC, respectively. The higher microwave absorption capacity could explain the higher temperature as was seen in our previous work (Pérez-Quirce et al., 2016). However, between 20s and 80s both samples showed similar temperature profile probably due to the fact that the higher energy absorption was compensated with the higher specific heat that makes this flour need more absorbed heat to get a similar change in temperature.

The boiling temperature of the flour with 20% initial moisture was higher than that of the flour with 30% because its water activity (Fig.1B), and in consequence its water vapor pressure, was lower in the former, which means a higher temperature needed to attain the external atmospheric pressure and to reach the boiling conditions. After 4min of MWT the water content of the flour was of 5–10% (Fig.2), near the value corresponding to the rice flour monolayer water content (Abebe & Ronda 2015) which corresponds to water tightly bound. The final long plateau of constant temperature probably corresponded to a period in which the heat absorbed by the sample (not too much as the water amount was really low) was equal to the heat lost by it towards the surrounding that increased with the temperature of the sample. In our previous work, where the sample was treated inside a hermetic container, a single and long temperature plateau was obtained below 100ºC, with water acting as 'protector' of the flour constituents, which was used to explain the low impact of the MWT in such conditions on flour functional properties (Pérez-Quirce et al., 2016), differently to what we have observed in this case.

3.3. Morphology of samples

From SEM images (Fig. 3) the important impact of MWT on flour particles macrostructure of samples moistened at 20% and 30% can be concluded. Rice starch differs by its shape from other cereals and the granules are polygonal and are packed very tightly in the rice grain cells being entwined with globular protein bodies and lipids (Nawaz et al., 2016). The starch granules form polygonal macrostructures often visible in SEM micrographs of rice kernel (Siruguri et al., 2009). After milling of rice

these macrostructures appear more or less intact in the flour (see Fig. 3-A1). With the increase of water content in flours treated with MW, the characteristic of particle size distribution was narrowed resulting in more homogenous one (see Fig. 3-B1-C1) which was probably caused by gluing the small particles together. The subsequent magnification revealed that primarily rough and frayed particle shapes in native flour become more rounded and ovoid (Fig. 3-A2-B2-C2) in treated samples which was also confirmed previously by Takahashi et al. (2005) for short grain rice autoclaved at 20% of moisture content. The particles' surface (Fig.3-A3-B3-C3) appears to be smoother and aligned similar as reported by other researchers for short rice grain flour (Majzoobi et al., 2016) and foxtail millet flour (Amadou et al., 2014) when were submitted to convectional hydrothermal treatments. At highest magnification starch granules clusters were visible covered by protein bodies spread and adhered over the surface (Fig. 3-A4-B4-C4). The resulting macrostructures (Fig. 3-A4-B4-C4) revealed that starch granules in clusters seem to be glued with neighboring ones and the slots between them visible at native flour were shallow and filled in MW-treated ones. Such sealing could be the result of amylose exudation during the thermal treatment of native flour as previously reported (Carrera et al., 2015). Such behavior was not reported for isolated starch treatment and presents the additional difference between HMT treatment of starch and flour.

3.4. X-ray diffraction and crystallinity of samples

The X-ray diffraction patterns of rice flour treated for 4 and 8 min are presented in Fig.4. All the rice flour samples presented an A-type diffraction pattern with crystalline peaks at 10°, 15°, 17°, 18°, 23° and 26°, maintained after the treatment. The reflection at 20°, which is usually connected with V-crystallinity, was also observed in all rice flour although the peak increased in the MW-treated samples about 14% in ones with the highest water content and the longest treatment (30%-8min).

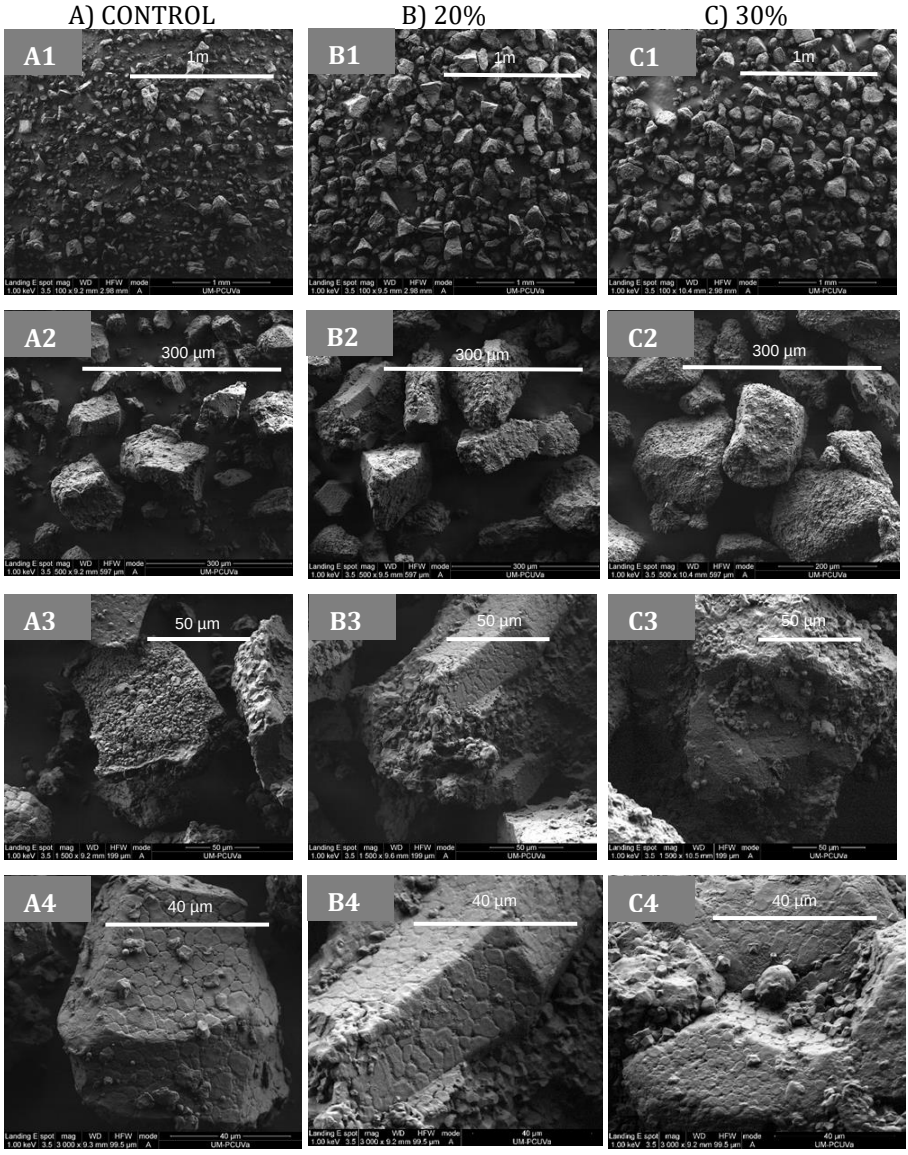

Figure 3. SEM pictures of rice flour particles at different magnifications (100x, 500x, 1500x and 3000x). A: untreated flour (control), B: flour with an initial water content of 20% treated by microwave radiation for 8 min, C: flour with an initial water content of 30% treated by microwave radiation for 8 min.

This suggests an increase in the amylose-lipid complex, although it did not result in an increase in the enthalpy of the complex dissociation obtained by DSC (see section 3.5). The duplex at 17° and 18° also changed the intensity of reflection revealing changes in crystallinity. The most intensive reflection was observed for the flour treated 8min at 20% moisture resulting in a crystallinity of 67.4% vs 61.5% for control, untreated flour. Usually, for HMT the relative crystallinity of the flours was lower than those from control (Zavareze and Dias, 2011; Silva et al, 2017).

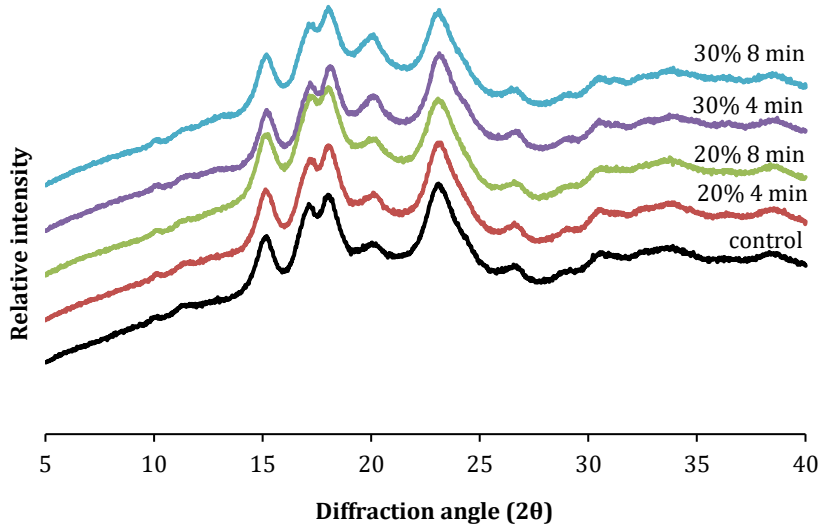

Figure 4. X-ray diffraction patterns of untreated rice flour (control) and microwaved (different treatment times) rice flours at initial moisture contents of 20% and 30%.

The conditions of typical HMT processes (high moisture, high temperature, long time) afford increased mobility to the starch chains and helical structures, resulting in structural changes in both the crystalline and amorphous regions of the starch granules. This mobility allows the disruption of the least stable structures of crystalline regions, altering organization of highly ordered helical structures and/or

crystallites within granules (BeMiller & Huber, 2015). However, MWT, due to much faster and uniform heat distribution as well as local superheating points and the loss of water during the process, can lead to other structure rearrangements reflecting in higher final crystallinity. Similar results were obtained by Qiu et al. (2015) during dry heat treatment of rice flour which resulted in higher crystallinity of both rice flour and starch. These authors suggested that dry heat treatment may contribute to the formation of new crystallites or recrystallization and perfection of the small crystalline regions of the starch granule.

3.5. Effect of microwave treatment on thermal properties of rice flour

The thermal properties of MW-treated rice flour are presented in Table 1. All the samples exhibited two endotherms. The first one, which appeared at 74–77ºC, corresponded to the melting of the amylopectin crystallites in the starch granules and the second one, at 96-97ºC, was due to the dissociation of the amylose-lipid complex (Eliasson, 1994). The effect of microwave treatment was significantly dependent on the initial water content of the flour. However, the treatment time, in the range studied, had no effect on any thermal property. The onset and peak temperature of starch gelatinization increased to 3ºC comparing to control when flours were moistened at 30%. While the gelatinization temperature range, quantified by Rgel = 2·(Tp – To), decreased from 12ºC to 10.5ºC denoting more perfect amylopectin crystallites in MW-treated flours. Chiu and Solarek (2009) also reported that after hydrothermal treatments, the starch gelatinization temperature was higher and the gelatinization endotherm more defined. We also observed when crystallites perfection is higher, greater gelatinization temperatures are required to melt starch crystallites (Ji et al. 2004). Therefore, the increase of gelatinization temperature observed in moistened treated flours would be in agreement with XRD results. The rise in gelatinization temperatures has also been associated with the formation of amylose-amylose and amylose-lipid complexes within the starch granule and an association and a more stable configuration in the granular structure (Lewandowicz et al. 2000).

Table 1. Thermal properties of aqueous dispersions (70 % solids) of rice flour treated by microwaves

IMC (% fb)	MWT time (min)	ΔH_{gel}- First (J/g db)	$T_{o\text{-}gel}$- First (^oC)	$T_{p\text{-}gel}$- First (^oC)	R_{gel}- First (^oC)	$\Delta H_{am\text{-}lip}$- First (J/g db)	$T_{p\text{-}am\text{-}lip}$- First ($^oC$)	$\Delta H_{am\text{-}lip}$- Second (J/g db)	$T_{p\text{-}am\text{-}lip}$- Second ($^oC$)	ΔH_{ret}- Second (J/g db)	$T_{p\text{-}ret}$- Second (^oC)
Control	0	10.3^{d}	68.1^{a}	74.0^{a}	11.8^{b}	1.1^{b}	96.0^{a}	2.8^{b}	96.7^{b}	4.5^{a}	55^{a}
20	4	9.6^{bc}	68.5^{a}	74.4^{b}	11.8^{b}	1.0^{b}	96.3^{ab}	2.4^{ab}	97.3^{b}	4.3^{a}	54^{a}
20	8	9.9^{cd}	68.1^{a}	74.4^{b}	12.6^{b}	1.1^{b}	96.6^{ab}	2.2^{a}	97.7^{b}	4.5^{a}	54^{a}
30	4	9.3^{ab}	71.3^{b}	76.5^{c}	10.4^{a}	0.8^{a}	96.1^{ab}	2.7^{ab}	95.6^{a}	6.1^{b}	53^{a}
30	8	8.9^{a}	71.5^{b}	76.8^{c}	10.6^{a}	0.8^{a}	97.0^{b}	2.8^{ab}	96.9^{b}	6.2^{b}	55^{a}
SE		0.25	0.27	0.10	0.40	0.10	0.35	0.20	0.32	0.67	1.23
Analysis of variance and significance (p-values)											
Moisture		0.022	0.000	0.000	0.008	0.028	0.765	0.065	0.047	0.006	0.632
Time		0.780	0.584	0.077	0.231	0.927	0.089	0.889	0.068	0.823	0.573
Moisture*Time		0.082	0.208	0.203	0.447	0.913	0.343	0.385	0.230	0.847	0.593

ΔH_{gel}, $\Delta H_{amyl\text{-}lipid}$ and ΔH_{ret}: Enthalpy associated to starch gelatinization, dissociation of amylose-lipid complex and melting of the recrystallized amylopectin; $T_{o\text{-}gel}$: onset temperature of gelatinization peak; $T_{p\text{-}gel}$, $T_{p\text{-}ret}$, $T_{p\text{-}amil}$: Peak Temperature of *gelatinization, retrogradation* and *amylose-lipid complex* dissociation peaks, respectively; $R_{gel}= 2\cdot(T_p\text{-}T_o)$ for the gelatinization peak; First: Scan carried out on native (un-gelatinized) sample. Second: Scan carried out on gelatinized samples after 7-days of storage. Mean values with different letters for the same parameter imply significant differences between means at $p<0.05$. SE: Pooled Standard Error obtained from ANOVA. In bold are highlighted the p-values < 0.05 that mean the effects are significant with a confidence level > 95%. MWT: microwave treatment, IMC: Initial Moisture Content

A slight but significant decrease in the gelatinization enthalpy of the microwaved-treated flours was observed probably because the samples underwent a partial gelatinization as a consequence of microwave treatment. The maximum reduction of ΔH_{gel} in the treated-flour samples with respect to the control flour was 14%, very low when compared with the values of 72% and 47% found by Lewandowicz et al. (2000) for wheat and corn starch after a MWT. Those authors related the higher effect of MWT on partial gelatinization of wheat starch with its lower initial gelatinization temperature, 54ºC versus 61ºC for corn starch. The higher gelatinization temperature of rice flour (Table 1), 71ºC, could explain its lower ΔH_{gel} reduction during MW treatment. Lewandowicz et al. (2000) also found that MW-treated waxy corn hardly changed its gelatinization enthalpy with respect to native corn starch; what means amylose content also plays an important role on this effect.

The second scan applied to gelatinized samples stored in the DSC pans at (4 ± 2)°C for 7 days (retrogradation scan) led also to two visible peaks. The first one, very wide, at a peak temperature of ~54ºC (Table 1), was related to the melting of the recrystallized amylopectin during the gel staling. The second peak was related to the amylose-lipid dissociation and appeared at the same temperature than in the first scan. As can be seen in Table 1, in the second scan the enthalpy of the amylose-lipid complex dissociation was similar for all the MW-treated samples and higher than in the first (gelatinization) scan. Eliasson (1994) reported that the increased values usually found during a second scan are probably due to better conditions for complex formation after the first heating because the leaking of amylose from granules can occurs at temperatures above the gelatinization temperature range. In the first scan, the samples treated with 30% water content led to a significantly smaller peak of amylose-lipid complex dissociation, with a reduction in enthalpy of 27% with respect to the control flour peak, which could be related to the higher difficulty in amylose leaching from the MW-treated granules. The amylopectin recrystallization extent was 35% higher in samples treated by MW with 30% initial water content than in native flour or in flours

treated at 20% moisture content. This fact could represent a drawback in the shelf life of MW-treated flour gels.

3.6. Effect of microwave treatment on pasting properties of rice flour

The impact of time and initial water content of MWT process on the RVA primary parameters is evidenced by the significant changes in the pasting and gelling behavior of the treated flours (Table 2). Major effects on cooking and cooling parameters were noted for the longest treatment (8min), resulting in increased paste temperature (8ºC and 11ºC for flours of 20% and 30% of initial moisture, respectively) and decreased peak (38% and 42%), final (13% and 25%), breakdown (81% and 86%) and setback (17% and 24%) viscosities with respect to the control, native, flour. These changes can be explained by associations between chains in the amorphous region of the granule as well to changes in crystallinity during hydrothermal treatment (Watcharatewinkul et al., 2009) as was confirmed by X-ray diffraction assays. Greater effect was always observed in flours with higher initial water content, similar to that reported by other authors with respect to HMT treatments (Zavareze and Dias, 2011). However, the important change that took place in the two last minutes of treatment, from 6 to 8min, when the moisture of samples was ±10% and the temperature ~150ºC makes us think that dry heat treatment had also a high impact on flour properties.

The MWT-induced reduction in the breakdown and setback of the flours (Table 2) shows that treated samples were more stable during heating and stirring and had a lower amylose retrogradation. The reduction in amylose leaching from starch granules as consequence of the promotion of amylose-amylose and/or amylose-amylopectin chain interactions could explain these results (Chung et al., 2009).

Table 2. Effect of microwave treatment on the viscometric parameters of rice flours

IMC (% fb)	MWT time (min)	Peak Viscosity (mPa·s)	Trough Viscosity (mPa·s)	Breakdown Viscosity (mPa·s)	Final Viscosity (mPa·s)	Setback Viscosity (mPa·s)	Peak time (min)	Pasting Temperature (°C)
Control	0	2580^{g}	1565^{c}	1015^{g}	3729^{d}	2163^{d}	5.60^{ab}	79.1^{a}
20	2	2410^{f}	1579^{cd}	831^{f}	3651^{d}	2072^{d}	5.55^{a}	83.6^{b}
20	4	2342^{f}	1622^{cd}	720^{e}	3733^{d}	2111^{d}	5.47^{a}	83.2^{b}
20	6	2096de	1626^{d}	471^{c}	3723^{d}	2098^{d}	5.49^{a}	84.5^{b}
20	8	1612^{b}	1423^{b}	189^{a}	3214^{b}	1791^{bc}	5.58^{a}	86.9^{c}
30	2	2120^{e}	1570^{c}	550^{d}	3431^{c}	1862^{c}	5.78^{c}	89.4^{d}
30	4	2002^{d}	1594^{cd}	408^{b}	3351^{bc}	1757^{bc}	5.77^{c}	88.9^{d}
30	6	1760^{c}	1561^{cd}	199^{a}	3245^{b}	1684^{bc}	5.74^{bc}	86.9^{c}
30	8	1499^{a}	1357^{a}	142^{a}	2786^{a}	1429^{a}	6.02^{d}	89.8^{d}
SE		48	26	29	70	52	0.05	0.57
Analysis of variance and significance (p-values)								
Moisture		0.000	0.005	0.000	0.000	0.000	0.000	0.000
Time		0.000	0.000	0.000	0.000	0.000	0.000	0.000
Moisture*Time		0.035	0.378	0.000	0.103	0.085	0.061	0.000

Mean values with different letters for the same parameter imply significant differences between means at $p<0.05$. SE: Pooled Standard Error obtained from ANOVA. In bold are highlighted the p-values < 0.05 that mean the effects are significant with a confidence level >95%. MWT: microwave treatment, IMC: Initial Moisture Content

4. Conclusions

The MW absorption capacity of rice flour exhibited a positive quadratic relationship with the water content while the flour dry matter did not show any radiation absorption capacity. However, the decrease in the specific heat of the flour associated with the loss of water was responsible for the significant change in the temperature of the samples, which reached 150ºC in 4min, although the water content of the flour decreased drastically during the treatment. The dry-heat treatment of the flour that took place in the last 4 min of the MWT could explain the increase in crystallinity observed in the treated samples. Microwave assisted thermal treatment of rice flour changed its thermal and pasting properties as morphological structure of flour particles and crystallinity/amorphous ratio. The changes observed in thermal properties of treated flour revealed the crucial impact of initial moisture content on the significant rising of gelatinization temperature, the decrease in the gelatinization enthalpy and the enhancement of the amylopectin recrystallization after seven days of storage. All those changes resulted in functional properties alteration represented by distinct pasting behavior of microwave irradiated rice flours. They showed higher pasting temperatures, lower peak, breakdown and setback viscosities than the native flour particularly when flour samples were treated at the highest (30%) initial moisture content. The results proved the efficiency of microwave assisted thermal treatment on physical changes of rice flour that took place in significantly shorter periods than conventional HMT. Further studies are needed to clarify the relative importance of hydro-thermal and dry-thermal stages in the MWT process. Also pending is the study of the ability of the MW-treated flours to improve the quality of gluten-free food products.

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CAPÍTULO VI

CHAPTER VI

IMPACTO DE LA RADIACIÓN MICROONDAS Y LA ADICIÓN DE PROTEÍNA SOBRE LA FUNCIONALIDAD Y LAS CARACTERÍSTICAS REOLÓGICAS DE GELES ELABORADOS CON ALMIDÓN DE ARROZ Y DE PATATA

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Microwave radiation and protein addition modulate hydration, pasting and gel rheological characteristics of rice and potato starches

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Abstract

This study evaluated for first time the effect of Microwave (MW) radiation on systems based on potato and rice starches supplemented with 5% of calcium caseinate (CA) or soy protein isolate (SPI). The goal of this treatment was the physical modification of these starch-based systems to provide ingredients of new functionalities. The hydration and pasting properties as well as gel viscoelastic features were evaluated. Dynamic oscillatory rheological tests were used. The effect of MW treatment (MWT) depended on the starch botanical origin and was significantly affected by protein presence and type. MWT of starch+protein blends revealed the most notable changes when SPI was added. Adding it to rice starch decreased swelling power (-45%), altered viscometric profiles and reinforced gel structure with important increases in both viscoelastic moduli (+160%-G' and +58%-G''). In blends with potato starch, MWT increased water absorption capacity (+115%), and decreased water solubility index (-82%). MWT of protein-potato blends promoted gel

stability, decreased their pasting profiles and resulted in enhanced viscoelastic moduli (+483-G' and 243%-G''). MWT combined with protein addition allows designing starch-based foods with tailored properties.

Keywords: Microwave; functional properties; gel rheology; potato starch; rice starch

1. Introduction

The native form of starch has limited applications in the food industry and is often modified physically, chemically or enzymatically to improve functional properties. Physically modified starch is considered a natural material and a highly safe ingredient, much safer and more predictable than chemically modified starch. So far, the usage of physically modified starch in food is not restricted by legislation, which is considered an outstanding advantage compared to chemically- or enzymatically-modified starch applications (Klein et al., 2013).

Starch, as a macro-constituent of many raw materials and processed foods, contributes to their nutritional and functional characteristics, as well as plays the pivotal role as a texture modifier (Walkenström and Hermansson, 1998) and influences many quality attributes of processed foods (Chen et al., 2015). Rice and potato starches are among the most commonly used for texture development, particularly in gluten-free (GF) products. During microwave (MW) radiation exposure, energy is delivered directly to the material, heating the entire sample in bulk at a faster heating rate than conventional heating (Bilbao-Sáinz et al., 2007). Therefore, MW radiation-induced heating is suitable for modifying starches physically and improving their functional properties. In moistened starches, the intermolecular structure changes from MW-delivered energy lead to changes in water absorption ability, solubility and swelling power, as well as in starch gelatinization, syneresis and paste viscosity (Brasoveanu and Nemtanu, 2014). There are many factors related to both starch characteristics (botanical origin, water content, density, dielectric properties, etc.) and microwave irradiation conditions

(frequency, power and exposure time) that significantly affect how granular starch responds to MW treatment (MWT) (Brasoveanu and Nemtanu, 2014). Zavareze et al. (2010) showed that microwave irradiation affected the pasting properties of high-amylose rice starches more intensely than in those with lower amylose content. Vermeylen et al. (2006) concluded that higher treatment temperatures and moisture contents generally caused the largest changes in starches. As applied temperature and microwave power level increased, the peak viscosity of potato starch decreased (Nadiah et al., 2015), while the gelatinization temperature increased (Nadiah et al., 2015; Vermeylen et al., 2006). Villanueva et al. (2018a) concluded that the MW radiation absorption capacity of rice flour exhibited a positive quadratic relationship with water content, while the flour dry matter did not show any radiation absorption capacity. They also observed changes in physical properties of treated rice flour, such as rice flour particle morphology or the crystallinity/amorphous region ratio.

Beyond physico-chemical and functional considerations, starchy gluten-free foods are often considered nutritionally poor compared to wheat-based counterparts due to their deficient protein/starch ratio (Villanueva et al., 2015). The addition of protein is a viable alternative to improve the nutritional properties of these products. Proteins are also known to modify structure and texture in foods (Villanueva et al., 2018b). Soy proteins isolate (SPI) and calcium caseinate (CA) have been investigated in GF applications because of their effect on nutrition and structure of products (Krupa-Kozak et al., 2011; Ronda et al., 2014). Starch-protein interactions affect the rheological, pasting, gelatinization, textural and physicochemical properties of food systems (Gallagher et al., 2003; Ronda et al., 2011; Ronda et al., 2014; Villanueva et al., 2015). As far as we know, how MWT affects protein-starch mixtures has not been studied so far, in spite of how important endogenous proteins seem to be in the effect of heat-moisture treatment (HMT) on treated-flour properties (Puncha-arnon and Uttapap, 2013). As protein and starch blends can serve as model systems for the study of more complex matrices such as flour, the impact of their interactions on changes appearing in MW

treated samples needs to be better understood. The main objective of this study was to investigate the combined effect of microwave radiation and protein addition (5% CA or SPI) on the hydration, pasting properties and gel viscoelastic properties of potato and rice starches. The ultimate goal of this study is to provide starch-based ingredients with tailored functional properties.

2. Material and methods

2.1. Materials

Rice starch and potato starch were supplied by Ferrer Alimentación S.A. (Barcelona, Spain). Soybean protein isolate (SPI) Supro 500-E IP (purity ~ 90%) was obtained from Proveedora hispano-holandesa S.A. (Barcelona, Spain), and calcium caseinate (CA) (purity ~ 96%) from Armor Protéines (Saint-Brice-en-Coglès, France). Protein added in all the starch+protein blends was 5 g/100 g blend. Twelve different samples were investigated: native and MW-treated rice and potato starches alone and blended with 5 g/100 g of CA or SPI proteins.

2.2. Microwave treatment of starch samples

Samples were treated by MW radiation at initial moisture content of 30%. The moisture content of the samples was measured following the American Association of Cereal Chemists (AACC) 44–19 method and the water necessary to adjust to 30% moisture content was sprayed onto the samples while they were mixed in a Bear Teddy Mixer (Bear 5L Teddy, Swansea, UK) for 15 min. Samples were then hermetically sealed in bags and held 24 h at 4 ± 2°C for the moisture equilibration.

Hydrated samples (100.0 g each) were heated in a SHARP R-342 (Osaka, Japan) microwave oven in a cylindrical polyethylene container closed with needle-punched, microwave-safe food grade film. The homogenous distribution of radiation on the sample is of crucial importance. So, the container was stirred constantly to ensure a uniform energy and temperature distribution during treatment as described in Villanueva et al. (2018a). MW radiation frequency was 2450 MHz and the power, 900W. MW was applied in cycles of 20 s radiation with 40 s downtime,

for a total of 32 min and 640 s of MWT; afterwards, samples were left to cool for 5 minutes. Agglomerates formed during treatment were manually disintegrated in a laboratory mortar to < 0.5 mm. Sample temperature during MWT was measured with Testoterm thermometer strips of different scales, and 0.5ºC accuracy (Instrumentos Testo S.A., Barcelona, Spain), as described in Villanueva et al. (2018a). Each measurement was made in duplicate. The temperature evolution curves obtained for the potato and rice starch systems studied were equivalent to those reported for 30% moistened-rice flour in our previous work (Villanueva et al., 2018a). The temperature reached and maintained after 4 min of treatment was 157±5ºC for all samples.

2.3. Amylose content

The amylose content of the starch samples was determined using a colorimetric assay kit for amylose/amylopectin ratio determination in starch (Megazyme International Ireland Ltd., Ireland), according to the manufacturer's procedure based on Gibson et al. (1997) method. The method determines the soluble amylose in acetate/salt solution susceptible to α-amylase/ amyloglucosidase digestion. Each sample was analysed at least in duplicate.

2.4. Functional properties

Water absorption capacity (WAC) of the samples was determined by the centrifugation method described by Abebe et al. (2015). Two grams of sample (w_s) were mixed with 20 mL of distilled water in 50 mL centrifuge tubes. The dispersions were hold at room temperature for 30 min with occasionally vortexed (Heidolph Reax, Schwabach, Germany) and followed by centrifugation for 30 min at 3000×g (Thermo Fisher Scientific, Waltham, USA). The supernatant was removed and weighed (w_{s+w}) and results were expressed as grams of water retained per gram of sample. Water absorption index (WAI) and water solubility index (WSI) were measured with slight modification of the method used by Abebe et al. (2015). Each sample of 2.5 g (w_0) was dispersed in 30 ml of distilled water in tared centrifuge tubes. After cooking for 10 min in a 90ºC water bath, samples were cooled to room temperature and

centrifuged at 4000xg for 10 min. The supernatant was poured into a pre-weighed evaporating capsule to determine the solid content and the sediment was weighed (w_{ss}). The weight of soluble solids was recovered by evaporating the supernatant overnight at 110ºC (w_{ds}). WAC, WAI, WSI and swelling power (SP) were calculated using the following equations:

$$WAC\ \left(\frac{g}{g}\right) = \frac{w_{s+w} - w_s}{w_s} \qquad (1)$$

$$WAI\ \left(\frac{g}{g}\right) = \frac{w_{ss}}{w_0} \qquad (2)$$

$$WSI\ \left(\frac{g}{100g}\right) = \frac{w_{ds}}{w_0} \times 100 \qquad (3)$$

$$SP\ \left(\frac{g}{g}\right) = \frac{w_{ss}}{(w_0 - w_{ds})} \qquad (4)$$

All results were referred to dry matter to avoid the effect of different water content in the samples.

2.5. Thermoviscous test: Viscometric profile

Following AACC International Method 76-21.01 Standard 2, viscometric profiles of MW-treated and untreated samples were obtained using a Kinexus Pro+ rfheometer (Malvern Instruments Ltd., Malvern, UK) supplied with starch pasting cell and controlled by rSpace software. Samples of rice starch (3 g, 14% moisture-based) or potato starch (2 g, 14% moisture-based) with or without added protein were transferred to a canister where 25 mL±0.1 mL of distilled water was added. Each starch suspension was equilibrated at 50 ºC for 1 min, heated to 95 ºC at a rate of 6 ºC/min, maintained at 95 ºC for 5 min, then cooled to 50 ºC at a rate of 6 ºC/min, and maintained at 50 ºC for 2 min. Paddle speed was set at 960 rpm for the first 10 s and then 160 rpm for the rest of the analysis. Each sample was analyzed at least in duplicate. Parameters calculated from the pasting profiles were pasting temperature (PT), peak viscosity (PV), trough viscosity (TV), breakdown (BD), final viscosity (FV), setback (ST) and peak time.

2.6. Rheological measurements

Dynamic oscillatory tests of the twelve potato and rice starch gels were performed with Kinexus Pro+ rheometer (Malvern Instruments Ltd., Malvern, UK) with parallel plate geometry (40 mm diameter) of serrated surface and with 1 mm of working gap. The gel samples were made following the same procedure described for thermoviscous tests. Different concentrations of rice and potato starches were used to prepare their gels, so that the gels produced from native starches were of similar consistencies. Just at the end of the pasting test the gel was removed from the canister and placed between the plates, the sample excess was removed and the sample was left to rest for 5 min to allow relaxation. Temperature was stabilized at 25ºC with a Peltier plate controller. Stress sweeps were performed from 0.1 to 500 Pa at a constant 1 Hz frequency. Frequency sweeps were carried out from 10 to 1 Hz in the linear viscoelastic region (at a constant value of 1 Pa). Frequency sweep data were fitted to potential equations as described by Ronda et al. (2014). All gels were prepared at least in duplicate and rheological tests were also performed in duplicate.

2.7. Statistical analysis

Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) was used for multifactor analysis of variance (ANOVA) of the data. The Least Significant Difference test was used to evaluate significant differences ($p < 0.05$) between samples.

3. Results and discussion

3.1. Amylose content

The amylose/amylopectin ratio of starch, which greatly affects starch functional properties, is attributed to different factors such as botanical source, soil type and climatic conditions during plant growth (Jane et al., 1999; Singh et al., 2006). The amylose contents in our native samples were 16±1% and 20±1% for rice and potato starch, respectively. MWT reduced the amylose content in the potato starch to 15±1% (significantly, $p<0.05$), while no change was observed for rice starch. This result is coherent and confirms that amylose-amylopectin (AM-AMP) interactions

promoted by MW-assisted HMT reduced amylose solubility of treated potato starch, as previously reported by Varatharajan et al. (2010). The method used for amylose quantification includes a previous separation step with Concanavalin-A (Con-A) that specifically complexes branched polysaccharides of amylopectin starch components (Gibson et al., 1997). The AM-AMP interaction could lead to a partial co-precipitation of amylose, leading to a reduction in the final amylose content. These interactions must have been weaker or less efficient in rice starch, given that amylose content results were hardly affected by MWT compared to potato starch. The same happened with the other properties measured in the treated mixtures, as shown below.

3.2. Hydration properties

Hydration properties were affected by all the factors studied: starch and protein type as well as MW treatment (see Table 1). WAC depends on starch structure, the degree of association to form hydrogen bonds between starch chains, internal forces controlling granule structure and availability of water binding sites (Gani et al., 2017). This would justify the differences found between the different native starches tested. Rice starch WAC was 26% higher than that of potato starch. The addition of 5% SPI to native starches improved their water retention properties and increased potato starch WAC by up to 42%. Similar results were reported by Chinma et al. (2013) when SPI was added to cassava starch. However, adding CA had no significant effect ($p>0.05$) on the WAC of the blends regardless of the starch to which it was added. Protein characteristics have been found to influence functional behaviour (Cornejo & Rosell, 2015). Polar amino acids have been shown to be primary sites for protein interaction in water, so the increased availability of these amino acids in SPI may explain the WAC increases observed when this protein was added (Li et al., 2010). WAC increased 37% in MW-treated rice starch and 117% in MW-treated potato starch (Table 1). High temperature applied to the moistened sample during MWT yields a high level of damaged starch (Pinkrova et al., 2003). This would explain the water absorption capacity increase. In addition, the internal granule structure collapses and the crystallites are disrupted during HMT (Hoover, 2010); this could

justify the fact that the starches uptake more water. MWT had no effect on rice starch WAC in presence of proteins, while a significant effect on potato starch was observed. Especially for SPI, WAC increased ~200% with respect to native protein-free potato starch.

Water absorption index (WAI) and swelling power (SP) values are shown in Table 1. These parameters depend on the interaction between starch chains within the amorphous and crystalline domains; amylose and amylopectin content, molecular weight distribution and branching length and degree, phosphate groups and starch molecule conformation all influence WAI and SP (Ratnayakea et al., 2002). The difference in tuber and cereal starch structures, especially the higher amylose and monoester phosphate content in tubers, explains the great difference in their WAI, WSI and SP values. It also explains the different effects that protein addition and microwave treatment had on them. The WAI and SP values of native rice were almost twice those of native potato starch. The low swelling power in potato starch can result from formation of stable amylose–phosphate group complexes (Kong et al., 2015) and from the weak internal organization caused by negatively-charged phosphate groups within the potato starch granules (Singh et al., 2003).

Adding proteins to native rice starch did not greatly influence WAI and SP values. However, MWT significantly decreased swelling power and ability to maintain gel structure after centrifugation. WAI decreases from MWT were 45% in non-protein rice starch, and 30% and 45% in CA- and SPI-rice starch blends, with respect to untreated counterparts. This WAI decrease could be attributed to increased crystallinity and interactions between amylose and amylopectin molecules strengthening intramolecular bonds, the formation of amylose-lipid complexes and crystalline region rearrangement resulting from HMT treatment (Zavareze and Dias, 2011).

Table 1. Effect of microwave treatment and protein presence on the hydration properties of starch samples. All values refer to sample dry matter.

Starch	Protein	MW treatment	WAC (g/g)	WAI(g/g)	WSI(g/100g)	SP(g/g)
Rice	0	0	1.15 ab	13.42 cd	2.77 a	13.76 cd
		1	1.57 c	7.29 a	2.97 a	7.50 a
	CA	0	1.04 a	12.98 c	4.55 b	13.52 c
		1	0.91 a	9.00 b	6.92 c	9.62 b
	SPI	0	1.41 bc	13.77 d	2.94 a	14.14 d
		1	1.49 c	7.57 a	3.08 a	7.80 a
Analysis of variance and significance (p-values)						
Factor 1 (protein type)			***	**	***	***
Factor 2 (MW treatment)			ns	***	***	***
Factor 1x2			*	***	***	***
Potato	0	0	0.91 a	6.75 a	3.57 b	6.96 a
		1	1.98 c	9.50 d	0.99 a	9.59 d
	CA	0	0.91 a	7.44 b	6.52 c	7.88 b
		1	1.27 b	8.72 c	3.12 b	8.97 c
	SPI	0	1.29 b	8.39 c	6.40 c	8.88 c
		1	2.77 d	9.37 d	1.15 a	9.47 d
Analysis of variance and significance (p-values)						
Factor 1 (protein type)			***	***	***	***
Factor 2 (MW treatment)			***	***	***	***
Factor 1x2			***	***	***	***

Protein: 0: without protein; CA: 5% calcium caseinate; SPI: 5% soy protein isolate. MW treatment: 0: without treatment; 1: with treatment. WAC: Water absorption capacity; WAI: Water absorption index; WSI: Water solubility index; SP: Swelling power. The different letters in the corresponding column within each starch type indicate statistically significant differences between means at $p<0.05$. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant.

In contrast to what occurred in rice starch, WAI and SP values increased (10% and 24% for CA and SPI, respectively) when protein was added to potato starch. Similar results were observed by Chinma et al. (2013) when soy protein concentrate was added to another tuber starch such as tapioca. They attributed this increase to the reduced amylose content of the mixture from the decrease in the starch content, as amylose acts as a diluent and inhibits swelling. MWT also increased the WAI and SP in our potato samples. The largest increase was seen in protein-free potato starch, although WAI and SP also rose in protein-fortified samples as a result of MWT (41% [protein-free] versus 17% [CA] and 12% [SPI]). These increases could be related to the decrease already described in the potato starch amylose content from MWT and the dissociation of phosphate groups and amylose complexes through the high temperatures reached during MWT (Thomas and Atwell, 1999).

Water solubility index (WSI) represents the amount of solubilized starch molecules and is often used as an indicator of degradation and dextrinization of starch molecules (Jogihalli et al., 2017). Native potato starch WSI was significantly (data not shown) higher than that of rice. MWT did not affect solubility in protein-free rice starch, while it markedly decreased it in potato starch (Table 1). Similar results were reported by Nadiah et al. (2015) from microwaved potato and tapioca starches. The interaction between amylose and amylopectin branches detected in potato starch and the consequent denser granule structure formed would explain the drop in potato starch solubility. How adding protein affected WSI depended on the nature of the protein and also on the type of starch. Using a soluble form of casein, such as calcium caseinate, increased the solubility of the mixtures with both starches, (with increases of 64% and 82% for rice and potato starches, respectively). The solubility increased even more after MW treatment of the rice starch-CA blend (52%). However, adding SPI protein only increased native potato starch WSI (85%), while had no effect on rice starch. MWT decreased the solubility of protein-enriched potato starch blends, leading to WSI reductions of 52% (CA) and 82% (SPI) (Table 1). These decreases indicate that MWT

alters the interactions between potato starch and proteins, greatly reducing the water solubility of the blends.

3.3. Viscometer profile

Pasting viscosity profiles of rice and potato starches, both native and microwave-treated, with and without proteins are shown in Fig. 1 and the results are summarized in Table 2. Microwave treatment greatly changed the pasting profile of the samples, which are likewise affected by starch granule size and amylose, lipid and phosphorous content (Jane et al., 1999). Pasting curves reflect the molecular phenomena that happen in starch granules during the heating cycle and provide a means of comparing the behaviour of potato and rice starches during cooking. The differences in pasting temperature between starches (Table 2) was due to the characteristic absence of lipids and phospholipids and the lower degree of crystallinity of tuber starches compared to cereals (Jane et al., 1999). In particular, MWT increased the potato starch PT by 27% in our study. Nadiah et al. (2015) also reported increased PT values for MW-heated potato and tapioca starches. The rise of PT (also found in HMT processes) has been associated to the fact that stronger bonds and cross-links between chains appear within the starch granule during MWT, requiring a higher temperature for structural disintegration and paste formation (Zavareze and Dias, 2011). No statistical differences in PT were observed between the native and the MW-treated rice starch samples, in contrast to what was previously observed with rice flour (Villanueva et al., 2018a), where PT increased ~10ºC. No PT changes were obtained either when glutinous rice starch was processed by dry heating at 130ºC (Qin et al., 2016). Puncha-arnon and Uttapap (2013) also found that treating rice starch with heat and moisture (at 100ºC and 20% moisture content for 16 h) increased PT only slightly, in contrast to the effect observed in treated-rice flour.

Native potato starch had a very high PV because of its high phosphate monoester content and long branch chains (Jane et al., 1999). The lower PV observed in rice starch is related to its high concentration of lipids and phospholipids linked to amylose and long chains of amylopectin that restrict swelling of granules (McPherson, 1999). We found a significant decrease in PV in both native starches when proteins were added (Table 2).

Table 2. Effect of microwave treatment and protein presence on the pasting properties of starch samples.

Starch	Protein	MW treatment	PT(ºC)	PV (Pa·s)	TV (Pa·s)	BD (Pa·s)	FV (Pa·s)	ST (Pa·s)	Peak time (s)
Rice	0	0	74.6 ab	2.55 c	1.25 c	1.30 b	2.24 c	0.99 d	665 d
		1	73.9 a	2.73 d	1.23 c	1.49 c	2.29 c	1.06 e	627 ab
	CA	0	75.8 b	2.09 a	1.02 a	1.08 a	1.82 a	0.80 b	650 c
		1	74.6 ab	2.27 b	1.11 b	1.16 a	2.00 b	0.88 c	635 b
	SPI	0	73.9 a	2.32 b	1.03 a	1.30 b	1.72 a	0.69 a	618 a
		1	74.3 a	2.68 cd	1.35 d	1.33 b	2.31 c	0.96 d	620 a
Analysis of variance and significance (p-values)									
Factor 1 (protein type)			*	***	***	***	***	***	***
Factor 2 (MW treatment)			ns	***	***	**	***	***	***
Factor 1x2			ns	ns	***	ns	***	***	**
Potato	0	0	70.0 a	6.88 e	2.06 b	4.82 d	2.84 ab	0.78 a	358 a
		1	76.2 b	1.85 b	2.11 b	0.00 a	3.70 b	1.59 b	687 c
	CA	0	71.4 a	3.15 d	1.66 a	1.49 c	2.34 a	0.67 a	518 b
		1	79.0 c	1.60 a	1.59 a	0.00 a	2.43 a	0.84 a	860 d
	SPI	0	70.5 a	2.72 c	1.50 a	1.22 b	2.39 a	0.89 ab	538 b
		1	78.1 c	1.92 b	2.01 b	0.00 a	3.29 ab	1.28 ab	762 cd
Analysis of variance and significance (p-values)									
Factor 1 (protein type)			**	***	**	***	*	ns	**
Factor 2 (MW treatment)			***	***	*	***	*	*	***
Factor 1x2			ns	***	**	***	ns	ns	ns

Protein: 0: without protein; CA: 5% calcium caseinate; SPI: 5% soy protein isolate. MW treatment: 0: without treatment; 1: with treatment. PT: pasting temperature, PV: peak viscosity, TV: trough viscosity, BD: breakdown, FV: final viscosity, ST: setback. The different letters in the corresponding column within each starch type indicate statistically significant differences between means at $p<0.05$. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant.

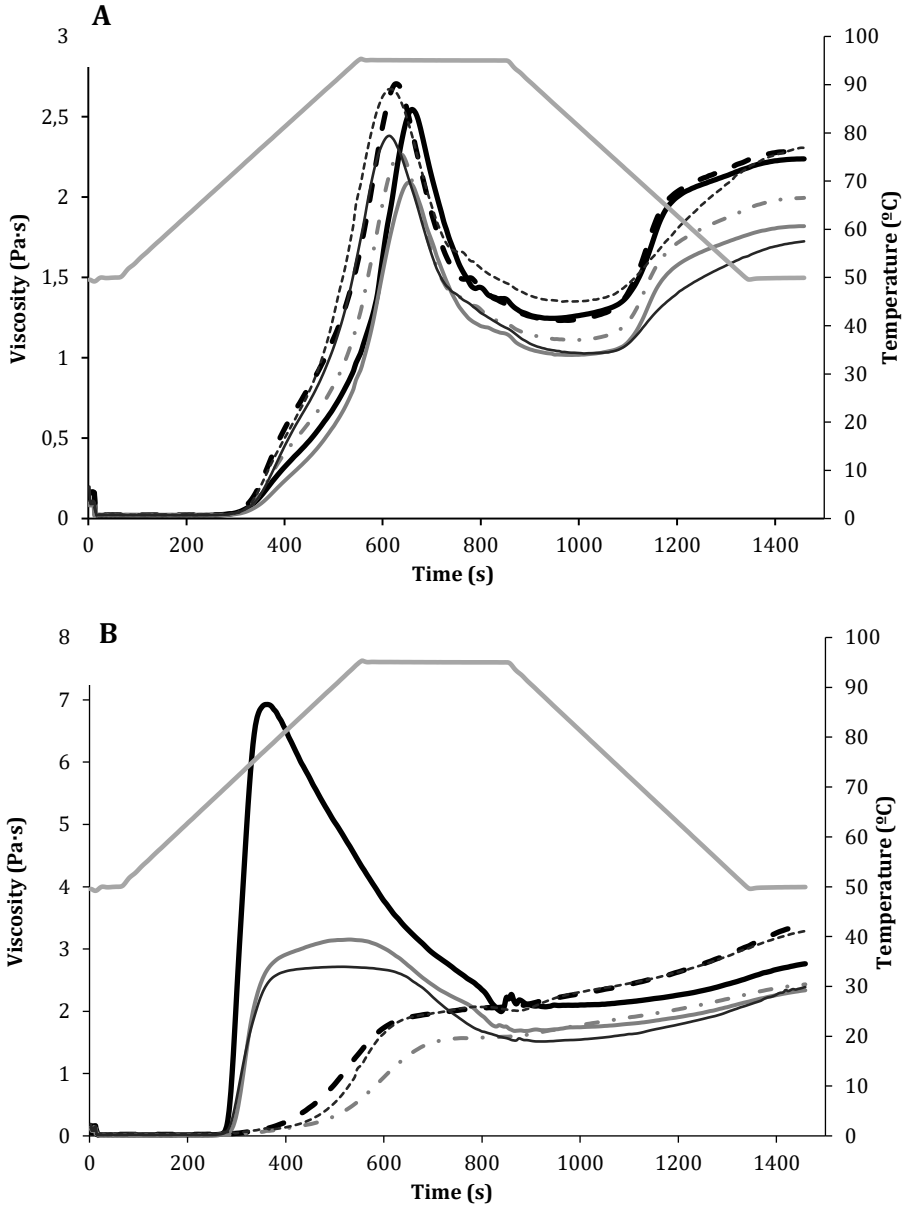

Figure 1. Pasting profiles of native and modified samples of rice starch (A) and potato starch (B). Starch without protein are represented by ——, with 5% calcium caseinate by ——, and with 5% soy protein isolate by ——. Microwave-treated protein-free samples are represented by – – –, microwave-treated samples with 5% calcium caseinate by – · – · and with 5% soy protein isolate by -------. The temperature profile is represented by —— in the second axis.

Ronda et al. (2014) observed that the reduction of starch due to the replacement of proteins caused lower pasting viscosity values. These proteins can retain water from the starch granules and consequently reduce initial starch granule swelling. Applying MWT also reduced potato starch PV, from 6.88 to 1.85 Pa·s. This result has previously been reported for both potato flour and potato starch; it was attributed to a possible reduction of amylose leaching as result of MWT (Varatharajan et al., 2010). The opposite effect was observed in rice starch, with a slight although significant increase in PV (7%) after treatment. Other studies reported significant PV decreases when rice flour was treated with HMT (Puncha-arnon and Uttapap, 2013) or MW radiation (Villanueva et al., 2018a). Proteins must play an important role in the effect of thermal treatments on rice flour. They can be denatured by heat treatment and some changes or interactions might occur between them during heat treatment (Puncha-arnon and Uttapap, 2013). Qiu et al. (2016) observed an increase in PV when a dry heating treatment was applied to rice flour or starch. The fact that MW treatment combines a high humidity heating period with a dry heating period could explain the intermediate behaviour that we observed in rice starch with respect to that obtained by Puncha-arnon and Uttapap (2013) and Qiu et al. (2016). The low peak time of native potato starch increased significantly with MWT, reaching the peak values of rice starch. Native potato starch showed a much higher BD than rice starch, 4.82 versus 1.30 Pa·s (Table 2). A low BD value means high paste stability versus heat and shear, and is related to granule rigidity and high lipid content (Singh et al., 2003). Adding protein increased the paste stability of potato starch, decreasing the BD value by 69% and 75% for CA and SPI, respectively. However, in the case of rice starch, only CA had an effect and it was much slighter. MWT hardly affected the BD value of the rice starch samples. However, the treatment did reduce potato starch BD to zero, regardless of the presence of proteins. This reflects the ability of MWT to increase potato starch gel stability versus heating and shearing. This behaviour has already been observed in tuber starches (potato and tapioca) when they were moistened and thermally modified either by convection heat transfer

(Klein et al., 2013) or MW radiation (Nadiah et al., 2015). ANOVA analysis (Table 2) revealed that protein type and MW treatment had a singularly strong effect on PT, PV and BD values of rice starch, without any mutual interaction of these factors. However, potato starch was highly susceptible, not only to protein type supplementation or MWT but also to the double interaction of the two factors on PV and BD. The final viscosity (FV) of the gels formed after subsequent cooling decreased in presence of either animal or vegetal protein. The same had previously been reported by Ronda et al. (2014). The effect was more pronounced for rice starch gels than for potato gels. MWT increased the FV of rice+protein gel, while potato starch FV was unaffected. ANOVA analysis showed that each of the main factors influenced final viscosity, but the double effect was found only in rice starch. Setback viscosity (ST), mainly related to the amylose leached from starch granules and their tendency to reorganise after gelatinisation (Miles et al., 1985), was equivalent for both native starches in our study. Adding proteins significantly ($p<0.001$) decreased this value in rice starch blends, while potato values remained unaffected. MWT increased ST in all rice starch-based formulation and protein-free potato starch. In the case of potato starch the increase was from 0.78 to 1.59 Pa·s. The ANOVA results confirmed that the simple and double-factor effects on rice starch ST were highly significant ($p < 0.001$). However, except for MWT, there were almost no effects on potato starch.

3.4. Gel rheological properties

The viscoelastic properties of gel samples were studied by dynamic oscillatory tests, applying a sinusoidal stress to the samples. The obtained viscoelastic moduli as a function of stress are shown in Figure 2. The parameters obtained from fitting the mechanical spectra to power law are summarized in Table 3. The high R^2 values obtained, always above 0.998 for all samples, demonstrate that the systems studied adjusted well to the power law model. Stress sweeps made it possible to establish the linear viscoelastic region (LVR) by identifying the maximum stress (τmax) that samples could tolerate conserving their structure. These values are also included in Table 3.

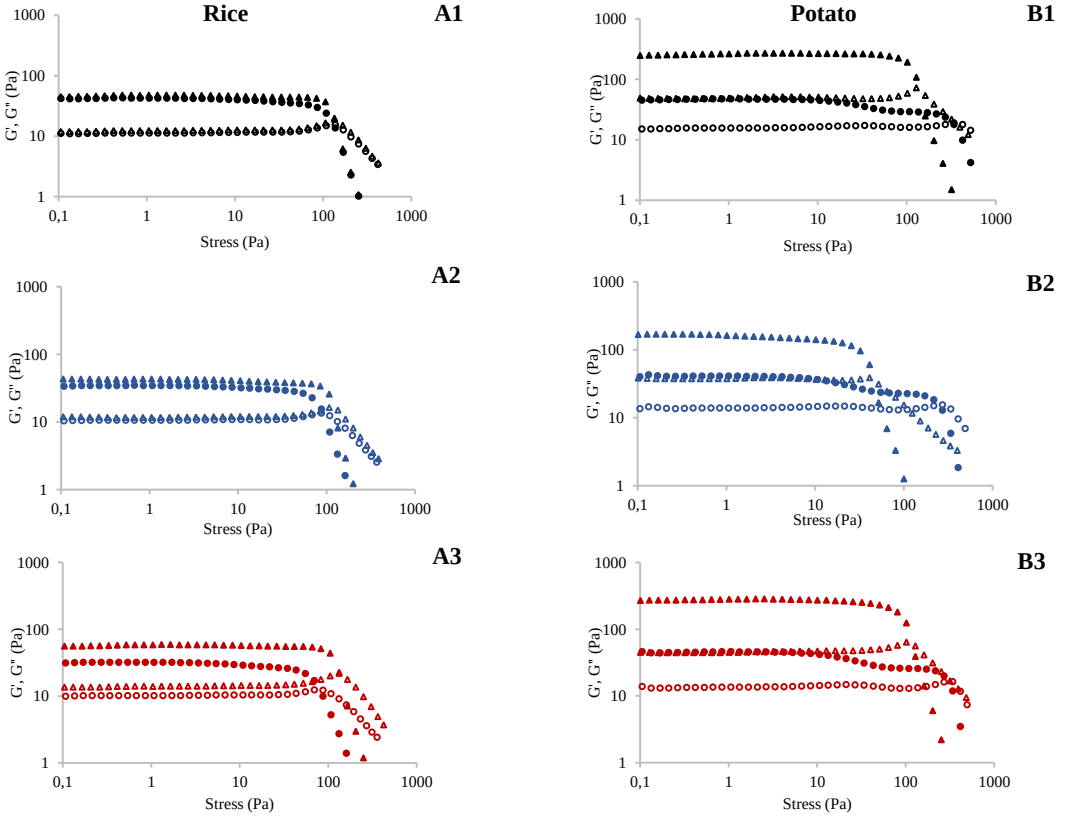

Figure 2. Strain sweeps of rice (A) and potato (B) starch gels without protein addition (A1 and B1), with 5% calcium caseinate (A2 and B2) or 5% soy protein isolate (A3 and B3). Non-treated samples are represented with circles and MW-treated samples with triangles. The elastic modulus, G', is represented using solid symbols and the viscous modulus, G'', with open symbols.

Table 3. Effect of microwave treatment and protein presence on the dynamic rheological characteristics of starch gels.

Starch	Protein	MW treatment	G'_1 (Pa)	a	G''_1 (Pa)	b	$(\tan\delta)_1$	c	τ_{max} (Pa)
Rice	0	0	52 ab	0.17 b	14 ab	0.36 a	0.27 bc	0.18 ab	108 c
		1	74 bc	0.15 a	17 ab	0.35 a	0.23 ab	0.20 b	119 c
	CA	0	41 a	0.18 b	12 a	0.36 a	0.29 c	0.18 a	69 ab
		1	47 a	0.17 b	13 ab	0.35 a	0.27 bc	0.18 ab	85 b
	SPI	0	39 a	0.19 b	12 a	0.36 a	0.30 c	0.17 a	55 a
		1	88 c	0.14 a	19 b	0.35 a	0.22 a	0.20 b	105 c
Analysis of variance and significance (p-values)									
Factor 1 (protein type)			ns	ns	ns	ns	ns	ns	**
Factor 2 (MW treatment)			**	**	*	ns	**	*	**
Factor 1x2			ns	ns	ns	ns	ns	ns	*
Potato	0	0	53 a	0.181 e	16 a	0.29 b	0.31 e	0.11 ab	267 d
		1	421 d	0.077 a	51 b	0.22 a	0.12 a	0.15 c	101 b
	CA	0	46 a	0.184 e	14 a	0.28 b	0.31 e	0.10 a	216 c
		1	171 b	0.141 c	39 b	0.28 b	0.23 c	0.14 bc	26 a
	SPI	0	53 a	0.164 d	14 a	0.28 b	0.27 d	0.12 abc	304 d
		1	309 c	0.099 b	48 b	0.23 a	0.15 b	0.14 bc	84 b
Analysis of variance and significance (p-values)									
Factor 1 (protein type)			***	***	ns	*	***	ns	**
Factor 2 (MW treatment)			***	***	***	**	***	**	***
Factor 1x2			***	***	ns	*	***	ns	ns

Protein: 0: without protein; CA: 5% calcium caseinate; SPI: 5% soy protein isolate. MW treatment: 0: without treatment; 1: with treatment. G'_1, G''_1, and $(\tan\delta)_1$, represent the elastic and viscous moduli and the loss tangent at a frequency of 1 Hz. The a, b and c exponents quantify the dependence degree of dynamic moduli and the loss tangent with the oscillation frequency, ω. τmax represents the maximum stress that GF matrices can tolerate in the LVR. The different letters in the corresponding column within each starch type indicate statistically significant differences between means at $p<0.05$. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant.

The elastic modulus, G', of gels made from untreated starches started to decrease at 108 and 267 Pa for rice and potato starch, respectively. This indicated that starch sample structures had different resistances to stress-induced rupture. According to Villanueva et al. (2018b), potato gels preserved their structure beyond rice starch gels, meaning that potato gels had good mechanical resistance and hardness. Adding proteins to rice starch gels reduced their resistance to breakage and led to values of τmax 36% and 49% lower than those of protein-free gels for CA and SPI, respectively. The effect on potato starch gels differed depending on protein type: CA decreased τmax by 19%, while SPI increased it by 14%, compared to protein-free potato gels. MWT increased the strength of rice gels, although this effect was only significant in SPI-enriched rice gels. The effect of MW radiation on potato gels was the opposite and stronger, being much more remarkable in the presence of proteins: τmax decreased by 60% in gels without protein, 88% in CA protein-enriched gels and 72% in SPI-enriched gels.

In all samples, G' and G" increased with frequency, G' being higher than G", which indicated that elastic character prevailed over viscous factor (Xie et al., 2013). The gel viscoelastic moduli G_1' and G_1'' and the loss tangent at 1 Hz were the same for the two native starches (potato and rice) regardless of protein presence or type. However, MWT always increased gel consistency, this impact was dependent on protein presence and type and starch type. The effect of MWT on gel rheological properties was also strongly dependent on the botanical origin of the starches. Both viscoelastic moduli increased in MW-treated potato starch samples, while only SPI addition affected the moduli in treated rice ones. The G_1' and G_1'' increases from MWT of potato gels were related to the gelatinization level and the network development of chains leached from the starch granules during the MW treatment (Xie et al., 2013). As a consequence of MWT, the increase in the elastic modulus was always more noticeable than in the viscous modulus. Consequently, MWT was always accompanied by a drop in the loss tangent, which meant a reinforcement of the elastic character of gels made from MW-treated starches or starch+protein blends compared to the untreated

counterparts. Similar results have been reported for thermally-modified potato starch (Gryszkin et al., 2014). Tan δ value was very low for all samples (always < 0.4), which corresponded with a well cross-linked network. Adding SPI decreased the gel loss tangent of potato starch, but rice gels were unaffected by protein addition. Villanueva et al. (2018b) reported that the rheological properties of rice and potato starch gels fortified with egg albumen or SPI depended significantly on the double interaction (starch x protein), concluding that the same protein exerted a different effect depending on the starch source. The values of the "a" exponent were always below the exponent "b". This meant that G'' increased with frequency faster than G'. This yielded an increase in tan with frequency, which could also be confirmed from the positive value of exponent "c" (Table 3). Consequently, gels increased their viscous behaviour with frequency.

4. Conclusions

MWT has been shown to be successful in modifying starches and their protein mixtures and has proven to be efficient in altering the physical properties of products made from them. It is particularly useful in the development of protein-enriched GF products. The results showed that the effect of MWT is dependent on starch source and protein type. MWT changed the hydration properties and enhanced water absorption index and swelling power in potato samples, while it decreased them in rice starch samples, regardless of type of protein added. MW radiation influenced mainly the pasting properties of potato starch, increasing pasting temperature and setback, and decreasing peak viscosity and breakdown. The treatment increased the viscoelastic moduli significantly and decreased the loss tangent of potato gels both with and without proteins. In general, the inclusion of proteins increased WAC, WAI, SP and WSI, decreased the viscosity of gels and increased their stability, being the effect more marked for SPI incorporation.

This research helps understand the changes that occur during MW treatments of model systems and can help design and improve the quality

of new products. MW-assisted heating is an innovative flour treatment method and can be used as an alternative to chemical modification.

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CAPÍTULO VII

CHAPTER VII

IMPACTO DEL TRATAMIENTO HIDROTÉRMICO MEDIANTE ENERGÍA MICROONDAS SOBRE LA HARINA DE ARROZ Y SU EFECTO EN LAS PROPIEDADES REOLÓGICAS DE MASAS Y LA CALIDAD DEL PAN SIN GLUTEN

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Microwave assisted heat moisture treatment of rice flour improves the viscoelastic behavior of doughs and its bread-making performance

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Abstract

Microwave radiation of rice flour and its effect on the rheological and pasting properties of gluten-free doughs and the physical quality of their resulting breads was investigated. Two levels of flour initial moisture content, 20% (MW-20%) and 30% (MW-30%) and two levels of its addition (30% and 50%) to the dough were evaluated to assess the potential of the physical treatment to modify dough viscoelasticity and bread-making ability. MW-30% treated rice flour showed the most notable results. It provided enhanced dough viscoelasticity *vs* the control (100% native rice flour), increasing the dough G1' modulus up to 69% and 135% for the treated flour additions of 30% and 50% of MW-30% respectively. The treated flour increased the resistance of doughs to deformation and enhanced their elastic behavior and recovery capacity up to 170% when compared to the control dough. The major effects on pasting parameters were also obtained for the doughs formulated with

MW-30% flour at the maximum substitution level (50%). It delayed the pasting temperature, decreased the peak, trough and final viscosities with respect to the control dough. Both MW-treated rice flours (MW-20% and MW-30%) led to breads with higher-specific volume, softer crumb and delayed bread staling. The MW assisted heat moisture treatment of rice flour seems to be a valuable procedure to improve the viscoelastic behavior and bread-making performance of gluten-free doughs.

Keywords: Microwave treatment, Rice flour, Dough rheology, Pasting properties, Gluten-free bread.

1. Introduction

Rice flour is one of the most suitable ingredients for GF bakery formulations due to its hypoallergenic properties, bland taste and white colour. Other attributes such as the low protein and sodium content, as well as the presence of easily digested carbohydrates, are additional benefits of this raw material (Rosell et al., 2014). These characteristics encompass simultaneously some structural problems such as weak protein-starch network building capacity and the inability to sufficiently retain gas bubbles during fermentation. Several strategies have been developed to alleviate the rice flour dough formation problems, such as the addition of structure creating hydrocolloids (Ronda et al., 2013), nutritionally relevant fibers (Pérez-Quirce et al., 2014), external proteins (Crockett et al., 2011; Ziobro et al., 2013), emulsifiers (Demirkesen et al., 2010), enzymes (Renzetti and Rosell, 2016; Amin et al., 2017), dough acidification (Villanueva et al., 2015; Ronda et al., 2014) or emulsion formation (Yano et al., 2017). Other methods focus on physical modification of functional properties of rice flour by heat moisture treatment (HMT) that consists of heat undergoing different moisture levels (Puncha-Arnon and Uttapap, 2013; Qin et al., 2016; Bourekoua et al., 2016) or heat-pressure treatment (Cappa et al., 2016; Xu et al., 2016).

Thermal treatments deliver the possibility to impact both nutritional and functional properties during processing, as changing moisture and thermal conditions create an environment for different micro and macroscopic changes of complex matrix of flour (BeMiller and Huber, 2015). However, typical heat application results in high potential costs of industrial scaling up. Meanwhile application of microwaves (MW) as heat providing media seems to be reasonable from both cost and functionality effectiveness change perspectives. Our previous works had studied the application of microwave energy to rice flour for β-glucanase inactivation and enhancement of β-glucans bioactivity of fortified rice-based gluten-free breads (Pérez-Quirce et al., 2017). Bread physical quality was hardly affected by flour MW pretreatment. In fact, only a slightly higher loaf specific volume was noted for breads made from the most intensively treated flour (4 min of MW treatment at 25% moisture content and 96ºC maximum temperature reached by flour). No significant change was observed in the pasting properties of the treated flours.

It is known that more intense microwave assisted heat moisture treatment can change the functional properties of starches (Anderson and Guruya, 2006, Villanueva et al., 2018c) but since there are substantial differences between a starch and a flour, in a previous study we have investigated its impact on rice flour (Villanueva et al., 2018a). The effect of microwave assisted thermal treatment was studied in relation to the initial moisture content (20% and 30%) of treated-rice flour. The microwave radiation absorption capacity of flour, the moisture change during the treatment, the particle morphological structure as well as crystallinity/amorphous region ratio and flour thermal properties were studied revealing significant gelatinization temperature rise and the amylopectin retrogradation extent in treated-flours. The treatment resulted in lower viscometric profiles, amylose retrogradation and higher pasting temperatures (Villanueva et al. 2018a). The influence of this microwaved-treated rice flour on the viscoelastic behavior and bread-making performance of gluten-free rice based doughs seems necessary to be determined and has not been studied so far.

The objectives of this study were to evaluate the changes in fundamental rheological properties of doughs in which native rice flour was partially substituted by microwave-treated one as well as the impact of this new ingredient on the quality of the gluten-free bread. These results will complement our previous study (Villanueva et al., 2018a) and provide well-structured knowledge on how the microwave-assisted heat-moisture treatment of rice flours changes its bread-making properties.

2. Materials and Methods

2.1. Rice flour

Indica rice variety (long grain) flour provided by Herba NAT 300 (Herba Ricemills S.L.U., Valencia, Spain) was used in this work. The moisture content was 13%, ash <1.0%, protein: 8.13 %, fiber < 1%; fat < 1%. The granulometry of flour was as follows: 1% > 250 μm, 250 μm > 6.1% >210 μm, 210 μm > 36.1% > 150 μm, 150 μm > 33.4% >100 μm and 26.6% < 100 μm (data provided by manufacturer).

2.2. Flour preparation and microwave treatment

Native rice flour water content was measured with Official Method AACC 44-19.01 (AACC, 2010) and the amount of water for reaching 20% and 30% of initial moisture content (IMC) levels was added. The flour preparation and the procedure used to perform the microwave treatment are described in Villanueva et al. (2018a). The microwave treatment time was 8 min applied in cycles of 20 s of exposure and 40 s of rest. The temperature evolution curves obtained for the rice flour studied were equivalent to those reported for 20 and 30% moistened-rice flour in our previous work (Villanueva et al., 2018a). The temperature reached and maintained after 8 min of MW-treatment was 157±5ºC for all samples. Depending on the IMC of the treated rice flour (20% or 30%), the MW treatment resulted in two different modified rice flours that were called MW-20% and MW-30%. They were further added to the dough in substitution of native rice flour at two different levels: 30% and 50% of to the total amount of flour used in the dough formulation.

2.3. Dough preparation and bread-making

A straight dough process was performed using the following formula on a 100 g rice flour (13% moisture) basis: 1.5% salt, 2% HPMC, 5% sucrose, 6% oil and 95% water. Additional 3% dried yeast dispersed in the water was used in the bread-making process. The GF dough and bread-making procedures are described in detail elsewhere (Pérez-Quirce et al., 2017). After baking, the breads (~200 g) were removed from the pans and left for 1 h at room temperature before any analysis.

2.4. Dough measurements

Oscillatory and creep recovery tests

Oscillatory and creep-recovery tests were carried out with a Kinexus Pro+ rheometer (Malvern Instruments Ltd, UK) with parallel plate geometry (40 mm diameter) of serrated surface and with 1 mm gap. The excess of batter was removed and vaseline oil was applied to cover the exposed sample surfaces. Before the measurement, the dough was rested for 5 min to allow relaxation. Frequency sweeps were carried out from 20 to 0.1 Hz in the linear viscoelastic region (LVR) previously established for each batter by means of stress sweeps from 0.1 to 500 Pa at 1 Hz. The frequency sweeps of all batters were carried out at stress values of 1 Pa. Temperature was 25 ºC. Frequency sweep data were fitted to the power law model as in previous works (Ronda et al., 2013).

The coefficients G'_1, G''_1 and $(\tan \delta)_1$, represent the elastic and viscous moduli and the loss tangent at a frequency of 1 Hz. The a, b and c exponents quantify the dependence degree of dynamic moduli and the loss tangent with the oscillation frequency, ω. Creep tests were performed by imposing a sudden step shear stress in the LVR and outside the linear viscoelastic region (OLVR). For the creep study in the LVR, a constant shear stress of 1 Pa was applied for 150 s, while in the recovery phase the stress was suddenly removed and the sample was allowed for 300 s to recover the elastic (instantaneous and retarded) part of the deformation. For the study OLVR, a constant shear stress of 50 Pa was applied for 60 s and the sample was allowed to recover for 200 s after removing the load. The data from creep tests were modelled to the 4-parameter Burgers model (Ronda, et al., 2014).

$$J_c(t) = J_{0c} + J_{1c}\left(1 - \exp\left(\frac{-t}{\lambda_{1c}}\right)\right) + \frac{t}{\mu_0} \quad (1)$$

In the equation, J_c (t) is the creep compliance (strain divided by stress), J_{0c} is the instantaneous compliance, J_{1c} is the retarded elastic compliance or viscoelastic compliances, λ_{1c} is the retardation time and μ_0 gives information about the steady state viscosity. Similar equations were used for the recovery compliance J_r (t). As there is no viscous flow in the recovery phase, equations consist only of parameters describing the elastic response after removal of the shear stress. The data from creep tests were modelled to the 3-parameter Burgers model given by:

$$J_r(t) = J_{\max} - J_{0r} - J_{1r}\left(1 - \exp\left(\frac{-t}{\lambda_{1r}}\right)\right) \quad (2)$$

J_{max} is the maximum creep compliance obtained at the end of the creep step. The steady-state compliance in recovery step, J_{steady}, was also calculated by subtracting the compliance value at the terminal region of curve (where dough recovery reached equilibrium) from the J_{max}. The ratio J_{steady}/J_{max} (elastic recovery) was also calculated and expressed as Recovery (%).

Each rheological test was performed in triplicate.

Pasting properties

Obtained doughs were lyophilized in Genesis Pilot Lyophilizer (SP Scientific, Pa, USA) and the resulting solids where manually comminuted in mortar. The pasting properties were studied using Kinexus Pro+ rheometer (Malvern Instruments Ltd, UK) with starch pasting cell geometry using Standard 2 method 76-21.01 (AACC, 2010). Rice flour samples (3 g, 14% moisture basis) were transferred into the canister where 25 mL± 0.1 mL of distilled water was added. Each sample was analyzed at least in duplicate. The rSpace ver. 1.72 software (Malvern Instruments Ltd, UK) was used to calculate the pasting temperature (PT), peak viscosity (PV), trough viscosity (TV), breakdown (BD), final viscosity (FV) and setback (ST=FV-TV).

2.5. Evaluation of bread quality

The volume of bread was determined from two replicates using a Volscan profiler 300 (Stable Microsystems, Surrey, UK) analyzer. The breads were weighed immediately after removal from the pan once cooled to determine the baking loss.

Crumb texture was determined in quadruplicate with a TA-XT2 texture analyzer (Stable Microsystems, Surrey, UK) provided with the software "Texture Expert". An aluminium 20 mm diameter cylindrical probe was used in a "Texture Profile Analysis" double compression test (TPA) to penetrate 50% depth, at 1 mm/s speed test, with a 30s delay between the first and second compression. Firmness (N), chewiness (N), cohesiveness, springiness and resilience were calculated from the TPA graphic. Analysis was carried out at (20±2) ºC from two bread slices of 20 mm thickness taken from the centre of the loaf. Moreover, the differences in firmness values of breads between the fresh products and those after storage of 7 days (ΔFirmness) at (4 ± 2) ºC in hermetic bags were taken as a staling index.

Photographs of slices and side whole loafs were taken with PowerShot SX410 IS camera (Canon, Japan). Colour was measured with a Minolta spectrophotometer CN-508i (Minolta, Co.LTD, Japan). Results were obtained in the CIE L*a*b* and CIE L*C*h coordinates using the D65 standard illuminant, and the 2º standard observer. Colour determinations were made 5x5 times: bread crumb and crust colors were checked at five different points on each bread and every point was measured five times.

2.6. Statistical analysis

Statgraphics Centurion v.6 (Bitstream, Cambridge, MN, USA) was used for multivariate non-linear regression and Pearson correlation matrix. STATISTICA package (Tulsa, OK, USA) v.6, allowed performance of multi-factor analysis of variance (ANOVA) and LSD (Least Significant Difference) test was used to evaluate significant differences ($p<0.05$) between samples.

3. Results and Discussion

3.1. Effect of MW-treated flour on dough properties

Dynamic oscillatory tests on doughs

The impact of MW treated-rice flour substitution level (30% and 50% of the total amount of rice flour in the bread dough formulation) and its IMC before the MW treatment (20% and 30%) on doughs was studied by small amplitude oscillatory (SAOS) test. Table 1 summarizes the coefficients G'_1, G''_1 and $(\tan\delta)_1$, as well as the exponents *a*, *b* and *c* obtained by fitting the power-law model to frequency sweep data. The high r^2 values demonstrate the good adjustment of the systems studied to the model (r^2>0.997). For all the studied samples the elastic modulus G' resulted in higher values than the viscous modulus G'' providing values of tanδ that ranged 0.49 – 0.64. The ANOVA study indicated a significant effect ($p<0.001$) of the initial moisture content on G'_1, G''_1 and $(\tan\delta)_1$. However, the level of native rice flour substitution by MW treated flour did not have any significant effect. The interaction (level x IMC) affected significantly on G'_1 which explains the significant increase of the elastic moduli with the level of substitution of MW-30% flour while no effect was observed with MW-20%.

The control dough, made from 100% native rice flour, provided the lowest G'_1 and G''_1 values, nevertheless, they were not significantly different from the moduli of doughs made with MW-20% rice flour regardless its substitution level. However, the addition of MW-30% flour led to an increase in G'_1 of 69% and 135%, compared to the control dough, for the 30% and 50% addition level respectively.

Table 1. Effect of MW treated-rice flour substitution level (30% and 50% with respect to the total rice flour amount) and the initial moisture content of the flour before the treatment (MW-20% and MW-30%) on bread doughs viscoelastic properties obtained from oscillatory tests.

Viscoelastic properties	Level (%):	Control 0	MW-20% 30	MW-20% 50	MW-30% 30	MW-30% 50	SE	Factor 1 Level	Factor 2 IMC	Factor 1 x 2 Level x IMC
G'_1 (Pa)		1379a	1517a	1404a	2329b	3238c	268	ns	***	*
a		0.35b	0.35b	0.37b	0.31a	0.29a	0.020	ns	***	ns
r^2		0.999	0.999	0.9992	0.997	0.997				
G''_1 (Pa)		833a	946a	886a	1264b	1485b	139	ns	***	ns
b		0.37b	0.35a	0.39b	0.33a	0.34a	0.020	ns	*	ns
r^2		0.999	0.999	0.999	0.998	0.999				
$(tan\delta)_1$		0.64b	0.63b	0.63b	0.55a	0.49a	0.030	ns	***	ns
c		0.027a	0.005a	0.017a	0.023a	-0.015a	0.048	ns	ns	ns
τ_{max} (Pa)		5.42a	10.36a	8.98a	11.53a	21.25b	4.5	ns	ns	ns

IMC: Initial Moisture Content of the treated flour. MW-20%: Rice flour treated at 20% of Initial Moisture Content; MW-30%: Rice flour treated at 30% of Initial Moisture Content. The power law model was fitted to experimental results from frequency sweeps. $G' = G'_1 \cdot \omega^a$; $G'' = G''_1 \cdot \omega^b$; $\tan\delta = (\tan\delta)_1 \cdot \cdot \omega^c$). $(\tan\delta)_1$ was obtained from the quotient G''_1 / G'_1 and *c* from *b-a*. τ_{max} was obtained from stress sweeps. Different letters in the corresponding row indicate statistically significant differences between means at $p<0.05$. SE: Pooled standard error obtained from ANOVA analysis. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant.

The viscous modulus, G''_1, of doughs made with MW-30% flour also increased (up to 78%) *vs.* the control doughs and doughs with MW-20%, although no significant differences were observed between the doughs with addition level (30% or 50%). Similar effects were noted on the loss tangent at 1 Hz. The $(\tan\delta)_1$ values only varied significantly with respect to the control dough when MW-30% treated-flour was added, regardless the addition level used. The loss tangent decreased in these doughs up to 24% denoting the ability of this MW-treated flour to increase the elastic behavior of bread doughs.

Both moduli slightly increased with frequency in all the dough samples. The dependence of viscoelastic moduli on angular frequency, which is quantified by a and b exponents, decreased significantly as result of MW-30% addition (see Table 1) denoting more stable dough structures, regardless the addition level. However, the addition of MW-20% flour did not have any effect on these values. The stronger structure of dough obtained with MW-30% flour is coherent with the mayor changes observed in the functional properties of flour versus treated at higher initial moisture content (Villanueva et al., 2018a). Pinkrova et al. (2003) also reported minimal changes resulting in MW treatment of rice flour at moisture below 23% while obtained significant reduction in the pasting peak viscosity when the moisture was 30%. Authors have related this result with the significant increase of damaged starch found in this flour as a result of the MW energy and the temperature reached during treatment. The increase of damaged starch would also explain an increase in the flour's water absorption capacity (Villanueva et al., 2018c) and the concomitant increase in dough consistency. Puncha-Arnon and Uttapap (2013) concluded a reordering of amylose and amylopectin within starch granules during heat-moisture treatments (at 100ºC for 16 h at 20-30% moisture) of rice starch and flour. They also proposed the reinforcement of interactions between starch granules and proteins, denatured by heat. These molecular changes would justify the observed strengthening in bread doughs structure denoted by the viscoelastic moduli increase and the decrease of loss tangent.

The stress sweeps provide the τ_{max} value or maximum stress that doughs were able to stand before the structure broke. The τ_{max} values for all the doughs ranged from 5.4 to 21.3 Pa (Table 1). The Fig. 1a shows the stress sweeps of the control dough (100% native rice flour) and the dough with 50% of MW-30% treated rice flour. These two samples showed the lowest and highest values of τ_{max} respectively and again confirmed the stronger structuring effect of MW-30% flour over MW-20% flour. Table 1 shows that only when MW-30% was added at 50% level the effect on τ_{max} was significant. In general, as the initial moisture content and rate of addition increased, a greater structuring effect of the dough was determined. This could be due to the changes observed on structure, crystallinity, thermal and pasting properties of microwave treated rice flour samples, which were more pronounced with the higher levels of initial moisture content in the treatment (Villanueva et al., 2018a). Our previous results confirmed the suggestion made by Lewandowicz et al. (2000) that a higher gelatinization temperature of microwave irradiated starches may also indicate an association and a more stable configuration in a granular structure, resulting in higher values of G'_1, G''_1 and τ_{max}.

Creep-recovery tests

Creep-recovery tests were carried out at both 1 Pa, within the linear viscoelastic region (LVR), and 50 Pa, outside the linear viscoelastic region (OLVR). The Burgers model parameters obtained from these tests are summarized in Table 2. The stress values used for creep-recovery test in the LVR are often inadequate to real dough processing conditions because they are carried out in stress ranges very far from those experienced by the dough during processing or baking expansion. However, those measurements are of great value in studying the influence of ingredients (Ronda et al., 2017). Creep-recovery curves of GF doughs exhibited a typical viscoelastic behavior combining both viscous and elastic components (Figure 1b), similar to the corresponding curves previously obtained for rice flour (Sivaramakrishnan et al., 2004) and other gluten-free doughs (Lazaridou et al., 2007; Ronda et al., 2015; Villanueva et al., 2018b).

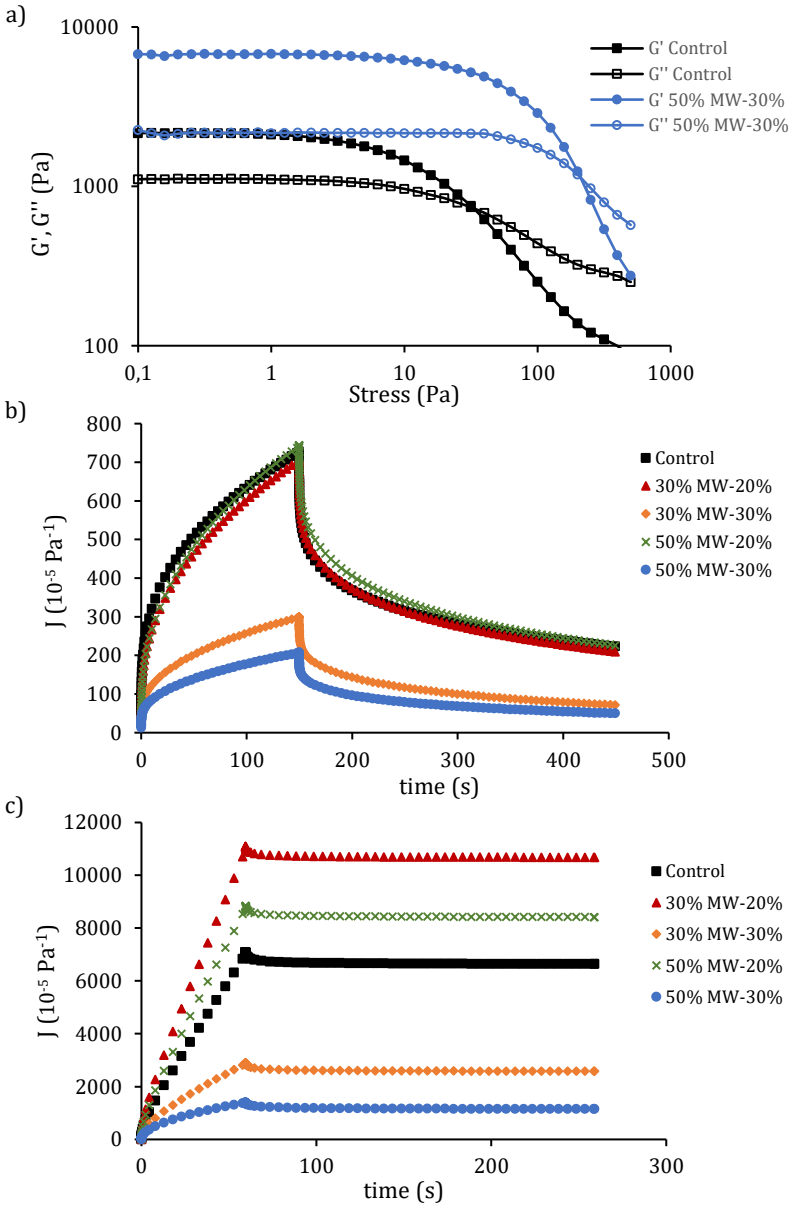

Figure 1. Effect of MW treated-rice flour substitution level (30% and 50% with respect to the total rice flour amount) and the initial moisture content (IMC) of the flour before the treatment: 20% (MW-20%) and 30% (MW-30%) on stress sweeps (a) and creep-recovery test of samples in the LVR (b) and OLVR (c) of bread doughs. ■ Control. ▲30% of MW treated-rice flour addition with MW-20%, ♦, 30% MW treated rice flour addition with MW-30%, × 50% MW treated rice flour addition with MW-20%, ● 50% MW treated rice flour addition with MW-30%. In stress sweeps, elastic modulus G', is represented by solid points and the viscous modulus G'', by void points.

Table 2. Effect of MW treated-rice flour substitution level (30% and 50% with respect to the total rice flour amount) and the initial moisture content of the flour before the treatment (MW-20% and MW-30%) on bread doughs viscoelastic properties obtained from creep-recovery tests measured in the linear viscoelastic region (LVR) and outside the linear viscoelastic region (OLVR).

Viscoelastic properties		Level (%):	Control 0	MW-20% 30	MW-20% 50	MW-30% 30	MW-30% 50	SE	Factor 1 Level	Factor 2 IMC	Factor 1 x 2 Level x IMC
LVR Creep phase											
J_{0c}	**($10^{-5}Pa^{-1}$)**		74c	59b	62b	35a	26a	4.4	ns	***	ns
J_{1c}	**($10^{-5}Pa^{-1}$)**		258b	237b	267b	97a	71a	26	ns	***	ns
λ_c	**(s)**		3.8a	5.5b	7.1c	4.6ab	4.9ab	0.61	*	**	ns
μ_c	**(10^3 Pa·s)**		42.6a	42.7a	37.6a	94.9b	126.0c	5.9	*	***	***
J_{max}	**($10^{-5}Pa^{-1}$)**		726b	667b	744b	288a	210a	67	ns	***	ns
LVR Recovery phase											
J_{0r}	**($10^{-5}Pa^{-1}$)**		142c	122b	118b	61a	45a	6.8	ns	***	ns
J_{1r}	**($10^{-5}Pa^{-1}$)**		334b	338b	376b	142a	106a	26	ns	***	ns
λ_r	**(s)**		54.2ab	57.8ab	61.1b	52.7a	53.1a	3.2	ns	*	ns
Recovery	**(%)**		73a	74a	71a	74a	75a	3.3	ns	ns	ns
OLVR Creep phase											
J_{0c}	**($10^{-5}Pa^{-1}$)**		73bc	88c	81c	64b	48a	5.2	*	***	ns
J_{1c}	**($10^{-5}Pa^{-1}$)**		597a	944b	831b	567a	481a	51	ns	***	ns
λ_c	**(s)**		2.1a	2.1a	2.5a	4.1b	5.9c	0.28	**	***	*
μ_c	**(10^3 Pa·s)**		0.98ab	0.60a	0.78a	2.71b	7.19c	0.61	*	***	**
J_{max}	**($10^{-5}Pa^{-1}$)**		7094b	11098c	8837b	2903a	1403a	68	*	***	ns
OLVR Recovery phase											
J_{0r}	**($10^{-5}Pa^{-1}$)**		61d	51c	53c	42b	34a	1.8	ns	***	*
J_{1r}	**($10^{-5}Pa^{-1}$)**		368c	344c	339c	260b	202a	9.9	**	***	*
λ_r	**(s)**		4.9a	4.7a	4.9a	5.9a	10.1b	0.53	***	***	**
Recovery	**(%)**		6.7b	3.7a	4.8ab	11.2c	18.1d	0.90	***	***	**

IMC: Initial Moisture Content of the treated flour. MW-20%: Rice flour treated at 20% of Initial Moisture Content; MW-30%: Rice flour treated at 30% of Initial Moisture Content. J_0 and J_1 are the instantaneous and retarded elastic compliances; λ_1 is the retardation time and μ_0 the steady state viscosity. J_{max} is the maximum creep compliance obtained at the end of the creep step. Recovery is the elastic recovery obtained in the recovery phase expressed as percentage of the maximum compliance, J_{max}. Subscript c corresponds to parameters in the creep phase and subscript r, in the recovery phase. Different letters in the corresponding row indicate statistically significant differences between means at $p<0.05$. SE: Pooled standard error obtained from ANOVA analysis. Analysis of variance and significance: *** $p<0.001$, ** $p<0.01$, * $p<0.05$. ns: not significant.

In the LVR, a significant and strong correlation ($p<0.001$; $r=0.99$) was found for both compliance parameters obtained in the creep phase, J_{0c} and J_{1c}, and the equivalents from the recovery phase (J_{0r} and J_{1r}). Besides, it was observed that factors providing an increase in viscosity at the steady state, μ_0, decreased elastic and retarded elastic components, J_{0c} and J_{1c} ($p<0.05$ $r=-0.97$ and $p<0.01$; $r=-0.98$ respectively).

Burgers model parameters obtained in the LVR were unaffected by the MW-20% flour addition with the exception of the instantaneous elastic compliance, J_0, which decreased (on average ~18%) with respect to the control dough, and the retardation time in the creep phase, λ_c, which increased 45% and 87% for the addition levels of 30% and 50% respectively. A higher retardation time means more time needed to obtain the viscoelastic deformation of the dough, even though the final deformation values were the same (not significantly different) in the control and MW-20%-flour-added dough samples. However, the addition of MW-30% flour led to a decrease in all compliance values when compared to the control dough (59%, 67% and 65% on average for J_{0c}, J_{1c} and J_{max} respectively) and an increase in the steady viscosity μ_0 (126% and 196% for the addition levels of 30% and 50% respectively). These results denote that the addition of MW-30% treated flour increases the resistance of the bread doughs to deformation. The level of addition only affected significantly on the steady viscosity values of MW-30% flour added-doughs, leading to an additional increase of 33% when the substitution level increased from 30% to 50%.

Creep-recovery curves from OLVR tests are presented in Figure 1c. The curves obtained in this case correspond to materials with a much more predominant viscous component than the elastic one, as can be inferred from the almost direct proportionality between compliance and time in the creep phase, and the almost horizontal line obtained in the recovery step, particularly in the control dough and in the two MW-20% flour added-doughs. The dough made with MW-30% treated flour led to much lower curves, with higher elastic contribution than the control dough. Opposite to what was observed in the LVR, the OLVR curves allowed distinguishing among control and MW-20% flour added-doughs. As can

be seen in Table 2, the J_{max} value obtained in MW-20% flour added-doughs increased up to 56% with respect to the control dough, while the viscoelastic compliance in the creep phase, J_{1c}, increased up to 58%. As could be expected, outside the linear viscoelastic region, where the stress applied overpasses the maximum stress the dough can stand without breaking its structure, the compliances obtained in the recovery phase were not significantly correlated ($p>0.05$) with those of the creep phase.

The recovery capacity of doughs after releasing the applied stress, which is related to the contribution of the elastic deformation (the only one recoverable) with respect to the total deformation, decreased markedly from 71 – 74% in the LVR (without differences among the tested doughs) to 4 – 11%, when the tests were performed OLVR. The doughs made with 30% and 50% of MW-30% flour showed significantly higher elastic behavior and their recovery capacity increased 67% and 170% with respect to the control dough respectively. Since elasticity reflects the extent of bonding between the structural elements of the dough an increase in the elasticity could mean less deformation or breakage of the composite network, due to the MW-30% treated flour presence in the dough (Skendi et al., 2009).

Pasting properties

The impact of MW assisted treatment on the viscometric parameters of bread doughs containing 30% and 50 % of treated flours is shown in Figure 2 and Table 3. Quantitative viscometric profiles of the control dough (100% native rice flour) during pasting and gelling were systematically higher as compared to doughs with 30% and 50% of MW-treated flour substitution. The major effects on cooking and cooling parameters were obtained for the doughs formulated with flours treated at the highest IMC (30%) and at the maximum substitution level (50%). Similar findings were reported for flours treated by MW-assisted heat moisture treatment (Villanueva et al., 2018a) or conventional heat moisture treatments (HMT) (Zavareze and Dias, 2011).

Table 3. Effect of MW treated-rice flour substitution level (30% and 50% with respect to the total rice flour amount) and the initial moisture content of the flour before the treatment (MW-20% and MW-30%) on pasting properties of bread doughs.

Pasting properties	Level (%):	Control 0	MW-20% 30	MW-20% 50	MW-30% 30	MW-30% 50	SE	Factor 1 Level	Factor 2 IMC	Factor 1x2 Level x IMC
PV (10^{-3}Pa)		1607e	1457d	1373c	1315b	1167a	8.0	***	***	**
TV (10^{-3}Pa)		1090e	1051d	997b	1015c	905a	3.4	***	***	***
BD (10^{-3}Pa)		519e	406d	376c	300b	261a	8.0	***	**	ns
SB (10^{-3}Pa)		1102b	1157d	1086a	1128d	1165d	3.4	***	**	***
FV (10^{-3}Pa)		2189d	2208e	2083b	2143c	2070a	3.2	***	***	***
PT (ºC)		86.1a	89.7b	91.3c	90.2b	92.9d	0.30	**	***	ns

IMC: Initial Moisture Content of the treated flour. MW-20%: Rice flour treated at 20% of Initial Moisture Content; MW-30%: Rice flour treated at 30% of Initial Moisture Content.PV: peak viscosity; TV: trough viscosity; BD: breakdown viscosity, FV: final viscosity ST: setback viscosity (FV-TV). PT: pasting temperature. Different letters in the corresponding row indicate statistically significant differences between means at $p<0.05$. SE: Pooled standard error obtained from ANOVA analysis. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant.

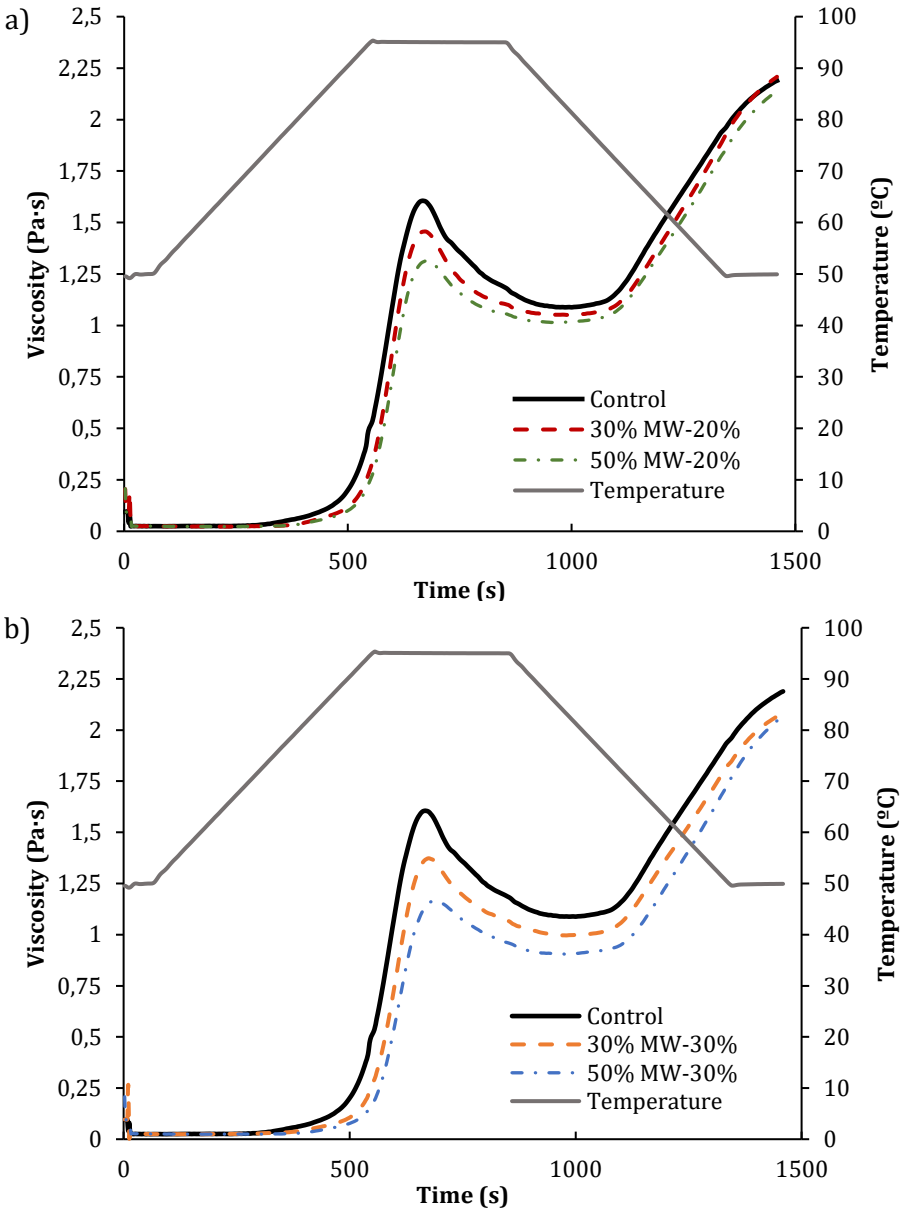

Figure 2. Pasting profiles of MW treated-rice flours depending on the treated-rice flour substitution level (30% and 50% with respect to the total rice flour amount) and the initial moisture content (IMC) of the flour before the treatment: a) IMC= 20% (MW-20%) and b) IMC= 30% (MW-30%). Control dough is represented by ——— , doughs with 30% of MW treated-flour by – – – , and doughs with 50% of MW treated-flour by – · – . The temperature profile is represented by ——— in the second axis.

The addition of treated flour delayed the pasting temperature (PT) up to 5ºC and 7ºC for MW-20% and MW-30% doughs, respectively. It decreased the peak (15% and 27%), trough (8% and 17%) and final (5% regardless the IMC of the treatment) viscosities with respect to the control dough. Such changes in pasting properties of doughs are due to heat-treated flour and have been attributed to associations between the polymeric chains in the amorphous regions of the starch granule as well as to changes in crystallinity caused by the hydrothermal treatment (Watcharatewinkul et al., 2009) which was confirmed by X-ray diffraction assays (Villanueva et al., 2018a). The structural modifications were found more pronounced as the flour moisture content increased before the hydrothermal treatment (Olayinka et al., 2008). As the intragranular chain interactions were strengthen (annealing effects), the reorganized starch structures required more heat energy for structural disintegration and paste formation; i.e., a higher pasting temperature, as found in the current study (Table 3), indicates a more dense cross-linking within the starch granules of doughs. The BD value decreased 28% and 49% for MW-20% and MW-30% respectively at the maximum level of addition (50%). This change denotes an enhanced stability of doughs versus heating and stirring. Such changes can be explained by associations between chains in the amorphous region of the granules of the treated flour as well to changes in crystallinity during hydrothermal treatment. These results are consistent with those previously found in MW-treated rice flour (Villanueva et al., 2018a): the MW treatment of rice flour at 20% and 30% of initial moisture content for 8 min resulted in past temperature increases of 8ºC and 11ºC and decreases of peak (38% and 42%), trough (9% and 13%), final (13% and 25%) and breakdown (81% and 86%) viscosities, with respect to native flour. Consequently, taking into account the dilution effect of the treated flour with native flour in the studied bread doughs, a similar effect can be expected for both the MW treatment and the IMC on the pasting properties of flours and doughs formulated from them. Of course, the quantitative viscometric profiles of studied doughs were always significantly lower as compared to those previously reported for flour suspensions (Villanueva et al. 2018a). This

is due to the presence of non-starch ingredients in the doughs, particularly HPMC and lipids. These ingredients, further its dilution effect on starch, can restrict swelling and gelatinization during cooking, in good agreement with the lower viscometric pattern observed in blended matrices (bread doughs) compared to native flours (Villanueva et al., 2018b). Similar increases of pasting temperatures and decreases of pasting viscosities were also reported by Puncha-arnon and Uttapap (2013) for rice flours from HMT at 20%, 25% and 30% of IMC and Majzoobi et al. (2016) for rice flour at 20% of IMC. Greater effects were always obtained at the highest moisture content of the flour during the treatment as we have observed in our formulated bread doughs.

3.2. Effect of MW-treated flour on bread quality

The effects of the addition of MW treated rice flour to bread dough formulation and the IMC of the flour before the treatment on the physical properties of breads are summarized in Table 4. The substitution of native rice flour by treated one always significantly improved bread specific volume which increased from 3.3 mL/g, for the control one (100% native rice flour), up to 4.6 mL/g for breads with 50% of MW-20% flour or 30% of MW-30% flour. The same tendency was previously observed by Pérez-Quirce et al. (2017) for breads made with rice flour irradiated with microwaves for beta-glucanase inactivation. In that case the increase in bread specific volume hardly reached 8% even in the most intense microwave treated flour because the maximum temperature reached by flour during those treatments was 96ºC, since the unique goal was the enzyme inactivation (instead of 157ºC as was reached in the present study). The addition level and the IMC of the treated flour, as well as its interaction (level x IMC), significantly affected ($p<0.001$) the bread specific volume. The bread volume increased with the substitution level in the case of MW-20% flour, while the opposite effect happened with the addition of MW-30% flour. The improvement in structural strength and bread volume can be related to the increase in dough viscosity as a result of the MW treatment (Marston et al., 2016).

Table 4. Effect of MW treated-rice flour substitution level (30% and 50% with respect to the total rice flour amount) and the initial moisture content of the flour before the treatment (MW-20% and MW-30%) on rice flour bread quality properties

Bread properties		Level (%):	Control 0	MW-20% 30	MW-20% 50	MW-30% 30	MW-30% 50	SE	Factor 1 Level	Factor 2 IMC	Factor 1 x 2 Level x IMC
Bake loss	**(%)**		19.13a	21.19b	22.44c	21.74b	21.54b	0.18	*	ns	**
Specific Volume	**(mL/g)**		3.31a	3.70b	4.61d	4.58d	4.28c	0.044	***	***	***
Firmness	**N**		0.712c	0.439b	0.345ab	0.281a	0.400ab	0.058	ns	ns	*
Springiness			0.918c	0.776b	0.626a	0.659a	0.596a	0.031	**	*	ns
Cohesiveness			0.448a	0.441a	0.447a	0.448a	0.479a	0.018	ns	ns	ns
Chewiness	**N**		0.290c	0.151b	0.100a	0.081a	0.107ab	0.018	ns	*	*
Resilience			0.230b	0.209a	0.218ab	0.204a	0.210ab	0.008	ns	ns	ns
ΔFirmness 7d	**N**		3.00c	2.18bc	1.30ab	1.41ab	0.91a	0.27	*	ns	ns
L^*_{crust}			53.2a	56.0ab	55.1a	58.7bc	60.5c	1.1	ns	***	ns
h_{crust}			60.5a	69.7c	70.9c	67.5b	69.8c	0.56	**	**	ns
C^*_{crust}			25.8a	25.0a	24.4a	29.1b	30.5b	0.64	ns	***	ns
L^*_{crumb}			66.4b	62.9a	61.2a	63.6a	67.9b	0.87	ns	***	**
h_{crumb}			95.3b	95.7b	96.8b	92.6a	92.7a	0.56	ns	***	ns
C^*_{crumb}			6.1b	6.1b	5.3a	6.5b	8.4c	0.21	*	***	***

IMC: Initial Moisture Content of the treated flour. MW-20%: Rice flour treated at 20% of Initial Moisture Content; MW-30%: Rice flour treated at 30% of Initial Moisture Content. Different letters in the corresponding row indicate statistically significant differences between means at $p<0.05$. SE: Pooled standard error obtained from ANOVA analysis. Analysis of variance and significance: *** $p<0.001$. ** $p<0.01$. * $p<0.05$. ns: not significant. L*: luminosity, h: hue, C*: chroma.

A greater consistency of the dough helps to retain the gas formed during fermentation and prevents its coalescence and loss during both fermentation and baking, allowing a higher volume of bread. However there is an optimal value of consistency. Excessive dough consistency may have detrimental effects and lead to smaller breads because the dough cannot sufficiently expand as a result of the pressure produced by the gas (Ronda et al., 2017). This would explain why doughs with 50% of MW-30% treated flour, having higher G'_1 and G''_1 moduli and steady viscosity μ_0 than doughs with 30% of the same flour, had led to breads of smaller specific volume. This explanation cannot be used to justify the effect of MW-20% treated flour on bread volume. Doughs made with MW-20% flour showed hardly any difference in viscoelastic moduli (Table 1), in compliance values or steady viscosities (Table 2) with respect to the control dough. The differences in pasting properties also explain the different bread volumes. The higher pasting temperature of doughs made with treated flour, including those with MW-20% flour, would allow a greater development of the dough during baking before the fixation of the crumb structure upon baking (Ronda et al., 2017). In fact, the Pearson coefficient revealed a significant positive correlation between specific volume of breads and dough pasting temperature ($p<0.01$; $r=0.78$). A significant negative correlation between dough breakdown and specific volume ($p<0.05$, $r=-0.74$) was also obtained. Similar correlation was found by Cornejo and Rosell (2015) from gluten-free breads obtained from different varieties of indica rice. From the previous section can be seen that BD values decreased gradually in doughs following the order: control > 30% MW-20% > 50% MW-20% > 30% MW-30% > 50% MW-30%. It would also contribute to explain the near opposite order in the bread volume evolution. A lower BD value means a higher stability of dough viscosity during heating, which also helps to retain the gas during baking, allowing a higher development, without prejudice to the foregoing.

The loss of weight during baking increased from 19% in the control bread, to 21-22% in breads made with treated flour regardless the IMC of the flour during the treatment. The bake loss was positively correlated to

bread specific volume ($p<0.01$; $r=0.89$). This relationship was previously found in rice based gluten-free breads where the effect of soluble fiber addition and dough hydration were studied (Pérez-Quirce et al., 2014). The higher development of the dough during baking of breads made with treated flour and the delayed pasting temperature of these doughs mean a higher surface exposed to dryness in the oven during a longer time, which could explain the positive correlation between baking loss and bread volume.

The use of MW treated rice flour led to breads of softer crumbs (Table 4). Crumb firmness decreased from 0.71 N, for the control bread, up to 0.3 N in breads made with 50% of MW-20% or 30% of MW-30% that showed similar values. The significant interactive effect (Level x IMC) on firmness gives account for the decrease in firmness with the addition level for the MW-20% treated flour while the addition of MW-30% flour led to an increase of firmness with the addition level. As expected, a strong negative correlation between crumb firmness and specific volume ($p < 0.01$; $r=-0.86$) was obtained. These two bread properties, volume and firmness, were found highly negatively correlated in other works (Pérez-Quirce et al., 2014; Ronda et al., 2015). A higher bread volume usually corresponds to higher amount of air retained in the dough structure during proofing and baking, which endorses a lower crumb firmness. Similar effect was observed for chewiness, probably because this parameter is mainly affected by hardness. Crumb springiness decreased with the addition of treated flours (Table 4). The decrease was dependent on the addition level and the IMC of the treated flour being more severe for the more intense treatment. However, resilience and cohesiveness, which relate to the bread crumb instant and retarded recovery capacity after a compression cycle, and are also desirable properties, were hardly affected by the flour treatment.

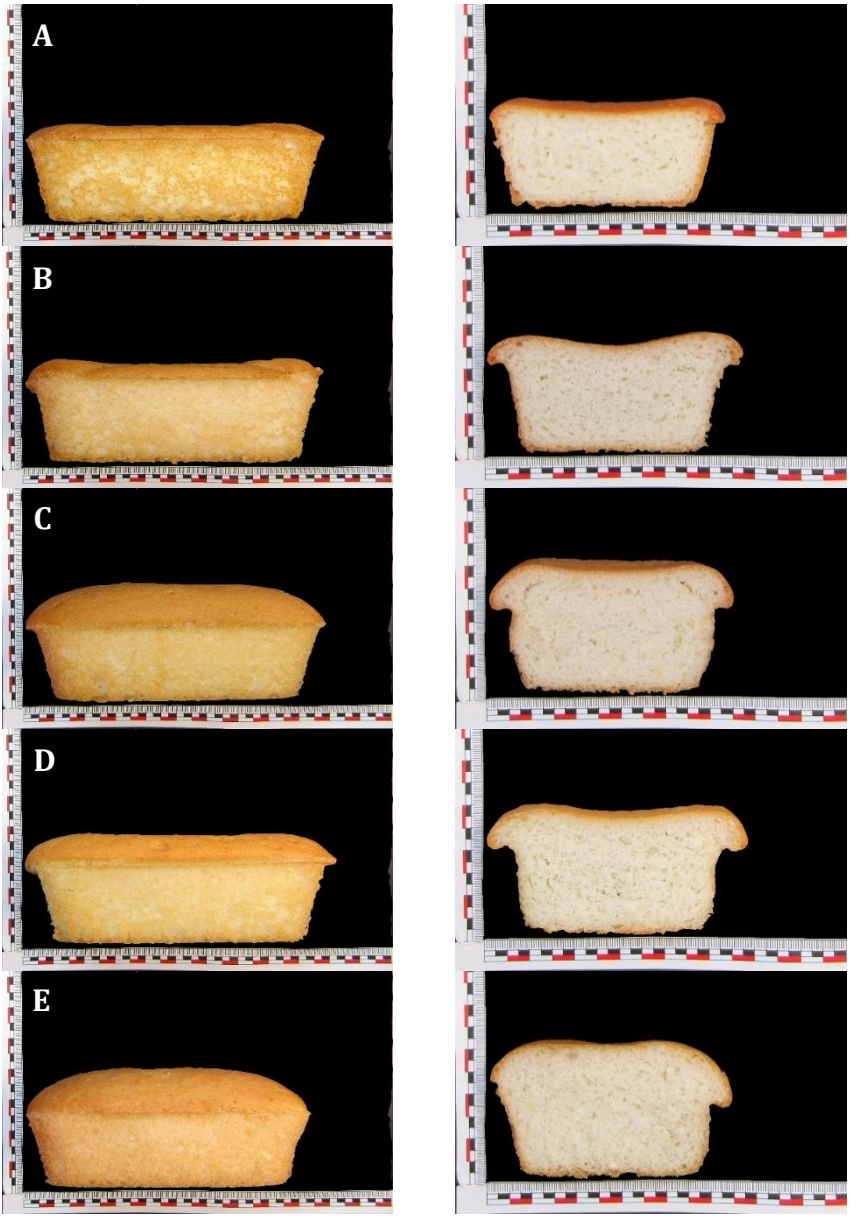

Figure 3. Effect of MW treated-rice flour addition on the external appearance and internal structures of gluten-free breads depending on the initial moisture content (IMC) of the flour before the treatment. A: Control 100% rice flour, B: 30% addition of treated-rice flour at 20% IMC, C: 30% addition of treated-rice flour at 30% IMC, D: 50% addition of treated-rice flour at 20% IMC, E: 50% addition of treated-rice flour at 30% IMC.

The bread hardening decreased significantly with the partial substitution of native flour by MW-treated rice flour. The change of the crumb firmness in 7 days decreased up to 70% for breads with 50% of MW-30% treated flour with respect to the control bread. Bread staling decreased significantly with the dose of MW treated flour regardless the IMC of the flour before the treatment. This is an important issue for coeliac patients, as gluten-free breads are generally more expensive and more difficult to access than traditional breads.

The breads made with MW-30% treated-rice flour showed crusts with significantly higher L*, h, and C* parameters than those of the control samples (Table 4). This means these breads were more yellowish, lighter and with more vivid colours (see Fig. 3). The breads made with MW-20% treated flour, also showed more yellow hues in the crust than the control bread, although their lightness and saturation were similar to the bread made with 100% native flour. The colour of the crust results from the Maillard reaction during baking (Purlis, 2010). However, the colour of the crumb is mainly related to the colour of the ingredients (Villanueva et al., 2015). The high similarity among the colour of crumbs of all formulated breads means the MW treatment did not alter the colour of rice flours (data not shown) and they did not suffer any burning as result of the treatment. The visual inspection of loaves (Fig. 3) confirms the different impact of IMC of MW treated flours resulting in more developed loafs without central part fall. The slices reveal a more uniform and fine crumb structure for breads made with MW-30% treated flour regardless its substitution level, while the bread made with 30% of MW-20% flour, revelead higher cells and less uniform granularity.

4. Conclusions

The MW assisted heat moisture treatment of rice flours resulted in substantial changes in dough viscoelastic and pasting properties. In consequence, treated flours showed a significantly different bread-making performance, improving all crucial parameters of bread quality. The doughs made from blends with MW treated flours reveled higher consistency, more elastic behavior and resistance to deformation versus

stress. All the breads obtained from treated-rice flour, regardless its IMC before the treatment and its level in substitution of native flour, resulted in higher specific volume, lower firmness and slower staling. The visual aspects, as bread loaf shape, crust colour or crumb cells distribution were substantially improved, particularly with the use of MW-treated flour at 30% of IMC. The initial moisture content of MW treated flours impacted significantly on all bread-making performance and the rheological parameters of doughs. The MW assisted heat moisture treatment seems to be a valuable alternative to other types of rice flour modification being both effective and scalable.

Acknowledgments

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CONCLUSIONES

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Esta Tesis Doctoral permite establecer como conclusión general la viabilidad de las estrategias estudiadas, uso de mezclas proteína-ácido y modificación física de las matrices almidón/harina mediante tratamiento hidrotérmico asistido con microondas, como herramientas de mejora de la estructura de los sistemas sin gluten, y de su capacidad para aumentar la calidad tecnológica, sensorial y nutricional de los productos sin gluten derivados.

A partir de esta conclusión general, se pueden extraer las siguientes conclusiones particulares de cada uno de los capítulos de la tesis:

• La fuente de almidón resultó un factor clave en las propiedades reológicas de las masas de pan elaboradas a partir de los sistemas almidón-proteína-ácido, que determinan su desempeño en la formulación de productos sin gluten. La incorporación de proteína fortaleció la estructura de la masa mientras que la acidificación la debilitó y permitió una mayor deformación de la masa para todos los almidones estudiados. La presencia de proteínas aumentó los perfiles de empastado, pero con diferencias asociadas a las proteínas estudiadas.

• La suplementación de masas de almidón de arroz con proteínas de diferentes orígenes resultó una estrategia viable. La incorporación de proteínas de origen vegetal dio lugar a masas de pan más estructuradas. Estos efectos se magnificaron con la dosis de proteína, y se vieron reducidos como consecuencia de la acidificación de la masa como con los almidones estudiados anteriormente. La incorporación de proteínas de origen animal dio lugar a diferentes comportamientos viscoelásticos según el tipo de proteína, la dosis y la acidificación, especialmente en el caso de caseína.

• Las proteínas de origen vegetal dieron lugar a panes de almidón de arroz con menor volumen específico y miga más dura, efectos que aumentaron con la dosis de proteína. La incorporación de proteínas de

origen animal dio lugar a comportamientos diferentes según el tipo de proteína, la dosis y la acidificación. En general, la acidificación de masas enriquecidas con proteínas aumentó el desarrollo de la masa durante la fermentación y por lo tanto el volumen del pan, disminuyó su dureza y aumentó la luminosidad de la corteza. Las propiedades físicas del pan se vieron significativa y altamente correlacionadas con las propiedades reológicas de la masa.

• Tanto el pH como la adición de proteínas exógenas son herramientas útiles para modificar las propiedades térmicas y reológicas de los geles de almidón de arroz, patata y de tapioca. Los geles preparados a 120 ºC fueron mucho más débiles que los formados a 90 ºC; siendo el almidón de arroz el más sensible al aumento de la temperatura y la patata el más resistente. En general, las proteínas funcionaron como agentes estructurantes a ambas temperaturas; aunque en los geles preparados a 120 ºC el efecto fortalecedor fue más pronunciado. Por el contrario, la acidificación debilitó la estructura de estos geles.

• El tratamiento térmico asistido por microondas de la harina de arroz resultó una herramienta capaz de cambiar sus propiedades térmicas y de empastado, así como la morfología de las partículas de harina y su cristalinidad. Los cambios observados dependieron significativamente del contenido inicial de humedad en la harina, manifestando alteraciones de las propiedades funcionales y de empastado de las muestras irradiadas con microondas, particularmente en las matrices de mayor contenido de humedad inicial (30%).

• El efecto del tratamiento de microondas de mezclas almidón-proteína dependió de la fuente de almidón y del tipo de proteína. La radiación de microondas influyó principalmente en las propiedades de empastado y reológicas del almidón de patata, con y sin proteína.

• El tratamiento térmico de la harina de arroz asistido por microondas resultó eficaz para la mejora de las propiedades físicas del pan sin gluten. Las masas elaboradas con harinas tratadas con microondas revelaron mayor consistencia, comportamiento más elástico y mayor resistencia a la deformación. Todos los panes obtenidos

presentaron mayor volumen específico, menor firmeza y ralentización del proceso de envejecimiento.

Los resultados de la presente Tesis Doctoral han contribuido a generar nuevos conocimientos tendentes al desarrollo y aumento de la calidad de los productos sin gluten panificados o basados en la formación de geles y a ampliar las opciones de alimentos al alcance de los consumidores.

CONCLUSIONS

The general conclusion drawn from the performance of the Doctoral Thesis endorsed the viability of the strategies studied -the use of protein-acid mixtures and the physical modification of the starch/flour matrices by means of hydrothermal treatment assisted by microwaves- as useful tools to improve the structure of the GF systems and their ability to increase the technological, sensory and nutritional quality of the resulting GF products.

From this general conclusion, the following particular conclusions can be derived from each of the chapters of the thesis:

• The source of starch was a key factor in the rheological properties of bread doughs made from starch-protein-acid systems, which determine their performance in the formulation of GF products. The incorporation of protein strengthened the structure of the dough while acidification weakened it and allowed for greater dough deformation for all the starches studied. The presence of proteins increased the pasting profiles, the extent depending on the type of protein.

• Supplementation of rice starch doughs with protein from different sources proved to be a viable strategy. The incorporation of vegetable proteins resulted in more structured bread doughs. These effects were magnified by the protein dose, and were reduced as a consequence of the acidification of the dough as with the starches studied previously. The incorporation of proteins of animal source gave rise to different viscoelastic behaviours depending on the type of protein, the dose and the acidification, especially in the case of casein.

• Vegetal proteins gave rise to rice starch breads with lower specific volume and harder crumb, effects that increased with the protein dose. The incorporation of animal proteins resulted in different behaviours depending on the type of protein, dose and acidification. In general, the acidification of protein-enriched doughs increased dough development

during fermentation and thus the volume of bread, reduced its hardness and increased the luminosity of the crust. The physical properties of the bread were significant and highly correlated with the rheological properties of the dough.

• Both pH and the addition of exogenous proteins are useful tools for modifying the thermal and rheological properties of rice, potato and tapioca starch gels. The gels prepared at 120°C were much weaker than those formed at 90°C; rice starch being the most sensitive to temperature increase and potato the most resistant. In general, proteins worked as structuring agents at both temperatures, although in gels prepared at 120ºC the strengthening effect was more pronounced. On the other hand, acidification weakened the structure of these gels.

• The hydrothermal treatment assisted by microwaves of rice flour was a tool capable of changing its thermal and pasting properties, as well as the morphology of the flour particles and their crystallinity. The changes observed depended significantly on the initial moisture content of the flour, showing changes in the functional and pasting properties of the microwave-irradiated samples, particularly in the matrices with the highest initial moisture content (30%).

• The effect of microwave treatment of starch-protein mixtures depended on both the starch source and type of protein. Microwave radiation mainly influenced the pasting and rheological properties of potato starch, with and without protein.

• The hydrotermal treatment assisted by microwaves of rice flour was effective in enhancing the physical properties of GF bread. The doughs made with microwave treated flours showed higher consistency, more elastic behaviour and higher resistance to deformation. All the breads obtained had a higher specific volume, less firmness and slower staling process.

The results of this Doctoral Thesis have contributed to the generation of new knowledge aimed at developing and increasing the quality of gluten-free products that are baked or based on the formation of gels, and at expanding the food options available to consumers.