PROGRAMA DE DOCTORADO EN CIENCIA, TECNOLOGÍA Y GESTIÓN ALIMENTARIA

Análisis estructural de los productos derivados de cereales y su aplicación en la optimización de procesos y productos

Presentada por:

Raquel Garzón Lloría

Dirigida por:

Prof. Cristina M. Rosell

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CSIC Consejo Superior de Investigaciones Científicas

iata Instituto de Agroquímica y Tecnología de Alimentos

CRISTINA MOLINA ROSELL, Profesora de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de Alimentos (IATA)

CERTIFICA:

Que la presente Tesis Doctoral, titulada " **Análisis estructural de los productos derivados de cereales y su aplicación en la optimización de procesos y productos"**, ha sido realizada bajo su dirección en el Departamento de Ciencia de Alimentos del Instituto de Agroquímica y Tecnología de Alimentos, por RAQUEL GARZÓN LLORÍA; y que, habiendo revisado el trabajo, considera que reúne las condiciones necesarias para optar al grado de Doctor en Ciencia, Tecnología y Gestión Alimentaria.

Y para que así conste a los efectos oportunos, se expide el presente escrito.

Valencia, a 8 de diciembre de 2020

MOLINA ROSELL M.CRISTINA - DNI 20794643K

Digitally signed by MOLINA ROSELL M.CRISTINA - DNI 20794643K
Date: 2020.12.14 20:40:12 +01'00'

Fdo: CRISTINA MOLINA ROSELL

Siempre que tengo una tesis en mis manos me paro a leer la parte de los **agradecimientos**, es increíble la cantidad de gente que se necesita para recorrer este camino. Estoy segura de que por mucho que me pare a pensar en este momento, habrá gente a la que debería haber agradecido por participar directa o indirectamente y no lo voy a nombrar, lo que no significa que no hayan tenido un papel protagonista para poder llegar al final, solo que el tiempo lo desfigura todo. Así que para todos los no nombrados gracias.

Desde luego, si hay una persona a la que no voy a olvidar agradecerle su esfuerzo es a mi directora de tesis, Cristina. Llamarle directora es quedarse muy muy corto, ya que no solo tengo que agradecerle la oportunidad de hacer la tesis, si no la mayor parte de lo que soy profesionalmente. No sé si existe un término correcto que pueda recoger lo que me has aportado todos estos años, aun quedándose lejos de la realidad, creo que se ajustaría mejor decir que has sido mi mentora. Es una suerte trabajar y formarse a tú lado. Por todo esto y mucho más, gracias.

Un laboratorio no sirve de nada sin la gente que lo ocupa y el entorno que lo rodea. En los años que llevo han pasado muchísimas personas y creo que con cada una de ellas he tenido la oportunidad de aprender y crecer un poco más. Hay gente con la que he pasado momentos geniales y que han hecho que los años pasen volando y otras que acaban de llegar, pero pisando fuerte. Como he dicho, va a ser imposible nombrar a todxs porque la lista sería eterna, así que aquí dejo una pequeña representación de la gente que ha ido moldeando mi camino: Paola, Rossana, María R., María S., Jehannara, Andrea, Carmen, Carla, Cris, Yaiza, Johanan, Ángela, Fede, Souad, Agustín, Javi… No puedo dejar pasar la oportunidad de dar unas gracias infinitas a Mariví, que me ha aguantado, ayudado, enseñado y calmado en incontables ocasiones, y que tanto su llegada como su partida del lab dejaron huella, menos mal que te tenemos localizada. A Nico que, aunque han sido solo los últimos años es como si fuera el doble de tiempo ya que las bromas, risas y enfados han sido dentro y fuera del trabajo. Y por supuesto no me he olvidado de vosotros, ir cada día con energía es mérito vuestro, Judit y Antonio, que hacéis que el trayecto hasta el trabajo sea pensando en las risas del desayuno.

Por supuesto, uno siempre necesita tener una base antes de embarcarse en este camino. Llegar hasta aquí, o hasta donde llegue, es y será siempre mérito de todos ellxs, de mi familia, la de sangre y la elegida. A Majo, Marta, Toni e Inma, por haber creado tantos recuerdos juntos estos años. Agradezco el día en el que decidimos irnos a comer juntas. A mi padre, mi madre, mi hermana, a Diego, a Nahia y a Juan, gracias por hacerme creer que puedo hacer lo que me proponga, incluso cuando sabéis que será difícil, y animarme a seguir cuando soy yo la que se da cuenta de que será difícil. ¡Mil gracias!

Abstract

Visual inspection is a quality parameter that describes both the external appearance as the internal structure in cereal-based products. Digital image analysis (DIA) is a tool widely used in several fields to process and analyse objects visually observed by the human eye. However, despite of its potential, it has been barely explored for food analysis purposes. Crumb structure in cereal-derived products is one of the most characteristic parameters in bread. Due to this, following up the structural changes caused by the process or by the formulation changes, is of fundamental importance. For that reason, the main target of this thesis was the definition of the relationship between the structure and the quality parameters of dough and baked products. Moreover, extending the DIA utility and its use to improve process and mixes in baked products represented other main objective of this work. To do that, first, the optimal capture conditions were established and the way to improve the edge detection was studied. Afterwards, image analysis was evaluated to quantify the structural changes that happened during the starch gels formation in a fast force analyser. Then, the impact on the structure by adding or replacing different ingredients or additives in gluten-free breads was studied. Finally, the possible correlations between the results obtained from technological characterization (texture profile, crumb structure, moisture content) versus sensory perception (bolus properties, chewing and swallowing) were also investigated. DIA allowed the quantification of the different structures obtained from different starches. In addition, the study revealed the differences in time needed to form a homogeneous and well-distributed structure, which was dependent on the origin of the starch. Formulation modification in bread with or without gluten required a process adjustment that allowed to obtain different structures depending on the ingredients / additives studied. DIA has been proved to be an effective tool for detecting and quantify changes originated at the structural, dough and final product levels. In addition, the changes quantified by DIA obtained statistically significant correlations with several parameters studied for the sensory perception, such as bolus adhesion and total time to chew and swallow the sample. Therefore, DIA was a sufficiently specific and non-destructive tool, which allowed to characterize the impact of the formulation change on the bread cell distribution with and without gluten. There being a relationship

between this, and the parameters usually used in cereal products characterization, as well as sensory perception.

Resumen

La inspección visual es un parámetro de calidad que describe tanto el aspecto externo como la estructura interna en los productos derivados de cereales. El análisis digital de imagen (ADI) es una herramienta ampliamente utilizada en diversos campos para procesar y analizar los objetos que observamos visualmente. Sin embargo, a pesar de su potencial, ha sido escasamente explotado en el ámbito de alimentos. La estructura de la miga de los productos derivados de cereales es uno de los parámetros característicos del pan y en este contexto es fundamental realizar el seguimiento de los cambios estructurales que se producen durante el proceso o debido a las modificaciones en la formulación. Por ello, el objetivo principal de la presente tesis doctoral fue definir la interrelación entre la estructura y parámetros de calidad de masas y productos horneados, así como extender la utilidad del ADI y su uso para optimizar los procesos y las formulaciones en productos horneados. Para ello, en primer lugar, se establecieron las condiciones óptimas de captura y se estudió la forma de mejorar la detección de bordes. Posteriormente se evaluó el potencial del análisis de imagen para cuantificar los cambios estructurales ocurridos en la formación de geles de almidón en un analizador rápido de fuerza. Seguidamente, se estudió el impacto en la estructura de la adición o sustitución de distintos ingredientes o aditivos en panes con y sin gluten. Y finalmente se investigaron las posibles correlaciones entre los resultados obtenidos de la caracterización tecnológica (perfil de textura, estructura de la miga, humedad) frente a la percepción sensorial (propiedades del bolo, masticación y deglución). El ADI permitió cuantificar las diferentes estructuras obtenidas con los distintos almidones. Además, el estudio reveló las diferencias en el tiempo necesario para formar una estructura homogénea y bien distribuida, que fue dependiente del origen del almidón. La modificación de la formulación de panes con o sin gluten requirió de la adaptación del proceso y permitió obtener diferentes estructuras atendiendo a los ingredientes /aditivos estudiados. El ADI resultó ser una herramienta eficaz para detectar y cuantificar los cambios originados a nivel estructural, en masa y producto final. Además, los cambios cuantificados mediante ADI obtuvieron correlaciones estadísticamente significativas con varios de los parámetros estudiados

de percepción sensorial, como son la adhesividad del bolo y el tiempo total para masticar y deglutir una muestra. Por lo tanto, el ADI resultó una herramienta suficientemente específica y no destructiva, que permitió caracterizar el impacto del cambio de la formulación en la distribución alveolar de panes con y sin gluten. Existiendo relación entre este y los parámetros utilizados habitualmente en la caracterización de productos derivados de cereales, así como con la percepción sensorial.

Resum

La inspecció visual és un paràmetre de qualitat que descriu tant l'aspecte extern com l'estructura interna en els productes derivats de cereals. L'anàlisi digital d'imatge (ADI) és una eina àmpliament utilitzada en diversos camps per a processar i analitzar els objectes que observem visualment. No obstant això, malgrat el seu potencial, ha sigut escassament explotat en l'àmbit d'aliments. L'estructura de la molla dels productes derivats de cereals és un dels paràmetres característics del pa i en aquest context és fonamental realitzar el seguiment dels canvis estructurals que es produeixen durant el procés o a causa de les modificacions en la formulació. Per això, l'objectiu principal de la present tesi doctoral va ser definir la interrelació entre l'estructura i paràmetres de qualitat dels diferents tipus de masses i productes enfornats, així com estendre la utilitat de l'ADI i el seu ús per a optimitzar els processos i les formulacions en productes enfornats. Per a això, en primer lloc, es van establir les condicions òptimes de captura i es va estudiar la manera de millorar la detecció de les vores. Posteriorment es va avaluar el potencial de l'anàlisi d'imatge per a quantificar els canvis estructurals ocorreguts en la formació de gels de midó en un analitzador ràpid de força. Seguidament, es va estudiar l'impacte en l'estructura de l'addició o substitució de diferents ingredients o additius en pans amb glútens i sense glútens. I finalment es van investigar les possibles correlacions entre els resultats obtinguts de la caracterització tecnològica (perfil de textura, estructura de la molla, humitat) enfront de la percepció sensorial (propietats del bol, masticació i deglució). L'ADI va permetre quantificar les diferents estructures obtingudes amb els diferents midons. A més, l'estudi va revelar les diferències en el temps necessari per a formar una estructura homogènia i ben distribuïda, que fou dependent de l'origen del midó. La modificació de la formulació de pans amb gluten o sense va requerir de l'adaptació del procés i va permetre obtindre diferents estructures atesos els ingredients /additius estudiats. L'ADI va resultar ser una eina eficaç per a detectar i quantificar els canvis originats a l'entorn estructural, en massa i producte final. A més, els canvis quantificats mitjançant ADI van obtindre correlacions estadísticament significatives amb els diversos paràmetres estudiats de percepció sensorial, com són l'adhesivitat del bol i el temps total per a mastegar i deglutir una mostra. Per tant, l'ADI va resultar una eina prou específica

i no destructiva, que va permetre caracteritzar l'impacte del canvi de la formulació en la distribució alveolar de pans amb glutens i sense. Tot i existint relació entre aquest i els paràmetres utilitzats habitualment en la caracterització de productes derivats de cereals, així com amb la percepció sensorial.

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DISCUSIÓN GENERAL

ESQUEMA DE LA TESIS

La Tesis Doctoral está organizada en cinco secciones: **Introducción**, **Objetivos**, **Resultados (Capítulos 1 al 4)**, **Discusión General** y **Conclusiones**.

La **Introducción** pone en antecedentes el uso del análisis digital de imagen (ADI) como herramienta científica. A lo largo de la primera parte de la introducción se detalla que se considera el ADI, así como los puntos principales del procesado de imágenes digitales. Además, se citan algunas de las principales aplicaciones de esta herramienta. En la segunda sección de la introducción se ha incluido un capítulo de libro que detalla la formación de la estructura (miga y corteza) a lo largo del proceso de los productos derivados de cereales, así como los inicios de la utilización del ADI en este tipo de productos a nivel de microestructura y estructura de la miga. Seguidamente se presenta el Objetivo general y los específicos de la esta tesis.

El apartado de **Resultados** incluye cuatro capítulos. El **Capítulo 1** trata de explicar el efecto de las condiciones de captura y la forma de aplicar el umbral que dividirá las zonas a medir (alveolos) de las zonas descartadas (resto de la miga). Además, se realiza la validación del ADI para identificar cambios en estructuras de panes con y sin gluten a los que se les realiza modificaciones en la formulación. Por otra parte, debido a las necesidades de algunos estudios de minimizar la cantidad de ingrediente/ aditivo a utilizar, se realiza la validación de la producción de panes a pequeña escala, así como la adaptación de la metodología para obtener resultados extrapolables. El **Capítulo 2** trata de comprender la formación de la estructura de los geles de almidón, utilizando un equipo que permite realizar el ensayo en 90 segundos. Para ello se utiliza el análisis de imagen a nivel cualitativo y cuantitativo para describir y cuantificar los cambios en el almidón a lo largo del ensayo. El **Capítulo 3** pone en práctica lo aprendido del ADI durante el Capítulo 2. A lo largo de tres secciones se explica el uso de esta herramienta para estudiar la formación de la masa, la fermentación y/o a la estructura de la miga la adición o sustitución con otros ingredientes o aditivos y su uso en la evaluación de panes con y sin gluten. En el **Capítulo 3-I** se adiciona una microalga (*C. vulgaris*), para estudiar el efecto en el proceso y estructura y con el fin de la mejora nutricional (macrocomponenetes y compuestos bioactivos) del pan. Posteriormente, en el **Capítulo 3-II** se estudia la

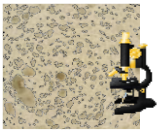
capacidad estructurante de una glicoproteína (β-conglicinina) en panes sin gluten. La última parte, **Capítulo 3-III**, estudia la capacidad de varios emulgentes utilizados en panadería para estabilizar las burbujas o núcleos formados durante el amasado, así como el efecto en la estructura final. Por último, en el **Capítulo 4** se busca la vinculación de los aspectos estructurales con parámetros sensoriales mediante correlaciones entre la caracterización tecnológica, específicamente de los parámetros obtenidos en el ADI, y la percepción sensorial (propiedades del bolo, masticación y deglución).

La sección de la **Discusión general** analiza desde una perspectiva global los resultados más relevantes referente a la aplicación del ADI, obtenidos en los cuatro bloques de Resultados.

Finalmente, las **Conclusiones** presentan los hallazgos más relevantes de la Tesis Doctoral.

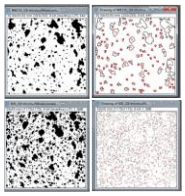
Análisis estructural de los productos derivados de cereales y su aplicación en la optimización de procesos y productos

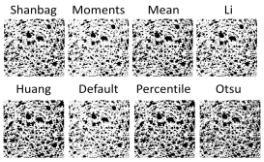
Capítulo 1. Optimización de las condiciones de captura y procesado de imagen para productos de panadería.



SECCIÓN I

Resolution

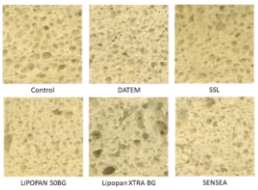




Algorithms

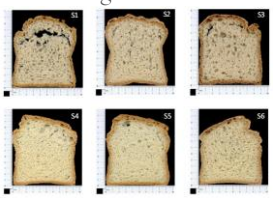
SECCIÓN II

Different wheat breads



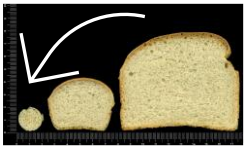
&

Different gluten free breads

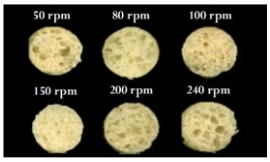


SECCIÓN III

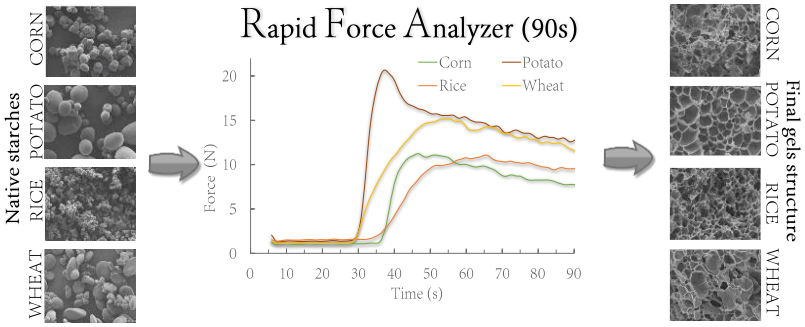
Scale down



≠ Mixing speed

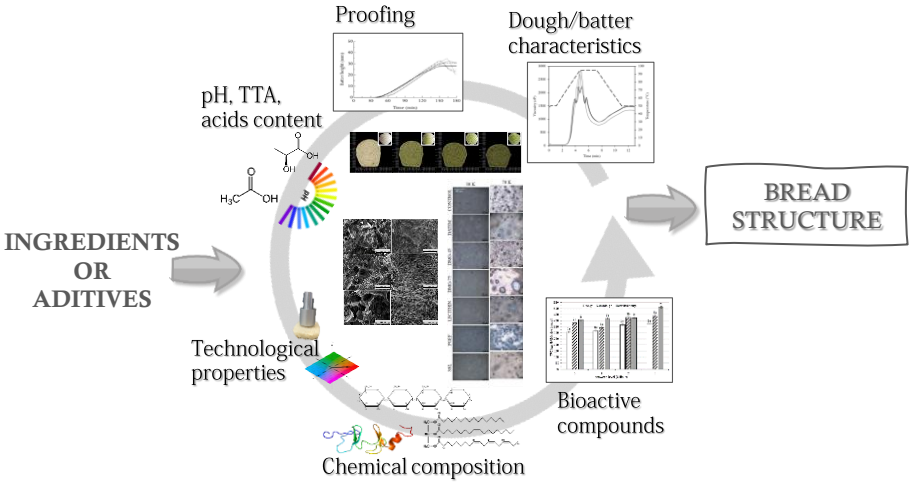


Capítulo 2. Entender los cambios estructurales del almidón durante la gelatinización y gelificación mediante un analizador rápido de fuerza (Amylab).

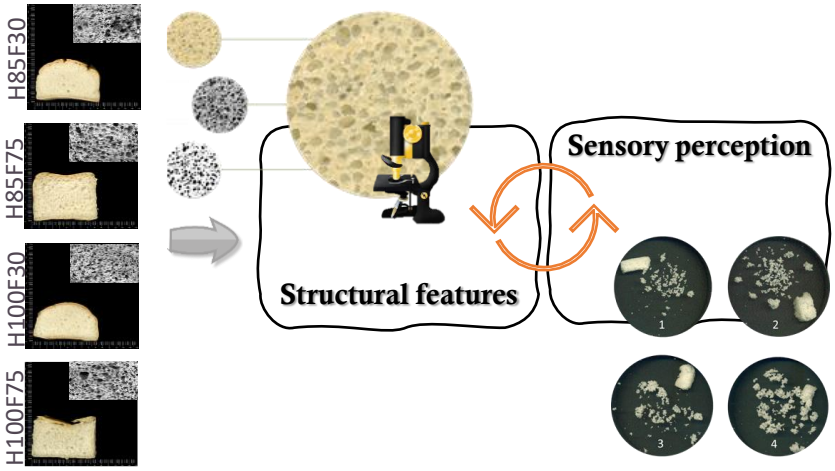


Análisis estructural de los productos derivados de cereales y su aplicación en la optimización de procesos y productos

Capítulo 3. Implantar el uso del análisis digital de imagen para evaluar el impacto de distintos ingredientes/aditivos panarios en la formación de la estructura de los productos resultantes.



Capítulo 4. Establecer posibles relaciones entre las características tecnológicas de los panes y las propiedades del bolo, la masticación y la deglución de la miga.



INTRODUCCIÓN

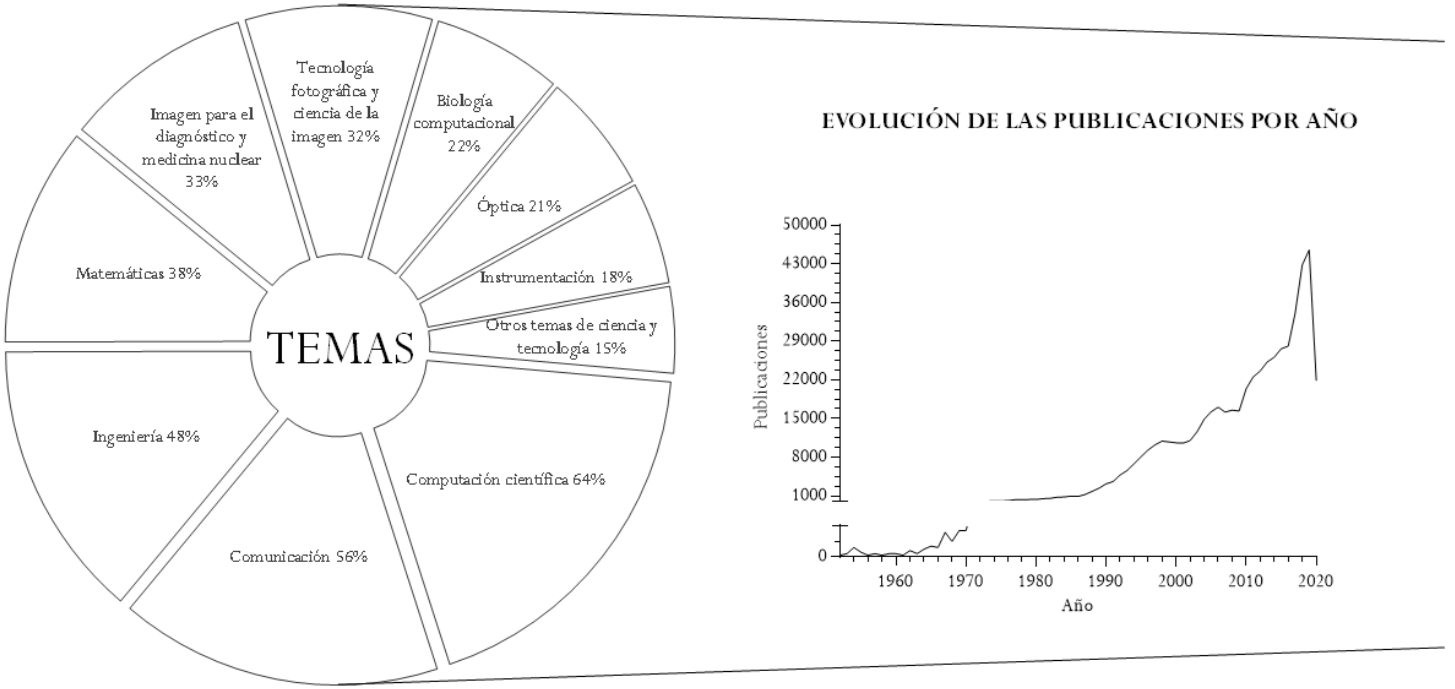
I. El análisis digital de imagen como herramienta científica.

II. Microestructura de productos de panadería y repostería.

I. El análisis digital de imagen como herramienta científica.

La visión humana es uno de los mecanismos más importantes y complejos que participan en la percepción sensorial. Mediante este sentido se percibe información utilizada para tareas sencillas como el reconocimiento de objetos o personas, pero su contribución es fundamental en tareas más complejas como el mapeo de zonas o la investigación científica. El análisis digital de imágenes (ADI) es una herramienta capaz de procesar y analizar los objetos que observamos visualmente en una imagen, cuantificando las cualidades de los objetos. El ADI es un método de evaluación no invasivo ni destructivo, lo que lo convierte en una herramienta útil y complementaria a muchas otras técnicas. Hoy en día, el análisis de resultados de la búsqueda de procesado o análisis de imagen en la "Web Of Science" (WOS), centrada en artículos científicos, libros y revisiones, origina más de medio millón de resultados (545.826 publicaciones), lo que pone de manifiesto el gran potencial de esta herramienta en diversas áreas de investigación. Debido a la complejidad del desarrollo de esta herramienta, las publicaciones se encuentran englobadas principalmente en el área temática de la computación científica, destacando su uso en comunicación, ingeniería, matemáticas, medicina y distintas ramas de ciencia y tecnología (Figura 1). La necesidad de convertir lo que se aprecia visualmente en datos objetivos que permitan simplificar y cuantificar los resultados obtenidos ha hecho que el procesamiento y análisis de imágenes se haya incrementado exponencialmente en las últimas décadas. La primera publicación registrada con el tema de análisis de imagen data de 1928 y se engloba dentro del área de ingeniería [1]. Debido a la fecha de la publicación no se trata de análisis de imagen por computadora como se entiende hoy en día, sino de la utilización de una fotografía aérea para realizar mediciones de forma manual sobre ella [1]. Sin embargo, a partir de finales de los años 80, la introducción de la forma computacional aumentó el número de publicaciones que incluyen esta herramienta llegando hasta las 45.538 en 2019 (Figura 1).

Figura 1. Análisis de resultados obtenidos en WOS (búsqueda: TS= ("imag* analysis" OR "imag* process*"); tipos de documentos: (article or book or other or review).



Una de las ventajas principales de la cuantificación de los objetos mediante ADI es la posibilidad de repetición de las tareas de manera objetiva. El procesamiento de imágenes incluye la transformación de una imagen a formato digital y su posterior procesado. Es decir, se trata de un conjunto de operaciones matemáticas que se efectúan a imágenes matriciales obtenidas con cualquier tipo de sensor o cámara y que permiten cuantificar lo apreciado visualmente [2]. Una imagen matricial se considera una imagen transformada en una matriz de números, los cuales pueden manipularse para que se cuantifique la parte deseada (Figura 2). Cuanto mayor sea la resolución de la imagen más fiel será la imagen matricial. En el ejemplo presentado las zonas sombreadas en gris serían las zonas negras que detecta el ojo humano y los 0 representarían el fondo blanco, debido a la baja resolución de la imagen mediante computación no se podrían definir mejor los bordes.

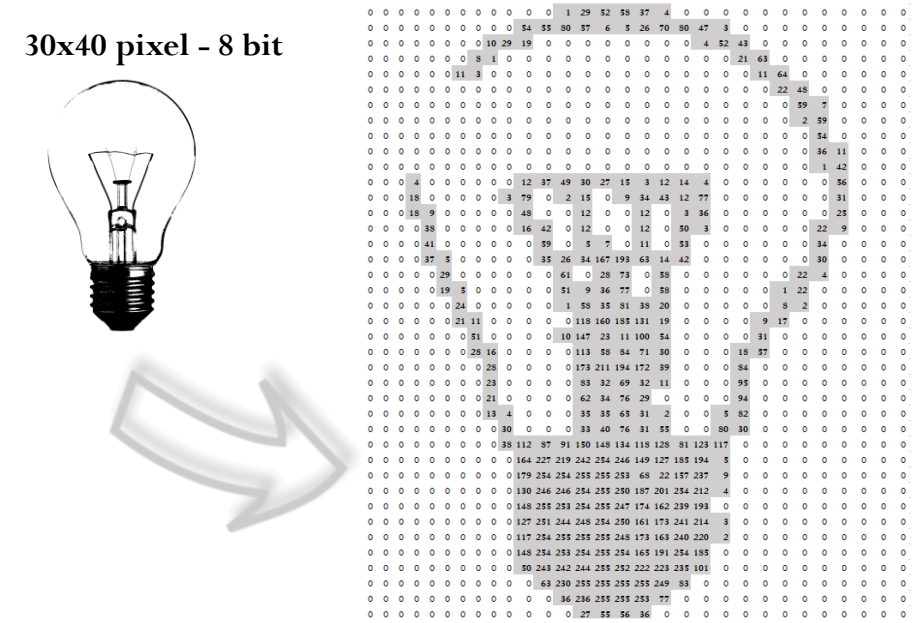


Figura 2. Imagen matricial de una bombilla con una resolución de 30 x 40 pixeles.

Tras la captura de la imagen el ADI se puede dividir en distintas etapas: procesado y/o restauración, segmentación e identificación de los objetos de la imagen [3] (Figura 3). Durante la mejora o pre-procesamiento se pretende optimizar la percepción de la información en las imágenes para el ojo humano, pero en el ADI se realiza principalmente para ayudar a diferenciar los objetos en las posteriores etapas del procesamiento. Por otra parte, la resolución de captura de las imágenes cada vez es mayor, y por lo tanto también la cantidad de información que debe procesarse, por ello a veces durante esta etapa es necesario realizar una compresión de imágenes con el fin de facilitar las tareas. Finalmente se realiza la diferenciación de los objetos de la imagen, para ello existen distintas herramientas como la segmentación de imágenes, la extracción de bordes, la detección del movimiento en vídeo, etc. Una vez segmentado solo queda identificar y cuantificar los objetos de estudio aplicando limitaciones o modelos que permitan extraer únicamente el objeto que se quiere cuantificar.

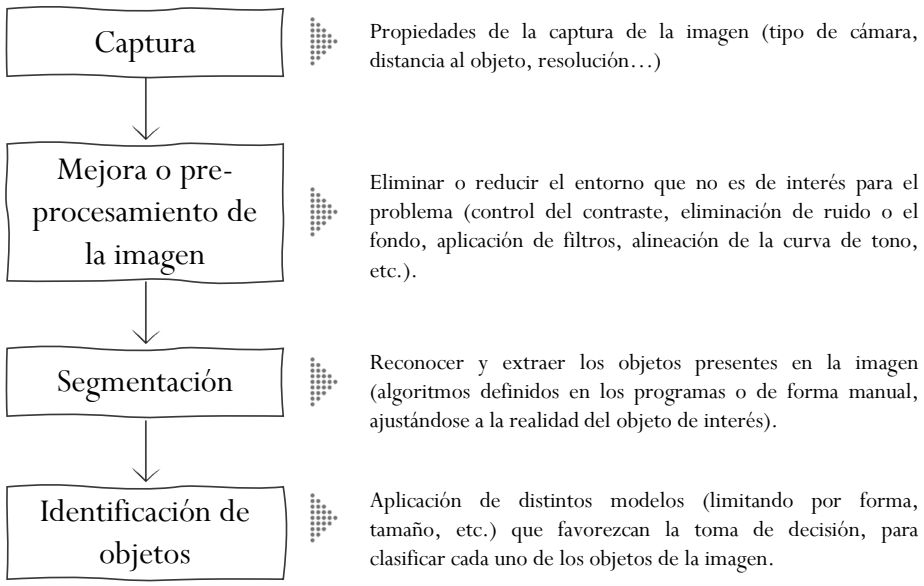


Figura 3. Esquema de las etapas generales del ADI.

Aplicaciones del procesado y cuantificación de la imagen digital

El procesado de imágenes digitales es una herramienta básica, que utilizamos de forma rutinaria gracias a la mejora y actualización de la tecnología. Por ejemplo, al aplicar a las fotografías los distintos filtros que ofrecen los programas de edición de imagen de las aplicaciones móviles, que permiten modificar el contraste, tonalidad, etc. Sin embargo, el aspecto que convierte al procesado o análisis de imágenes en una herramienta potencial en los campos de la ciencia y la tecnología, ingeniería o medicina, radica en la posibilidad de cuantificar los cambios. Por ello, el ADI se utiliza en múltiples aplicaciones como la navegación, robótica, videovigilancia inteligente, inspección industrial o la industria aeroespacial, tanto en investigación como para el diagnóstico médico, control industrial, etc.

Aplicaciones médicas

El uso de imágenes en el campo de la medicina permite observar el interior del cuerpo en busca de alguna afección médica, así como complemento para determinar el posible tratamiento. Por ello en medicina está muy extendido desde hace tiempo el uso de rayos X, resonancia magnética, tomografía computarizada, mamografía digital, ecografías y otras modalidades de imágenes [4]. Un claro ejemplo del progreso científico en cuanto a captura y procesado de las imágenes médicas, son las mejoras introducidas en los ecógrafos para el seguimiento del embarazo (Figura 4). Inicialmente las imágenes obtenidas eran capturadas en formato 2D, que permiten realizar mediciones (medición del tamaño, estimación del peso, observación del sexo, etc.). Posteriormente se pasó a lo que llaman ecografías 3D, que permiten observar la figura del bebé en 3 dimensiones, consiguiendo en un corto periodo de tiempo ecografías 4D que además permiten la grabación en vídeo. Finalmente, gracias al procesado de imágenes automatizado, hoy en día se ofrece la posibilidad de realizar ecografías 5D, que utilizando de base las anteriores tecnologías y con un aumento de la nitidez, resolución

y juego con los tonos y sombras logra un aspecto mucho más realista del aspecto del bebé.

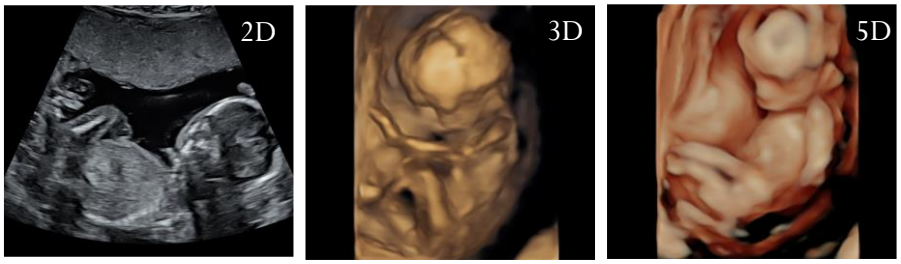


Figura 4. Evolución de las ecografías obtenidas del feto durante el embarazo. (Imágenes cedidas por M. Molina, semana 16 de gestación).

El número de investigaciones que combinan técnicas médicas y el análisis de imagen es numeroso. Ya en 1995 Nicolás Ayache [5] planteó la dificultad de la utilización del análisis de imagen en 3D, pero remarcó su utilidad. Un ejemplo de ello fue el éxito en la identificación de la zona a operar mediante ADI, determinando la posición exacta de los brazos del robot que realizaría la operación y llevando a cabo el proceso de forma rápida, segura y precisa [6]. También se ha aplicado para llevar a cabo el seguimiento mediante ecografía o resonancia magnética detectando problemas de forma más precisa durante el curso de una patología, detectando las zonas infectadas a tratar o mejorando la precisión del diagnóstico de cáncer de mama por histopatología [7-9]. Inicialmente la interpretación de imágenes médicas se ha realizado principalmente por expertos, como radiólogos o médicos. La complejidad de las imágenes médicas sumado a que cada vez es mayor el número de estas ha fomentado el interés por la automatización de su análisis y en consecuencia el estudio de modelos y algoritmos que permitan adaptarse a este tipo de imágenes[10,11].

Aplicaciones biológicas

El análisis de imagen es complemento indispensable cuando se utilizan imágenes fijas o vídeos de diversos fenómenos biológicos [12], como, por ejemplo, el estudio del movimiento de *Caenorhabditis elegans* para comprobar el efecto de un tratamiento en un organismo modelo [13]. Muchas veces las imágenes utilizadas son capturadas gracias al acople de

una cámara a un microscopio óptico, confocal, etc., aunque no son los únicos dispositivos de entrada ya que existen diferentes tipos de sensores como los fotomultiplicadores. Con el fin de eliminar el sesgo subjetivo en la observación de las imágenes y realizar el análisis de forma automática o semi automática, se utiliza el análisis de imagen digital para realizar por ejemplo conteo de microorganismos en placa, mediciones morfogeométricas y/o densiométricas más precisas y exactas de las imágenes digitalizadas [14-16]. Adicionalmente, también es posible detectar, registrar y cuantificar trayectorias, dietas y supervivencia de animales, empleados por ejemplo en el estudio de las aves acuáticas [17] o movimiento de bancos de peces. Esto permite predecir cuáles serán las migraciones, así como comprender mejor la elección de estas a la hora de desplazarse a una zona y otra.

Teledetección de superficies y objetos

La teledetección es la técnica que permite obtener información, mediante imágenes obtenidas a distancia. Para ello se utilizan sensores o cámaras capaces de enviar la información en forma de imágenes. Una de las herramientas de teledetección más extendidas es su uso en la agricultura para realizar el mapeo de las zonas. Debido a la compleja subdivisión de las tierras de cultivo existen herramientas diseñadas para poder identificar, localizar las coordenadas y medir parcelas en las zonas de cultivo. Un ejemplo de esto es la aplicación SIGPAC (Figura 5) del Ministerio de Agricultura Pesca y Alimentación, que permite mostrar imágenes de satélite con distintas capas (parcelas, croquis, zonas de pasto, recintos, etc.). El uso de la robótica en los huertos está aumentando, particularmente en la predicción del rendimiento [18] o para la recolección automatizada identificando por color, forma geométrica o textura en la imagen los frutos [19,20]. Estas técnicas no son aplicables exclusivamente en campo, la capacidad de poder eliminar frutos que no cumplen los requisitos de calidad ha hecho que se implante también en líneas en continuo, capaces de detectar frutos defectuosos y eliminarlos [21].

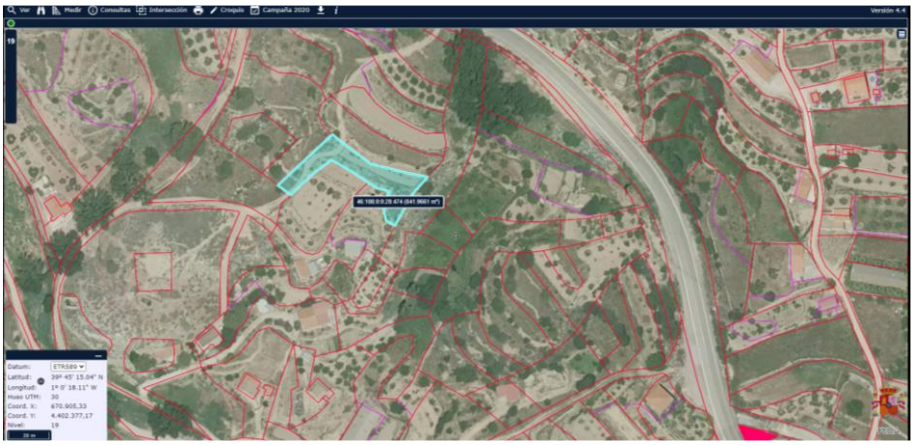


Figura 5. Imagen capturada en SIGPAC (MAPAMA), con la capa de parcelas y la medición de una de ellas.

La geomorfometría es la ciencia multidisciplinar (estudio del suelo, glaciología, volcanología, hidrología, etc.) del análisis cuantitativo del terreno utilizando modelos digitales [22] y ha sido uno de los campos que se han beneficiado de la teledetección. Gracias a la captura remota de las imágenes y el análisis de imagen hoy en día es posible cuantificar superficies o cambios en suelos, humedales, biomasa forestal, plantas submarinas, etc. sin necesidad de desplazarse hasta la zona y con la misma precisión en las mediciones, pudiendo alcanzar incluso zonas de difícil acceso [22-25].

Aplicación en ingeniería y automatización de procesos

Uno de los usos más extendidos del ADI es su aplicación en ingeniería civil como análisis no destructivo para la inspección con radares de tierra o radiografías industriales, comprobando el estado de las construcciones[26]. Por otro lado, es importante el control de la degradación de los materiales utilizados en ingeniería debido a las pérdidas económicas que pueden generarse si el material no es suficientemente resistente para la aplicación utilizada. Existen numerosas formas de control como técnicas por microscopía, inspección visual, por aumento o pérdida de peso, etc. Sin embargo, estos cambios también pueden visualizarse gracias a dispositivos de adquisición y procesamiento de imágenes que pueden dar información atendiendo al color, tamaño o forma del tipo y la extensión de la corrosión de forma automatizada,

teniendo control durante todo momento del desgaste producido en el material [27].

Asimismo, con la llegada de los detectores digitales de alta velocidad y las computadoras, actualmente es posible el procesamiento casi en tiempo real que involucra rutinas moderadamente complicadas. El ADI es una herramienta que puede ser empleada para automatizar algunas partes de control de las industrias o tareas que requieren de mucho tiempo al realizarse de forma manual. Stojanovic et al.[28] consiguieron sustituir el sistema visual subjetivo, automatizando un sistema de control que permitiese identificar defectos en la calidad de la tela, más concretamente en el patrón de esta. Otro ejemplo de su uso fue la mejora en la detección de los bordes de textos obtenidos a partir de imágenes o fotografías a color, permitiendo un reconocimiento total del texto y evitando la introducción de este de forma manual [29]. De igual forma la tecnología avanza a pasos agigantados y buena muestra de ello son los múltiples dispositivos que hoy en día necesitan el procesamiento de imágenes para llevar a cabo las funciones para los que están destinados. Los móviles, portátiles, etc. se desbloquean mediante reconocimiento biométrico lo que implica una constante mejora en los algoritmos utilizados con el fin de evitar ataques de suplantación de identidad [30]. Un ejemplo que combina la teledetección de objetos con la automatización de procesos es la creación de algoritmos que permitan diferenciar entre objetos similares entre sí, con el fin de poder distinguirlos, seleccionarlos y dispensarlos. Zhang et al.[31], consiguieron un algoritmo capaz de diferenciar entre diez tipos distintos de refrescos centrados en la posibilidad de automatizar la venta minorista sin necesidad de ser atendido por una persona.

Aplicaciones en alimentación

La aplicación del ADI en alimentación ha sido más tardía que en las áreas de aplicación mencionadas anteriormente. Sin embargo, debido a la versatilidad del ADI [32] cada vez es más habitual encontrar análisis rutinarios de calidad de alimentos que se han sustituido por técnicas de captura y procesado de imágenes. Uno de los aspectos visuales destacable en multitud de matrices alimentarias es la morfogeometría y volumen de los productos. Medir el volumen de los alimentos por geometría de manera manual es generalmente una tarea difícil y complicada debido a

su superficie heterogénea y a la variabilidad dentro de un mismo tipo de alimento, sobre todo si son frescos. El método más sencillo de medición del volumen se basa en el desplazamiento de agua o de semillas, siendo un método que debe realizarse de manera individual a cada producto y necesitando realizar muchas repeticiones para alcanzar el valor real. Por tanto, la combinación de captura de imagen del producto y procesado de esta ha sido objeto de estudio para intentar estimar el volumen de varios tipos de productos agrícolas como tomate [33], sandía [34], naranja [35], zanahoria [36], manzanas [37] u otro tipo de productos como moluscos [38], camarones [39], huevos [40] o jamones [41,42].

La frescura de las hortalizas, frutas, carnes, pescados, etc. es un parámetro importante que debe ser controlado, no solo por el aspecto visual y posible rechazo del consumidor, sino porque esta es indicativa de su calidad higiénico-sanitaria. Algunos de los últimos estudios permiten detectar diferencias en el grado de madurez o si no cumplen los requisitos de calidad por exceso de defectos [43], utilizándose incluso en granos de cereales pequeños como el sorgo y permitiendo calcular el porcentaje de adulteración [44]. Asimismo, existen estudios que demuestran la utilidad del ADI para determinar la frescura en carnes o pescado. Muchos de los cambios ocasionados durante el almacenamiento están relacionados con cambios en color, luminosidad, etc. de las piezas, por ello no es de extrañar que los estudios enfocados en este sentido sigan siendo de interés para la industria. Kunjulakshmi et al.[45] desarrollaron un sensor de mano, portátil, capaz de capturar y procesar la imagen obtenida de los ojos de la caballa y clasificar la frescura del producto *in situ*, pudiendo ser útil como herramienta para rechazar o no los lotes de producto fresco. Asimismo, se han desarrollado algoritmos que permiten diferenciar piezas de aves defectuosas (magulladas, desgarradas o con presencia de tumores) [46] para poder ser retiradas o clasificar peces por especies [47].

La manipulación y cambios ocurridos durante la etapa de transformación de los alimentos es responsable, en gran medida, de la calidad final de los productos obtenidos. Por ello, el desarrollo de herramientas que faciliten el control de estos se considera una de las líneas más importantes de innovación. Aunque la aplicación del ADI como herramienta de control es bastante incipiente, existen algunos ejemplos que validan su utilidad. Varias investigaciones confirman la validez del ADI para monitorizar la

estructura de la espuma, su distribución y morfología en diferentes matrices alimentarias, como bebidas, productos lácteos o postres [48-50]. Otras aplicaciones que se han propuesto recientemente incluyen el análisis de imagen para monitorizar y diferenciar la absorción de agua en distintas variedades de arroz [51], o para comprobar la retención de agua en yogures, analizada inicialmente mediante la sinéresis producida en el yogurt tras la centrifugación [52]. Aunque para esta última aplicación, es necesaria la combinación del ADI con la resonancia magnética nuclear de baja frecuencia.

Sin embargo, en los procesos de fabricación de alimentos, no solo es fundamental el control de los procesos, sino también el control de la calidad final del producto. Por ello muchos de los estudios que aplican esta herramienta han estado centrados en caracterizar, por ejemplo, la estructura interna de patatas fritas [53], así como el aspecto final de productos semielaborados [54]. Además, es una herramienta útil en la inspección de envases de alimentos, siendo una de las primeras aplicaciones alimentarias estudiadas. Los principales estudios se han centrado en la búsqueda de defectos en los envases (arrugas, abolladuras, defectos de rosca) que pudieran originar problemas de conservación o derrames del contenido, así como defectos visuales que hagan que el consumidor pueda rechazarlo (mala colocación de etiquetas, etc.) [55,56].

Como se ha visto el procesamiento de imágenes se utiliza en una amplia variedad de aplicaciones y el interés por esta herramienta no ha dejado de aumentar. Aunque su aplicación en el campo de la industria alimentaria ha sido más limitada, a pesar del gran potencial de esta herramienta en el control de procesos y calidad de ingredientes y productos. Particularmente, en los procesos de fabricación de productos horneados, la aplicación del ADI resultaría de gran interés para el seguimiento de los procesos de fermentación y horneado, así como la evaluación de la calidad de los productos horneados. Sin embargo, la aplicación de ADI en este sector requiere del desarrollo de metodología adecuada y su validación con los parámetros de calidad más habitualmente empleados.

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I. Microstructure of confectionary and bakery products[1]

*Cristina M. Rosell**, *Raquel Garzon*

Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain
**Corresponding author:* crosell@iata.csic.es

Abstract:
Bakery and confectionary foods cover an extensive number of specialties that can differ in the raw materials and making process, and both of them determine their sensory characteristics. One of the main attributes within the sensory characteristics of this kind of products is their texture that defines their quality and even freshness. Like any other food, in this type of products texture is largely connected to its mechanical properties, which in principle are directly derived from the internal food microstructure. Specifically, in the bakery and confectionary products microstructure is mainly governed by flour microstructure after being processed. This chapter compiles the information reported on microstructure of bakery and confectionary products, the main techniques used for assessing their microstructure as well as a brief mention of the ingredients and process and their impact on food microstructure. An attempt to show the relationship between crumb digital image analysis and quality parameters of breads (hardness and color) is also presented.

Key words: starch; gel; rapid force analyzer; microstructure; texture; Amylab

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Raquel Garzon: Investigation, Writing- Original Draft

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Introduction

Bakery and confectionary products play a main role in human nutrition worldwide. Bakery products constitute staple foods in many parts of the world, in some others they are used as nutrients carriers to balance nutritional deficiencies and they are even pleasure commodities. Conversely, confectionary products are mainly consumed for pleasure or convenience due to they are mostly high-caloric foods [1]. Whichever motivation explains their consumption, it is well-known that bakery and confectionary foods cover an extensive number of specialties that varied around the world, some of them have been extensively known but some other are autochthonous or country's specialties. Those products can differ in the raw materials and making process, and both of them determine their sensory characteristics. One of the main attributes within the sensory characteristics of this kind of products is their texture. In fact, one of the primary actions when buying those products is to check their softness by finger pressing the surface. Texture is an important component for the consumers' perception of baked goods quality. Texture is considered a multidimensional attribute that comprised a number of different textural properties [2]. In a more specific definition, texture is primarily the response of tactile senses to physical stimuli that result from contact between some part of the body and the food. In case of bakery and confectionary products, the textural attributes that better define its quality and even freshness are crispness and crumb firmness. Any food's texture is largely connected to its mechanical properties, which in principle are directly derived from the internal food microstructure. Despite the importance of microstructure of bakery and confectionary products in defining the quality, there is scarce information about the relationship between them. The quality of bakery and confectionary products is mainly assessed through volume and texture, and microstructure is only determined to complement those parameters or to support texture information.

The present chapter compiles the information reported on microstructure of bakery and confectionary products, the main techniques used for assessing their microstructure as well as a brief mention of the ingredients and process, their impact on food microstructure and some relationships identified between quality parameters and microstructure.

Microstructure evaluation techniques for bakery and confectionary products

The quality of bakery and confectionary products is determined by the quality and quantity of the raw materials and the processing characteristics. There are a number of parameters commonly related to food quality, particularly bakery and confectionary products. Volume, texture, color and moisture content are among the most used quality parameters. Food microstructure is defined as the spatial arrangement of its structural components and their interactions [3]. Food microstructure might have some impact in the physical, sensory and textural properties of bakery and confectionary products. Microstructure of bakery products determines the appearance, texture, shelf life, taste perception and rheology [4,5].

Different techniques have been applied to determine the microstructure of these types of products. The most used techniques are listed in Table 2.1. Confocal laser scanning microscopy (CLSM) has been very useful for assessing the distribution of macrocompounds after staining proteins, starch, lipids and carbohydrates [6,7,8]. This technique allows obtaining optical sections through a three-dimensional specimen [9]. Scanning electron microscopy (SEM) has been used for examining the surface and cross-section structure of cereal grains and also particle size distribution in flours [10,11,12], flour-water systems, the structure of doughs and gluten network [13,14], the influence of additives on crumb microstructure [15,16], the effect of process in bakery products [17,18]. Similarly, environmental scanning electron microscopy (ESEM) has been used to directly analyze bakery products, without the necessity of drying the samples [19,20].

Overall, those techniques provide very sensitive information of tiny sections of the food matrixes. In consequence, microscopy techniques have been mainly applied to identify interactions among ingredients or their distribution in the matrix. Generally, those techniques that do not require samples preparation are preferred over those that require some pretreatment. Consequently, SEM of freeze-dried samples are often reported, or cryo-SEM whenever possible. Only when the target is the functionality of specific biopolymers, CLSM is the election technique.

Table 1. The most used microscopy techniques in bakery and confectionary products.

Technique	Sample	Sample pretreatment	Stain	Coated	Magnification	Conditions	Reference
CLSM	gluten free bread	-	Nile Blue (0.1% w/w)	-	-	Excitation:488nm Argon laser (fat); 633 nm helium neon laser (protein and starch). Objective 63x	Alvarez-Jubete et al., 2010
CLSM	cake	-	Nile red & Rhodamine B (proteins & carbohydrates)	-	-	Excitation: Ar laser line: 488 nm	Rodriguez-Garcia et al., 2012
CLSM	gluten free bread	-	Fluorescence isothiocyanate (proteins and starch); aniline blue (β-glucan)	-	-	Excitation line: 405 and 488 nm. Objectives 10x &20x	Hager et al., 2011
CLSM	crumb bread	immersed in a 2% agar solution	Fuchsin acid (proteins)	-	-	Excitation 568 nm; emission 620 nm. Objective 10x &40 x water immersion	Renzetti et al., 2008
Cryo-SEM	dough, prefermented frozen dough, batter	immersed in liquid nitrogen & fixed with OTC compound	-	gold	-	10 kv	Baier-Schenk et al. 2005; Baixauli et al., 2007; Bonet et al. 2007
Cryo-SEM	wheat flour, dough, bread, cookies, cakes	immersed in liquid nitrogen & cryo fixed in slush nitrogen	-	gold	-	15 kv	Sarabhai & Prabhasankar, 2015; Rodriguez-Garcia et al., 2012; Bárcenas & Rosell 2005; Rojas et al., 2000
ESEM	dough	no- pretreatment	-		200x & 2000x	-	Létang et al., 1999
ESEM	oat cookies	no- pretreatment	-			25 kv	Duta & Culetu, 2015

Table 1. The most used microscopy techniques in bakery and confectionary products.

Technique	Sample	Sample pretreatment	Stain	Coated	Magnifica-tion	Conditions	Reference
SEM	Rice flour	-	-	gold	50x, 700x & 1000x	-	Ahmned, J et al., 2015
SEM	Wheat flour, crust layers	freeze-dried	-	gold	-	10 kv	Altamirano-Fortoul et al., 2015; Angelidis et al., 2015
SEM	pseudocereals and gluten free bread	fixed with aluminium specimen stub	-	-	-	1kv	Alvarez-Jubete et al., 2010
SEM	mixed dough, prefermented dough, bread	freeze-dried	-	gold	-	5-10kv	Bahal et al. (2013); Calderon-Dominguez et al., 2003; Brennan et al. 1997
SEM	cookies	freeze-dried	-	gold	1000x	15 kv	Dachana et al., 2010; Filipcev, et al. 2011
SEM	muffin crumb	-	-	-	-	15 kv	Jyotsna et al., 2011
SEM	gluten free bread	freeze-dried	-	platinum	50x, 150x, 500x, 2000x & 4500x	15 kv	Kawamura-Knish et al., 2013
SEM	gluten free cookies	-	-	gold	-	30 kv	Park et al., 2015
SEM	dough with celluloses	fixed in 10% glutaraldehyde, submerged in acetone	-	gold	-	-	Correa et al., 2010
Fluorescence microscope	-	embedded in Tissue-Tek O.C.T. compound & frozen, fixed in cryostat	acid magenta (10 min)	-	-	using WU (blue) range 330-385 & 420 nm	Maeda et al., 2015
Light microscopy	Bread	soaked 15 min under vacuum in issue-Tek O.C.T	Light Green (30 min)	-	-	-	Hug-Iten et al., 1999

Ingredients and processing of bakery and confectionary products

Therefore, to understand the basis of the quality of those products it becomes primordial to know the main ingredients and the changes that they undergo during processing. A very brief mention to ingredients and additives that could be used in the production of bakery and confectionary products in included in the next section.

Bakery and Confectionary ingredients

Flour is the major ingredient for making confectionary and bakery products and definitively the main responsible of texture and crumb structure. Water is essential for making bakery products, because it is responsible of many interactions between ingredients after its hydration. Some baked products require yeast as an essential ingredient. In leavened-products yeast is in charge of the production of carbon dioxide derived from the alcoholic fermentation of sugars, which, provides bread loaf volume, crumb structure and contributes to the typical bread flavor [21]. Other leavening agents that can be used to produce carbon dioxide are chemical leavenings. They are more useful for making cookies or cakes and can be divided into three groups depending on the type of gas producing: sodium bicarbonate, potassium bicarbonate and ammonium bicarbonate [22]. Among other ingredients it could be cited sweeteners as sugar, which affect the taste, color, texture, appearance and it also contributes to fermentation [23], and lipids (butter, vegetable shortenings, etc.), which impart sensory attributes such as mouthfeel, textural properties and structure [24]. Apart from the ones mentioned before, there are numerous functional additives used in bakery to facilitate processing, to compensate for variation in raw materials functionality, to guarantee constant quality and to preserve freshness and food properties. On this category would fall the enzymes or processing aids as amylases, proteases, oxidases and transglutaminase among others [25]. Transglutaminase, alpha-amylase, xylanase and proteases affect significantly the viscoelastic properties of dough and can change bread quality parameters as volume or crumb structure [26]. The industrialization of breadmaking process and the consumer demand for high quality and longer shelf life have increased the use of those processing aids and some other additives like emulsifiers

that affect starch and proteins, producing softness, longer shelf-life products and increase in the volume, stability and structure [27]. Finally, other improvers that are useful in the production of these products are hydrocolloids like xanthan gum, hydroxypropyl methylcellulose (HPMC), guar gum, etc. They modify the rheology and the texture of the products and their functionality is mainly related to their ability to bind water, and subsequently changes in dough rheology, freshness and shelf-life have been described [14,16,28]. Similarly, in special type of products, like gluten-free, the functionality of the hydrocolloids is essential because they are acting as gluten replacers and thus give the structure of the baked products [29-31].

Flour microstructure

Since flour is the main ingredient in the bakery and confectionary products, a special emphasis is given to it and all the changes that mechanical and temperature constrains induce in its structure. It must be stressed that the term flour is also very extensive, given the variety of sources and thus the differences in chemical composition. Nevertheless, when referring in general to flour attention drives to wheat flour, because it is the main cereal used for making baked goods. Because of that, this section will be referred to wheat flour, although changes induced in proteins and starch when subjected to mechanical or temperature stresses can be extrapolated to any other type of cereal flour.

The knowledge of the composition of wheat grain is important in order to understand wheat flour microstructure. Wheat grain is divided in three main parts, endosperm, peripheral layers and germ. Endosperm is the largest morphological component (82% of the grain). Peripheral layers or bran (15%) that surround the endosperm and germ (3% of the grain) are removed during the milling to obtained refined wheat flour [32]. Those parts are very well integrated in the kernel structure, in fact, scanning electronic microscopy (SEM) pictures of the wheat flour (Figure 1a) have revealed that endosperm tissue is composed of large aggregates (≥200 microns length) and protein matrix embedding endosperm starch granules [33,34] but it changes with intense milling. Nevertheless, wheat kernels are not consumed directly, they undergo milling in which the initially ordered structure becomes disorganized and broken in smaller

particles. The particle size affects the physicochemical properties of the flour. In fact, flour fractionation according to particle size distribution has been employed to obtain special flours for different end-use applications or for nutritional improvement. For instance, fine particle size flours have better protein quality, determined by sodium dodecyl sulfate (SDS) sedimentation value, higher damaged starch and falling number, and also lower ash content and improved baking performance for some type of products [35]. Sakhare, et al. [36] used microstructure analysis to study the distribution of major constituents in the flour mill streams during wheat milling. Their results revealed that as milling intensity increased, more deformed and damaged starch granules were obtained probably due to repetitive grinding. Besides, special techniques as jet milling treatment have been studied using different air pressure and observed that large aggregates were gradually reduced in size, depending on the intensity of the process, and starch granules were separated from the protein matrix [34]. The effect was also tested on whole-meal flour, in which aleurone layer was broken down to small particles (about 20-180 μm) [11]. The effect of jet milling on the whole wheat flour also has impact on the flour composition, because an increase in the total fiber content and digestible starch was observed as increase the severity of the jet milling treatment and also more starch granules were separated from the protein matrix [11].

Microstructure changes through processing

The making process to obtain bakery products is a dynamic system, where physical and chemical changes occur on the flour components and those changes will determine the final microstructure of the baked products [37]. To understand the microstructure of baked products, a very brief description of the processing is included. There are three important stages that involve the major part of the changes during processing, which include mixing, proofing and baking.

The first important stage is mixing. Even it is the first stage its incidence on the final structure of the food is crucial and undermixing or overmixing can make significant differences. During this stage occurs the hydration of the ingredients, their uniform distribution in the dough, dough development (gluten formation) and air incorporation into the

dough (Figure 1b). The creation of gas bubbles and their retention depends entirely on the mixing characteristics, this is necessary to define the cellular structure in baked products. Air incorporated into the dough, and more specifically oxygen, is responsible of the dough oxidation, which is needed to form disulfide bonds, linking protein chains and in consequence, increasing the strength of the gluten dough, which is crucial for the development of gluten network. To obtain a correct dough development it is necessary to adjust and optimize mixing time and hydration [38]. The structure of white bread dough with short mixing (undermixed) consists of large starch granules (over 10 μm) and smaller ones (less than 10 μm), a coarse veil (proteins) covering the structure and many intact flour particles [39]. When bread dough is well developed a compact system (Figure 1b) with small and big starch particles embedded and distributed on the surface (protein cover disappear) [40] and an even network of starch and proteins is obtained [33]. If mixing continues (overmixed) an open system with holes is obtained, in which the proteins and the starch particles are not embedded [19,41] (Figure 1c). The distribution and morphology of water-flour dough depends on the water content, the veil protein covering starch granules are much less visible in dough with less hydration [19]. The same trend has been observed even in more complex formulations. Calderon-Dominguez, et al. [41] observed that dough of sweet yeast bread containing flour, sugar, shortening, milk, yeast and water, exhibited the same structure observed with white bread dough during mixing.

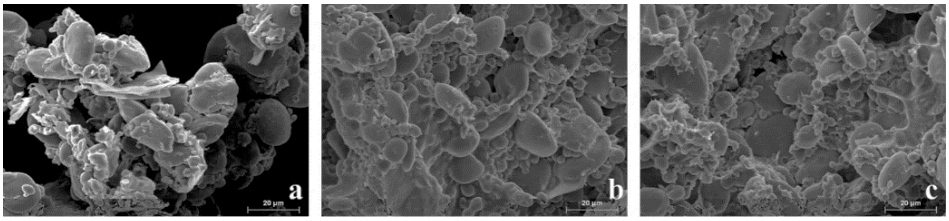

Figure 1. Scanning electron micrographs of flour (a), well developed dough (b) and overmixed dough from wheat.

There are products with high quantity of shortening like puff or short pastry, in which the distribution of gluten, starch and butter is very important. Maeda, et al. [40] visualized by fluorescence fingerprint the microstructure difference in puff and short pastry due to their different processing. Puff pastry exhibited a structure like gluten network spread

in the direction of dough extension, whereas in short pastry small and large clumps without a continuous gluten network was observed owing to fat avoid its formation.

The use of different additives to improve bakery products quality is a common practice in bakery and confectionary. For instance, some enzymes are added during mixing to improve dough development. Bahal, et al. [15] observed that addition of lypoxygenase had a more uniform dough surface, which persisted after fermentation and also improved gas retention. The use of glucose oxidase has been studied by Bonet, et al. [42] to improve dough obtained from damage wheat flour as an alternative to dough conditioners such ascorbic acid, azodicarbonamide or potassium bromate. These authors observed that untreated damage flour led to a discontinuous network with large and small fragments of gluten but the use of glucose oxidase induced the formation of a protein network with a continuous structure similar to that of the dough from sound wheat. Disrupted doughs resulted in softer doughs with low stability and to improve their performance the use of modified celluloses have been proposed [14]. Depending on the structure of the modified celluloses the dough characteristics can be modulated. The addition of microcrystaline cellulose resulted in a more disaggregated structure of bread dough, whereas carboxymethylcellulose made difficult the gluten film formation and some types of hydropropylmethylcellulose (HPMC) led to filamentous microstructure, resembling gluten network [14]. The addition of hydrocolloids (carrageenan, xanthan gum and HPMC) modifies the interaction between starch granules and protein matrix, being structures more closely linked in the presence of hydrocolloids (Figure 2).

In the case of the batters, after mixing the dosing type could even change the structure of muffins [18]. Batter resulting from manually dosing displayed two types of starch granules (rounds and lentil shape) immersed in the reticular structure of proteins and soluble solutes that form the matrix. Conversely, when the dosing was automatic greater compactness was exhibited and a decrease in the fat globules size [18].

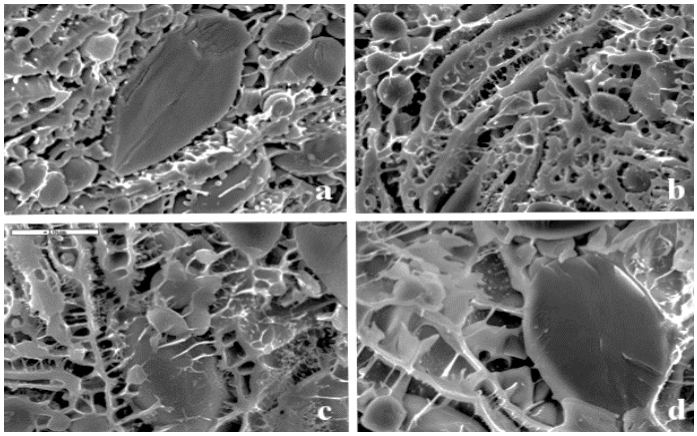

Figure 2. SEM micrographs of different doughs (3500x). Control dough (a); with addition of k-carrageenan (b), xanthan gum (c) and HPMC (d).

The second important stage is the fermentation or proofing. Fermentation aims to increase dough volume but also to generate small metabolites that will contribute to taste and flavor of final products. During fermentation sugars are transformed in carbon dioxide and alcohol due to the action of yeast, and also short fatty acids are released due to the contribution of lactic acid bacteria. In the absence of sugars, enzymes action on the starch polymer released sugars and dextrins (fermentable carbohydrates) that would be also substrates for yeast. The carbon dioxide released during the fermentation moves towards to the gas nuclei, formed during mixing, leading to dough expansion and consequently, volume increase of the dough (Figure 3). But there are also un-yeasted bakery and confectionary products, in which the gas required for volume increase is chemically produced from leavening agents like sodium bicarbonate, potassium bicarbonate and ammonium bicarbonate [22], among others.

Freezing has become a common practice in bakery and confectionary for extending the shelf-life or assisting bakeries in the process organization. This practice allows obtaining fresh products at any time of the day. In some cases, the bakery pieces are stored frozen after proofing. Micrographs of dough after proofing showed a porous structure and without the presence of ice crystals, but frozen storage might influence the dough properties and final quality [17]. In fact, after freezing dough cell walls exhibited small ice crystals (≤ 100 μm), but during storage

increased the number and size of crystals and they become more spherical [17,43,44]. To avoid the formation of ice crystals during frozen storage, Huang, et al. [45] proposed the addition of transglutaminase and observed that the gluten network was less fractured and disrupted than samples without transglutaminase. Other alternatives like the addition diacetyl tartaric acid ester of monoglycerides (DATEM) and guar gum were also studied, but larger amount of void among starch granules and gluten network were observed with DATEM, and more dense structure was obtained with guar gum [46].

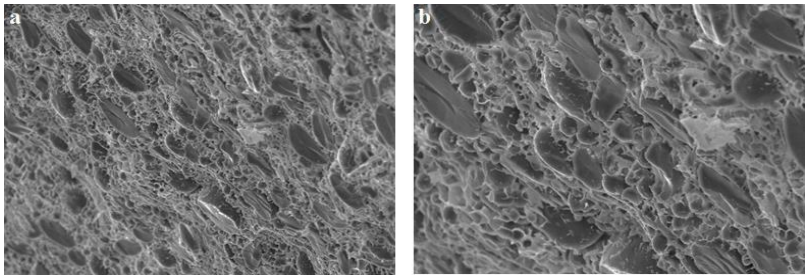

Figure 3. Cryo-SEM micrographs of fermented dough. Continuous and well-distributed matrix with firmly embedded starch granules (a: 750x and b: 1500x).

Finally, the last stage is baking, where dough is subjected to temperature increase resulting in gas expansion and a simultaneous stretch of the dough. Dough extensibility will depend on proteins coagulation and starch gelatinization, and the former will be influenced by water availability. When temperature is high enough, water evaporation takes places with the subsequent drying of the dough leading to a porous structure (crumb) [47]. Meanwhile, temperature on the dough surface reaches much higher values inducing caramelization and Maillard reactions that will lead to crust formation and released of flavor compounds [47].

Bakery and confectionary products

Product composition determines its microstructure, and one of the main differences in bakery and confectionary products is the presence or the absence of gluten. Gluten creates a viscoelastic matrix that allows dough expansion leading to aerated and open structures. Conversely, the

absence of gluten tends to produce uneven and collapsed structures [48]. Because of that this section will present separately the microstructure of gluten containing products and those which lack of gluten.

Gluten containing products

In the case of breads, it is very characteristic their soft internal structure, namely crumb, surrounded by a dried outer part, which in the surface is called crust. When going into detail through micrographs, it is observed a complex structure of an open sponge with numerous cavities inside large gas cells (Figure 4a). In that matrix starch granules are predominant showing a veil on the surface comprised by gelatinized starch and degraded proteins [15,16,49] (Figure 4b). Therefore, starch granules and proteins are the main players in defining the structure of bakery and confectionary products. Their structure commonly consists of a number of gelatinized starch granules that together with denatured proteins form a smooth gel covering intact starch granules. In that matrix are integrated the rest of ingredients and or additives.

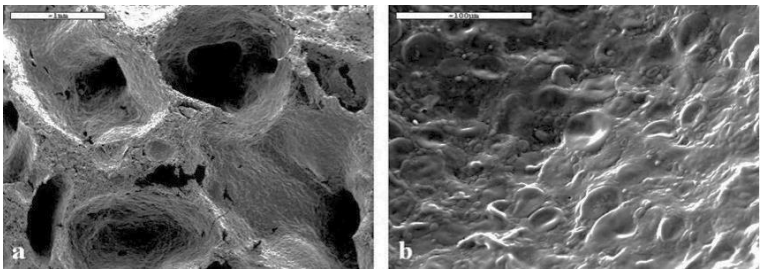

Figure 4. Scanning Electron Micrographs of wheat bread crumb at 35x (a) and cell gas surface at 500x (b).

Cookies and cakes have similar microstructure to gelatinized starch granules, protein aggregates, sugar phase and lipids embedding in the protein matrix [7,50,51]. Starch granules are more extensively gelatinized in the center of the cookie than in the bottom or the top of it [52,53]. Conversely, flat breads microstructure displays significant differences with other bakery products. No such gas cells could be observed in the microstructure of baked parotta (a type of flat bread), maybe because there is no fermentation and no gas formation in the process [54].

The addition of improvers changes bakery products microstructure. When guar galactomannan is added to doughs, the hydrocolloid is dispersed into the matrix and mixed with the starch granules and protein matrix [49]. Addition of lipoxygenase improved microstructure resulting in better network of gluten with embedding starch granules, moreover dough showed a more continuous and smooth surface that bread without enzyme [15]. Use of HPMC in bread formulation led to more continuous surface too, it seems that HPMC enfolds all the other bread constituents [16].

Apart from additives, there is a trend to improve the nutritional quality of bakery products adding other types of cereals or ingredients, which significantly affect the microstructure. The use of wholegrain buckwheat flour and rye in biscuits was studied by Filipcev, et al. [55] and they observed smooth and round spherical starch granules with different sizes. Other flours like the finger millet flour decreased the number of air cells that it is indicative of poor air incorporation during mixing [56]. In addition, the incorporation of wheat bran to improve fiber content of products induced the disruption of the protein matrix [53]. Flours from pulses have been also been incorporated with the objective of nutritional enrichment of cookies in proteins and fibers. Rajiv, et al. [51] studied the use of green gram flour for this purpose and observed that the protein matrix structure was disrupted when this ingredient was added to the formulations. In order to decrease the fat content in cakes, Rodriguez-Garcia, et al. [7] replaced part of the oil with inulin. These authors observed a continuous matrix with embedded starch granules and coated with oil; when fat replacements increased, starch granules appeared as separated structure. It seems that hydrophobic or hydrophilic ingredients can be incorporated in the dough or batter matrix up to a specific level but beyond that biphasic systems are obtained, which completely change their role and functionality in the dough or batter. This has been observed with inulin [7] and other hydrocolloids like xanthan gum, guar gum and HPMC [57].

Non gluten containing products

Products without gluten have very different microstructure, due to the absence of these proteins prevent the development of gluten-like network and dough consistency is more similar to a batter. There are several

alternative flours and starches for making gluten free bakery products, but to form a network-like structure and retain gases is necessary to use gluten mimetics like hydrocolloids. One of the most widely used gluten free flours is rice flour, owing to its hypoallergenic proteins and bland taste. Rice flour particles are irregular, polyhedral in shape and indeed, the average particle size of t rice flours is 40-125 μm [12,58]. The microstructure picture of rice flour-water dough shows the formation of a cohesive network but not enough to obtain acceptable bakery products. Dixit and Bhattacharya [58] added whey protein concentrate and xanthan gum for obtaining a more cohesive structure. The same approach was followed by Shanthilal and Bhattacharya [59] that used Arabic gum with rice flour, observing that the flour particles were amply coated with gluey material, which improved the particle binding. Rice based gluten free breads have a very irregular cellular structure, and different approaches have been reported for smoothing the structure. Kawamura-Konishi, et al. [60] used proteases to improve the quality of rice based. These enzymes induced protein hydrolysis decreasing the hydrophobic nature of the rice proteins, which seems to favor the connection between protein matrix and starch granules, as a result bread with a more regular structure and higher volume was obtained, with a subsequent decrease of the crumb hardness. Marco and Rosell [61] designed protein enriched gluten free bread, containing composite rice flour with soybean and in the presence of transglutaminase to promote protein crosslinking. Similarly, transglutaminase has been also added to other gluten free flours like buckwheat, brown rice and corn flour [62].

In the case of gluten-free cakes, also gluten mimetic is usually added as structuring agent. The addition of xanthan gum increased the size of the cells area in the cakes crumb compared either with sample without hydrocolloid or gums like guar, and kappa-carrageenan [63]. Not only the recipes affect the microstructure of cakes, also the process conditions can modulate the characteristics of the final product. For instance, cakes baked in conventional oven presented more deformed starches granules compared with cakes baked in IR-microwave combination oven [63].

Nutritional improvement has been also a concern in the case of gluten free bakery products. To improve fiber content in gluten-free bread and cookies, Hager, et al. [8] and Duta and Culetu [20] respectively, added oat β-

glucan. Micrographs of the final products showed that oat starch granules were rounded and with irregular shapes and sizes up to 10 μm. In cookies, the structure consisted of starch granules grouped in clusters with the protein and fiber matrix [20]. In the case of bread, confocal laser scanning microscopy revealed that proteins appeared like cloud and a big blue signal that confirmed the presence of β-glucan in the breads. Incorporation of finger millet flour, which is a good source of minerals, have been used to increase the nutritive value of gluten free muffins [56]. The presence of finger millet flour in muffins led to a few large air bubbles, indicating that the air incorporation was low and some of the gelatinized finger millet starch granules appeared in the form of a thin sheet (broken matrix) [56].

Pseudocereals are potential ingredients to obtain gluten-free bakery products and in the last decades have attracted much attention. Particle size distribution of the pseudocereals flours differ considerably. Buckwheat flour exhibits the smallest particle size followed by amaranth and quinoa; and all of them have smaller particle size than rice flour [6]. In amaranth and quinoa, the size of starch granules size is significantly smaller (<2 μm) than other studied flours, and their shape is polygonal [6]. Microstructure analysis of breads made with pseudocereal flours showed only partial gelatinization, with a great number of starch granules that retained their integrity but the overall structure was homogeneous with a well distribution of fat, proteins and starch, resembling the structure of gluten containing products [6]. Other alternative to make gluten-free products is the use of chestnut flour. Cookies dough from chestnut flour was characterized by the presence of small and large starch granules, which were enmeshed in a partially formed protein matrix [64]. Recently, with the same aim to improve nutritional values, Park, et al. [65] proposed the use of okara (by product of tofu manufacturing) to develop okara cookie. Those were made adding starch, soy flour and HPMC and micrographs of the okara cookie doughs showed a disrupted matrix structure.

Crust microstructure and changes due to processing and specific treatments

Crust is taken to refer to part of the bread near its surface, where the density is significantly higher than elsewhere [66]. When dough is placed into the oven water of the surface evaporates very fast, resulting in much lower water content than inside the product. The temperature in the crust can exceed 100 °C, and then Maillard reactions, responsible for the development of color and some flavors, is produced [67]. There are many bakery products with crispy crust that are sought by consumers for their appealing texture at the first bite. That sensory perception due to the low water content is felt when consuming cookies or crispy bread. A study carried out on the bread crust indicated that both cell size and shape observed by SEM was significantly related to crust crispiness [68]. The structure of the bread crust indicates a continuous gluten network with embedded non-gelatinized large and small starch granules (Figure 5a), maybe because the rapid water evaporation avoids to have the required water for starch gelatinization [69]. Furthermore, the size of gas cells in the crust is lower than in the crumb, because of the rapid loss of the extensibility of dough [67]. Pursuing the goal of extending the crispiness characteristics of the bread crust, some authors proposed the use of enzymes to change the crust microstructure. Primo-Martín, et al. [69] sprayed protease on the dough surface, and the resulting bread crust showed significantly lower water content indicating the presence of fractures that favored water diffusion. Other enzyme reported for increasing the crispness was amyloglucosidase [70]. When this enzyme was sprayed onto the surface of partially baked bread, the resulting bread had lower water activity and moisture content in its crust and SEM micrographs revealed a more disordered structure with small irregular voids and great cracks (Figure 5b). To understand the action of different additives on the crust texture, and thus on its microstructure, a crust model was proposed by [71]. Those authors tested the effect of glycerol, gluten, protease, DATEM, citric acid, linoleic acid, beeswax and HPMC on the mechanical crust properties and water vapor permeability of the model crust. The crust layer containing protease exhibited a compact structure, more disrupted gluten network an high deformation of starch granules; addition of HPMC revealed an irregular starch granules within a disrupted and discontinuous protein network; glycerol functionality

was greatly dependent on its concentration, with 1% glycerol the crust structure resembled a continuous and smooth gel, but with 10% of glycerol microstructure was rather compact; crust layer with DATEM showed starch granules covered with alternate continuous veil-like film and some cracks, finally addition of lipids gave smooth and non-porous structure without phase separation [71].

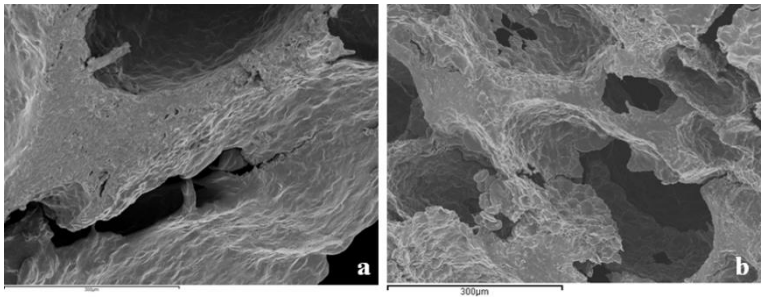

Figure 5. SEM micrographs of bread crust (a) and that resulting after spraying amyloglucosidase onto the dough surface (b).

Digital image analysis of bakery and confectionary products

In the last decades, great attention has been payed to gas cell distribution in the cross-section of baked products, owing to the relationship between that and volume or texture 72. At a macroscopic level crumb is comprised of two phases: a fluid (air) and a solid (cell wall material) 73. Crumb structure showed a gelatinized starch network associated with a protein network 74 and after baking show and open cell structure of medium and small size air cells with homogeneous distribution in it 69,75. Study of gas cells size, shape and distribution can be made by image digital analysis of cross-sections of the products. For that purpose, it is necessary to capture the image of the cross-section and then to use an image software to binarize images using an algorithm (Figure 6).

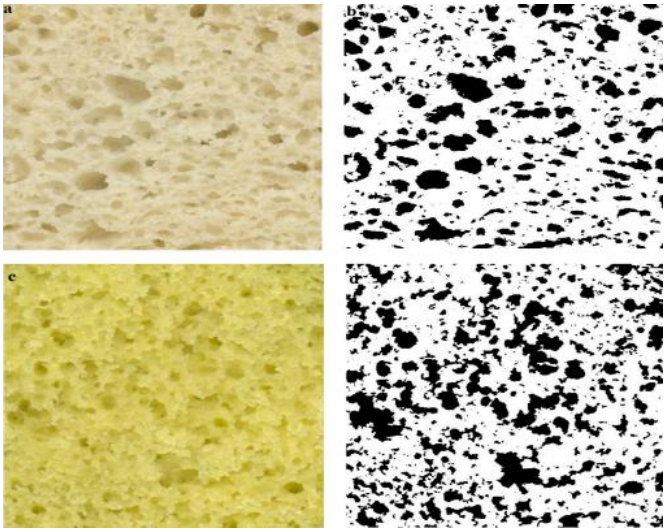

Figure 6. Cellular structure of the crumb of the products. Left column: scanned crumbs of gluten free bread and muffin respectively (a, c). Right column: binarized images of gluten free bread and muffin (b, d).

Different algorithms have been proposed for better understanding and quantification of the gas cells in bakery products [76], which could be very useful for assessing the effect of process, ingredients and additives. Digital image analysis have been used for determining differences in the gas cell distribution of different commercial types of breads, allowing to discriminate among them regarding number of cells, their surface, diameter and circularity [77]. The use of different flours could result in different crumb structure. The use of buckwheat and quinoa originated differences in the number of cells, cell volume and wall thickness, particularly buckwheat flour yielded the larger number of cells than quinoa [6]. Garzon and Rosell [78] studied the effect of rice based gluten-free bread formulation the role of vegetal protein, animal protein and different gums (xanthan and guar gum, UltraCel™ and HPMC on the structure of the bread crumb (Figure 6.2.). The digital image analysis of the bread crumbs allowed confirming that casein and albumin led to more homogeneous and smaller air cells than proteins from vegetal sources. Image digital analysis can be useful to study different parameters during breadmaking process. Nevertheless, no relationships have been reported between quality parameters and values derived from digital image analysis. An attempt for finding that type of correlation is presented in

Table 2. The digital image analysis of the cross section of breads showed in Figure 6 was correlated with some quality parameters, namely crumb hardness and color. Only significant correlation were observed between gas cell number per cm^2 and hardness with color parameters, specifically luminosity (L^*) and a^*. Gas cell number per cm^2 was positively correlated with L^* and negatively with a^*, and the opposite trend was observed with the hardness. It has been previously described that crumb structure has great influence on the texture properties and the luminosity of the crumb of gluten free breads [5,48].

Recently, Marston, et al. [79] use this tool to determine the effect of heat treatment of sorghum flour on the crumb structure, and observed that heat treatment of flour increased the number of crumb cells resulting in an irregular crumb structure compared with bread from non-treated flour.

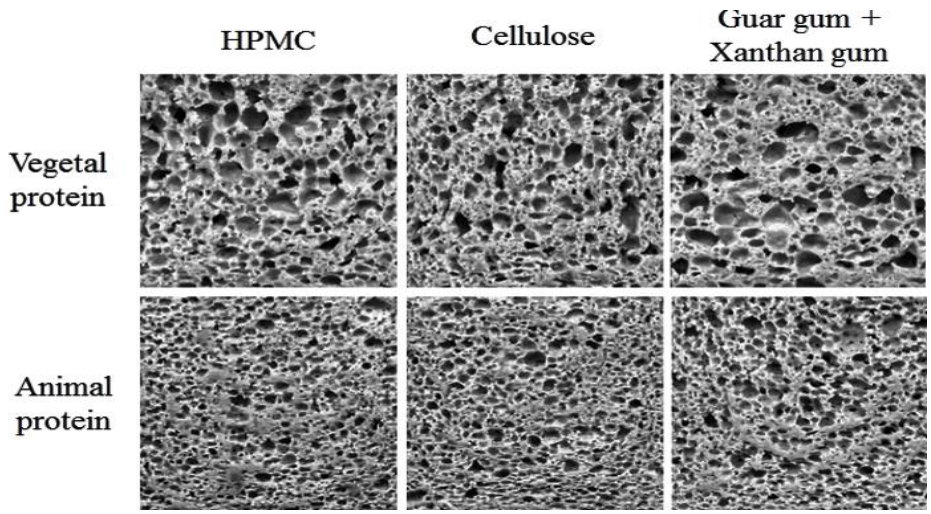

Figure 7. Digital image analysis of crumb cross sections from different gluten free breads. A comparison of the effect of protein origin (vegetal, animal) and the used of different hydrocolloids (HPMC, cellulose, xanthan gum and guar gum blend) is showed.

Table 2. Correlation matrix between bread quality (hardness and color) and parameters from crumb digital image analysis.

	*L**	*a**	*b**	Hardness (N)	Gas cells / cm^2	Cell average size (mm^2)	Total gas cell area (cm^2)	Circularity
*L**	-	-0.8909***	0.5335***	-0.3532**	0.7342**	-0.5829*	-	-
*a**	-	-	-0.6400***	0.3172**	-0.581*	-	-	0.6226*
*b**	-	-	-	-	-	-	-	-0.6156*
Hardness (N)	-	-	-	-	-	-	-	-
Gas cells/cm^2	-	-	-	-	-	-0.9477***	-	-
Cell average size (mm^2)	-	-	-	-	-	-	-	-
Total gas cell area (cm^2)	-	-	-	-	-	-	-	-
Circularity	-	-	-	-	-	-	-	-

Future trends

Microstructure analysis of bakery and confectionary products have been carried out for confirming purposes, but rarely as a main suggest of research. Nevertheless, powerful microstructure techniques have been developed and applied in different areas, which have been scarcely applied to these food products. Therefore, microstructure utility in bakery and confectionary products should be exploited in great extent, mainly with the purpose of better understanding the role of ingredients and additives, and even optimizing process conditions, and further defining rapid predictors of bread quality.

Further sources of information

Image J program for image analysis could be downloaded at: http://rsb.info.nih.gov/ij/download.html

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OBJETIVO

El **objetivo principal** de la tesis fue: Definir la interrelación entre la estructura y parámetros de calidad de masas y productos horneados, así como extender la utilidad del análisis digital de imagen y su uso para optimizar los procesos y las formulaciones en productos horneados.

Este objetivo se dividió en 4 objetivos específicos:

1. Evaluar el potencial del análisis de imagen en alimentos, y concretamente en productos fermentados y horneados mediante la optimización de las condiciones de captura y procesado de imagen para este tipo de productos.
2. Entender los cambios estructurales del almidón durante los procesos de gelatinización y gelificación mediante el equipo Amylab, de reciente lanzamiento en el mercado, extendiendo su actual aplicación al conocimiento fundamental del almidón.
3. Implantar el uso del análisis de imagen como tecnología analítica no destructiva para evaluar el impacto de distintos ingredientes y aditivos panarios en la calidad de productos horneados, y concretamente en la formación de la estructura de los productos resultantes.
4. Establecer posibles relaciones entre las características tecnológicas de los panes y las propiedades del bolo, la masticación y la deglución de la miga.

RESULTADOS

Capítulo 1

Optimización de las condiciones de captura y procesado de imagen para productos de panadería

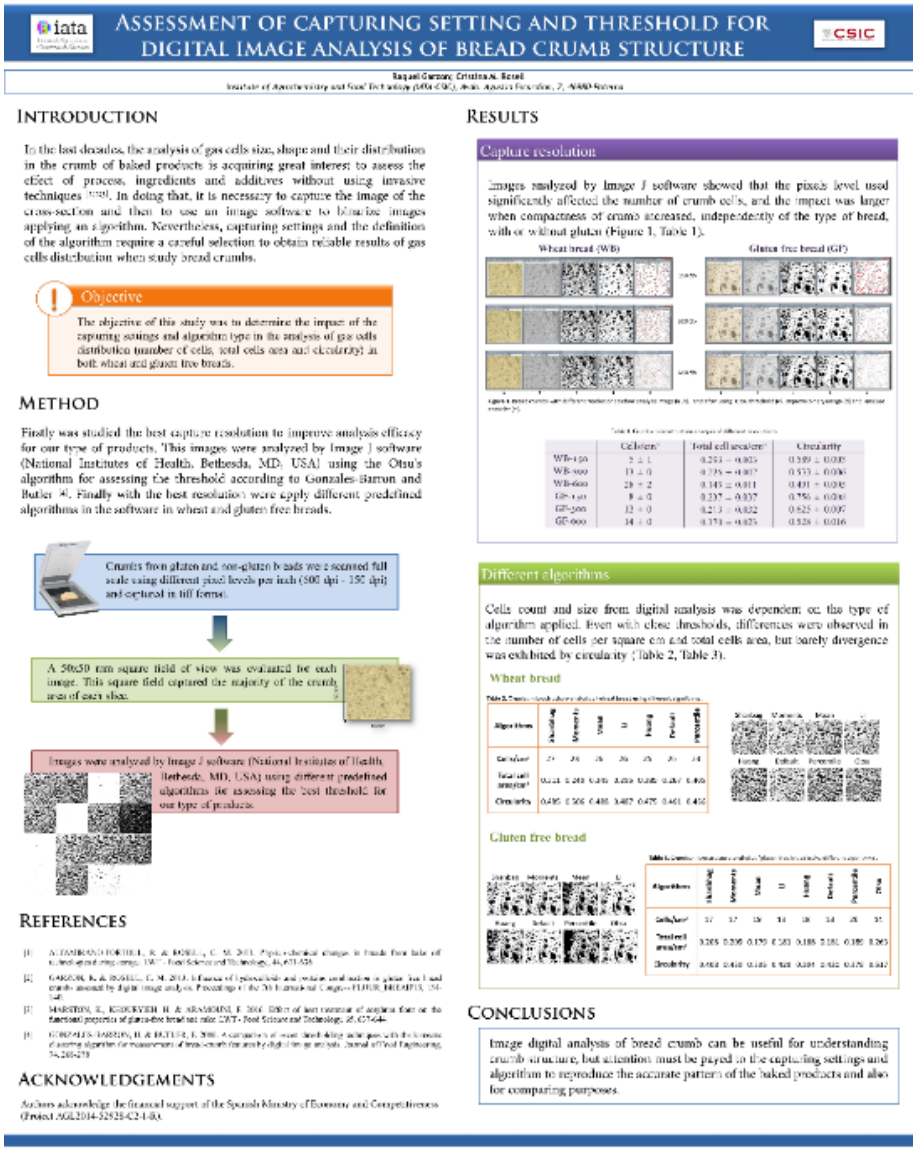
I. Estudio del impacto de la resolución de captura y el tipo de algoritmo en el análisis de la distribución alveolar del pan de trigo y el pan sin gluten.

II. Validación del ADI para cuantificar diferencias en la distribución alveolar de las migas de pan (con y sin gluten).

III. Puesta a punto y validación de la obtención de panes a pequeña escala (minipanes).

Para abordar el objetivo se han utilizado diferentes estudios preliminares que fueron divulgados en Congresos Científicos Internacionales.

- C.M. Rosell & R. Garzon. *Assessment of capturing setting and threshold for digital image analysis of bread crumb structure.* 2016. AACC International Annual Meeting, Savannah, Estados Unidos de América. Póster.



ASSESSMENT OF CAPTURING SETTING AND THRESHOLD FOR DIGITAL IMAGE ANALYSIS OF BREAD CRUMB STRUCTURE

INTRODUCTION

In the last decades, the analysis of gas cells size, shape and their distribution in the crumb of baked products is acquiring great interest to assess the effect of process, ingredients and additives without using invasive techniques [1-3]. In doing that, it is necessary to capture the image of the cross-section and then to use an image software to binarize images applying an algorithm. Nevertheless, capturing settings and the definition of the algorithm require a careful selection to obtain reliable results of gas cells distribution when study bread crumbs.

Objective

The objective of this study was to determine the impact of the capturing settings and algorithm type in the analysis of gas cells distribution (number of cells, total cells area and circularity) in both wheat and gluten free breads.

METHOD

Firstly was studied the best capture resolution to improve analysis efficacy for our type of products. Then images were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA) using the Otsu's algorithm for assessing the threshold according to Gonzales-Barron and Butler [4]. Finally with the best resolution were apply different predefined algorithms in the software in wheat and gluten free breads.

Crumbs from gluten and non-gluten breads were scanned full scale using different pixel levels per inch (300 dpi - 150 dpi) and captured in tiff format.

A 50x50 mm square field of view was evaluated for each image. This square field captured the majority of the crumb area of each slice.

Images were analyzed by Image J software (National Institutes of Health, Bethesda, MD, USA) using different predefined algorithms for assessing the best threshold for our type of products.

RESULTS

Capture resolution

Images analyzed by Image J software showed that the pixels level used significantly affected the number of crumb cells, and the impact was larger when compactness of crumb increased, independently of the type of bread, with or without gluten (Figure 1, Table 1).

Wheat bread (WB) | Gluten free bread (GF)

	Cells/cm²	Total cell area/cm²	Circularity
WB-150	5 ± 1	0.253 ± 0.005	0.389 ± 0.005
WB-300	13 ± 0	0.235 ± 0.007	0.533 ± 0.006
WB-600	28 ± 2	0.145 ± 0.011	0.491 ± 0.005
GF-150	8 ± 0	0.237 ± 0.037	0.756 ± 0.010
GF-300	12 ± 0	0.213 ± 0.022	0.625 ± 0.007
GF-600	14 ± 0	0.173 ± 0.025	0.528 ± 0.016

Different algorithms

Cells count and size from digital analysis was dependent on the type of algorithm applied. Even with close thresholds, differences were observed in the number of cells per square cm and total cells area, but barely divergence was exhibited by circularity (Table 2, Table 3).

Wheat bread

Gluten free bread

CONCLUSIONS

Image digital analysis of bread crumb can be useful for understanding crumb structure, but attention must be payed to the capturing settings and algorithm to reproduce the accurate pattern of the baked products and also for comparing purposes.

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ACKNOWLEDGEMENTS

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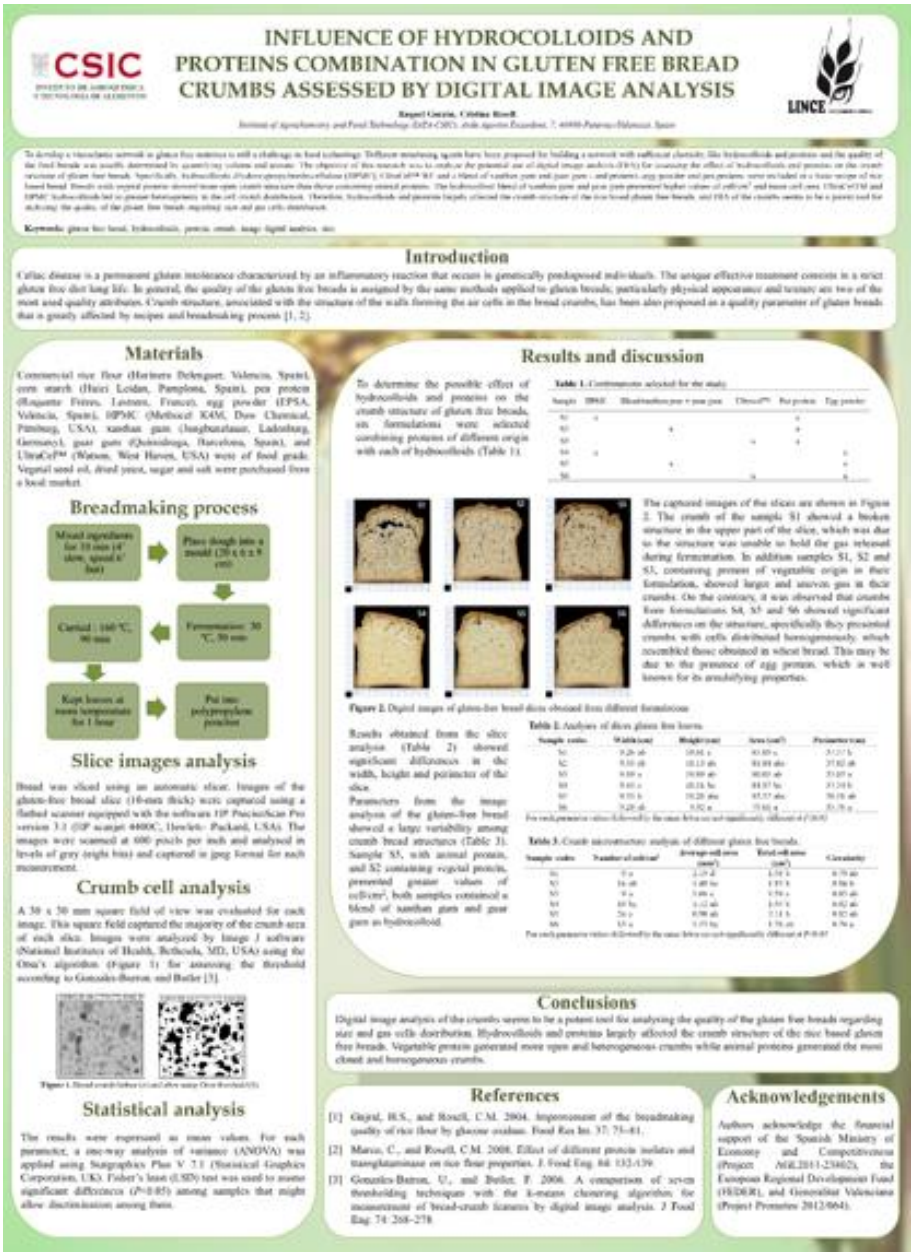

INFLUENCE OF HYDROCOLLOIDS AND PROTEINS COMBINATION IN GLUTEN FREE BREAD CRUMBS ASSESSED BY DIGITAL IMAGE ANALYSIS

Introduction

Materials

Breadmaking process

Slice images analysis

Crumb cell analysis

Statistical analysis

Results and discussion

Conclusions

References

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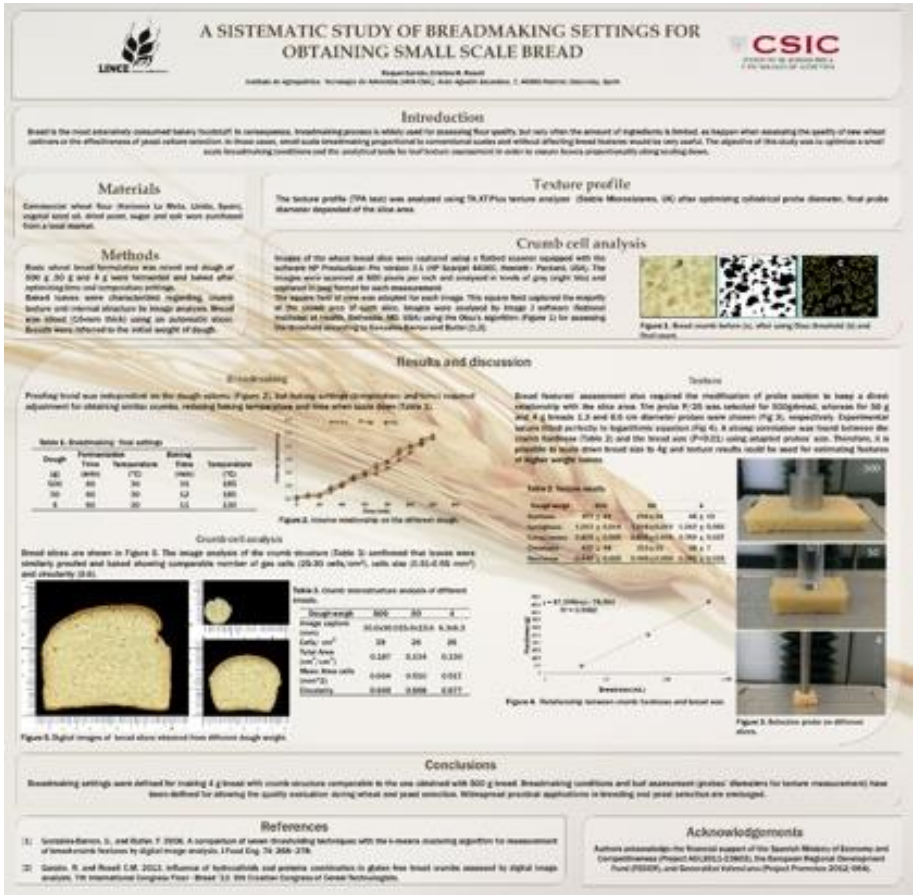

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Introducción

La inspección visual es un parámetro de calidad que describe tanto el aspecto externo como la estructura interna en los productos derivados de cereales, y se incluye habitualmente entre los descriptores utilizados en las evaluaciones sensoriales y de calidad[1]. Existen algunos estudios que evidencian la correlación entre la inspección visual en los análisis sensoriales y los parámetros de calidad instrumentales, como por ejemplo las propiedades mecánicas de los productos horneados y la estructura de estos [2,3]. No obstante, la evaluación del aspecto sigue realizándose mayoritariamente de manera manual por profesionales entrenados, lo cual es costoso y subjetivo. La cuantificación mediante el análisis digital de imagen (ADI) ofrece la posibilidad de caracterizar de forma objetiva la apreciación visual característica de cada producto[4], pudiendo incluso automatizar el análisis.

En los últimos años ha habido importantes avances para el procesado de imágenes digitales, incluso existen equipos desarrollados específicamente para caracterizar la estructura interna de este tipo de productos. Un ejemplo es el equipo C-Cell (Calibre Control International Ltd., Warrington, Reino Unido), capaz de cuantificar el número, tamaño y forma de los alveolos de la estructura interna realizando análisis en imágenes 3D. Sin embargo, existen numerosas formas de adquisición de imágenes digitales susceptibles de ser analizadas (cámaras digitales, microscopios, escáneres, etc.), con las que obtener imágenes 2D en las que poder realizar la cuantificación de los elementos a medir y que están fuertemente correlacionados con los resultados obtenidos en imágenes 3D[5], y por otro lado es necesaria la optimización de las condiciones de análisis de las imágenes. En los últimos años se han desarrollado diversos programas informáticos específicos para el ADI. En particular, el programa ImageJ [6] es muy versátil y de licencia abierta. Este permite desde la modificación y mejora de la imagen hasta la cuantificación de los distintos elementos que se pretenden medir.

Para poder cuantificar los elementos de una imagen es necesario segmentar la imagen digital, es decir, extraer la información que se quiere cuantificar de los datos totales que tiene la imagen. Es posible realizar la segmentación de distintas formas, siendo una de las más comunes el

establecimiento de un umbral a partir del cual los pixeles de la imagen serán considerados objetos. Esto se hace utilizando diferentes algoritmos matemáticos capaces de calcular el valor umbral (t) o bien estableciéndolo de manera manual gracias al histograma generado a partir de los pixeles de la imagen[7,8]. En los productos derivados de cereales establecer dicho umbral resulta complejo debido principalmente a las características de su estructura interna. Por ello es fundamental identificar los parámetros de definición de las imágenes previo a su análisis. Los parámetros que influyen principalmente en la calidad de la imagen binaria obtenida para poder realizar el ADI son: el equipo utilizado en la captura, la resolución, y la profundidad de color. Asimismo, es posible modificar dichos parámetros escogiendo la mejor combinación de estos para la posterior cuantificación.

Los productos derivados de cereales habitualmente se producen con fórmulas complejas que pueden incluir un amplio intervalo de ingredientes, aditivos y coadyuvantes (emulgentes, hidrocoloides, enzimas, etc.). Entre los ingredientes, el uso de masas madre se ha recomendado para mejorar las características sensoriales y retrasar el envejecimiento[11,12]. Los aditivos y coadyuvantes tecnológicos contribuyen a conseguir uniformidad en los procesos, asegurar una calidad constante o preservar la frescura de los alimentos[9,10]. Muchos de estos aditivos y coadyuvantes son utilizados en productos de panificación sin gluten con el fin de superar los problemas que la ausencia de gluten provoca sobre la calidad tecnológica o nutricional. Los agentes estructurantes como goma guar, goma xantana, hidroxipropilmetilcelulosa (HPMC), agar y almidones de maíz, yuca o patata se han utilizado para superar los problemas asociados a la ausencia de la viscoelasticidad de las masas sin gluten [13]. Otra de las alternativas aplicadas para mitigar este problema es la adición de proteínas (albúmina de huevo, proteína de soja, guisante o suero de leche, entre otras), estas se han utilizado para mejorar la incorporación de aire y el mantenimiento de la estructura durante la fermentación, además de incrementar el valor nutricional de los productos sin gluten [14,15]. La búsqueda de nuevos ingredientes, aditivos o coadyuvantes sigue siendo un reto para la industria y la investigación. No solo se buscan nuevos aditivos capaces de mejorar tecnológica o nutricionalmente los productos, sino también conseguir nuevos productos que sean atractivos para los consumidores.

Algunas veces los posibles candidatos a convertirse en potenciales ingredientes en panificación son ingredientes utilizados habitualmente en otro tipo de matrices y disponibles en el mercado[16]. Sin embargo, el mayor reto suele encontrarse en productos novedosos como componentes extraídos individualmente, materias primas, etc.[17,18] que se encuentran en cantidades muy limitadas. Por lo que el desarrollo de metodologías de panificación adaptadas a cantidades mínimas resulta de gran interés en investigación y en desarrollos tecnológicos. Algunos estudios se han realizado limitando la cantidad de masa y obteniendo productos más pequeños de lo encontrado normalmente en el mercado o marcado en las normas estándares de panificación[18]. Otra tendencia para la innovación en la industria de la panificación es la obtención de productos horneados sin incluir aditivos en su formulación es la modificación del proceso. El aumento de la tendencia de etiquetas limpias ("clean label"), ha hecho que haya aumentado el interés por la modificación del proceso para mejorar los productos sin el uso de aditivos. En este contexto resulta fundamental realizar el seguimiento de los cambios estructurales que se producen durante el proceso de panificación asociados a la incorporación de aire y crecimiento de núcleos de gas[19] y su impacto sobre las características finales del producto como el volumen o la textura[20].

Por lo general se utilizan métodos de análisis destructivos para estudiar el efecto de ingredientes y coadyuvantes o los cambios en el proceso de panificación. El análisis de imagen es una técnica no destructiva que permitiría reflejar los cambios estructurales provocados por formulaciones o procesos. De hecho, se ha observado un creciente interés por la utilización del análisis digital de imagen para cuantificar los cambios estructurales ocurridos en productos con distintas formulaciones [21-24] o procesos [25-28]. Sin embargo, en la literatura científica no existe un consenso sobre la metodología a utilizar que permita homogeneizar el análisis y que posibilite la comparación de los resultados. Por ello, el objetivo de este estudio fue evaluar y definir las condiciones para mejorar la captura y el procesado de imagen para la posterior cuantificación de los parámetros estructurales de la miga de productos horneados. Para abordar dicho objetivo el Capítulo 1 se dividió en 3 secciones:

- Sección I: Estudio del impacto de la resolución de captura y el tipo de algoritmo en el análisis de la distribución alveolar del pan de trigo y el pan sin gluten.
- Sección II: Validación del ADI para cuantificar diferencias en la distribución alveolar de las migas de pan (con y sin gluten).
- Sección III: Puesta a punto y validación de la obtención de panes a pequeña escala (minipanes).

I. Estudio del impacto de la resolución de captura y el tipo de algoritmo en el análisis de la distribución alveolar del pan de trigo y el pan sin gluten.

Las condiciones de captura y el procesado de la imagen influyen en el ADI, por ello el objetivo de esta sección fue estudiar el impacto de la resolución seleccionada durante la captura de la imagen, así como el tipo de algoritmo utilizado para la transformación en imagen binaria en pan de molde con y sin gluten.

Material y métodos

Impacto de la resolución en el análisis digital de imagen

En un primer lugar se definió la mejor resolución para dos tipos de pan con estructura diversa. Para ello, se adquirieron 2 tipos de panes de molde comerciales, un pan de trigo tradicional (T) y otro sin gluten (SG). Se escaneó la imagen de dos rebanadas de cada uno de ellos mediante un escáner plano HP SCANJET 2200C (Hewlett-Packard Co, Colorado, EE. UU.), utilizando diferentes niveles de pixeles por puntos (600-150 ppp) y se guardaron en formato "tiff".

Las imágenes fueron analizadas con el programa de licencia abierta ImageJ (National Institutes of Health, Bethesda, EE. UU.). Para ello la imagen se recortó, seleccionando una sección de 50x50 mm del centro de la rebanada escaneada, intentando abarcar la mayor área posible de la miga sin incluir corteza. Finalmente, las imágenes fueron convertidas a 8-bits, y se aplicó el algoritmo "Otsu" tal como recomiendan Gonzales-Barron y

Butler[29] para establecer el umbral de conversión de la imagen binaria. Posteriormente se erosionó la imagen una vez (eliminación de 1 pixel en cada uno de los bordes detectados), para eliminar el posible ruido ocasionado durante la captura. Finalmente, se cuantificaron los objetos excluyendo los alveolos truncados en los bordes de la imagen. Los parámetros registrados fueron: nº de alveolos por cm^2, área media de los alveolos (cm^2), porosidad (definida como el porcentaje de alveolos frente al área total analizada) y circularidad (1-círculo perfecto, 0-cuadrado perfecto). El análisis se realizó por duplicado para cada resolución y tipo de pan.

Impacto del algoritmo seleccionado para segmentar la imagen digital

Una vez seleccionada la resolución más fiel a la estructura de la miga estudiada, tanto en pan de trigo como en pan libre de gluten, se aplicaron diferentes algoritmos, predefinidos en el programa, con el fin de comprender el impacto de estos sobre los resultados y definir el umbral que mejor se ajustara a la imagen. El análisis de imagen se llevó a cabo como se ha descrito previamente y anotando además el umbral (t) calculado automáticamente en cada uno de los algoritmos probados. Los algoritmos predefinidos seleccionados en el estudio fueron: "Shanbhag[30], Moments[31], Mean[32], Li[33], Huang[34], IsoData[35], Percentile[36] y Otsu[37]".

Análisis de los resultados

Se realizó un análisis de la varianza multifactorial para cada uno de los parámetros estudiados mediante el software Statgraphics Centurion XVII versión 17.2 (Maryland, EE. UU.). Se consideraron como factores el tipo de pan y la resolución de la captura de la imagen.

Resultados y discusión

Impacto de la resolución en el análisis digital de imagen

La miga de pan de molde de trigo seleccionado se caracterizó por tener una porosidad elevada y con una estructura de miga homogénea (Figura 1), con un alveolado bien distribuido y pequeño representativo de este tipo de productos[38]. El pan sin gluten escogido presentó una miga

heterogénea, presentando alveolos de mayor y menor tamaño distribuidos de forma arbitraria. El pan sin gluten generalmente presenta estructuras muy diversas y menos uniformes que el pan de trigo tradicional[39]. A nivel visual, la resolución no afectó a la conversión de la imagen a binaria, siendo similares independientemente de los puntos por píxel utilizados en la captura. Sin embargo, al procesar la imagen binaria aplicando la erosión, la resolución a la que la imagen fue capturada afectó a las imágenes obtenidas. Es decir, el aumento de la resolución permitió definir mejor los contornos de los alveolos debido a la mayor precisión definida durante la captura.

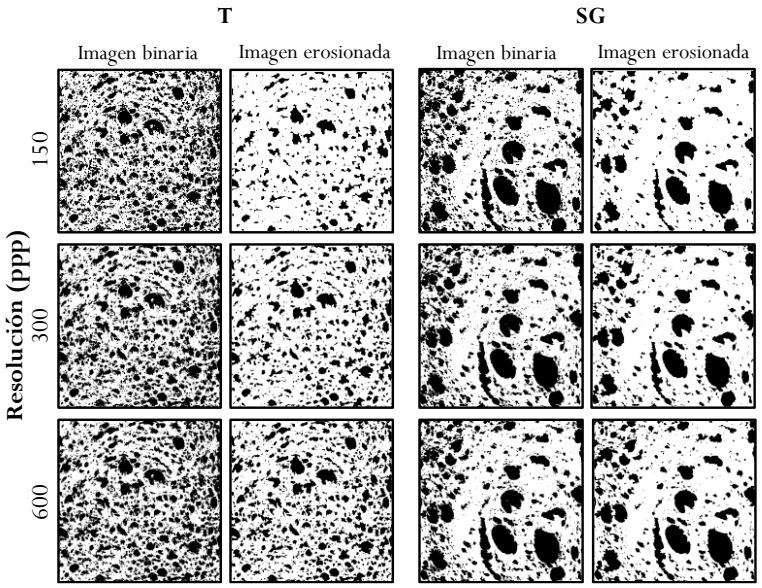

Figura 1. Imagen binaria y tras la erosión de sección de miga de pan de molde de trigo (T) y pan de molde sin gluten (SG).

La resolución afectó significativamente ($P<0,05$) en todos los parámetros de la estructura tanto en el pan de molde de trigo, como en el pan de molde sin gluten (Tabla 1). Sin embargo, el tipo de pan solo afectó significativamente a la circularidad. El recuento de alveolos por cm^2 aumentó a medida que se aumentó la resolución de la imagen en ambos tipos de pan, debido a la mejora en la definición de los contornos de estos y a la detección de alveolos de menor tamaño. Del mismo modo, se observó una disminución progresiva del área media de los alveolos y un

aumento de la porosidad. Farrera-Rebollo[7] encontraron resultados similares al analizar la miga de pan dulce, demostrando que el aumento de la resolución en la captura de la imagen permitió detectar un mayor número de alveolos de menor tamaño. Por otra parte, la circularidad disminuyó con la resolución, lo cual pudo atribuirse a que, a resoluciones bajas, el programa utilizado para el ADI detecta estructuras más redondeadas.

Tabla 1. Caracterización de la estructura de la miga de pan de molde de trigo (T) y pan de molde sin gluten (SG), capturada con distintas resoluciones.

Pan	Resolución (ppp)	Alveolos /cm2	Área media alveolo (mm2)	Porosidad (%)	Circularidad
T	**150**	5 ± 1^{e}	2,93 ± 0,79^{a}	14,22 ± 1,12^{d}	0,59 ± 0,00^{c}
	300	13 ± 0^{bc}	1,80 ± 0,11^{bc}	22,59 ± 0,77^{bc}	0,53 ± 0,01^{d}
	600	28 ± 2^{a}	1,06 ± 0,07^{c}	29,22 ± 0,26^{a}	0,49 ± 0,00^{e}
SG	**150**	8 ± 0^{d}	2,16 ± 0,39^{ab}	16,93 ± 2,33^{cd}	0,76 ± 0,01^{a}
	300	12 ± 0^{c}	1,85 ± 0,35^{bc}	21,28 ± 3,24^{bc}	0,63 ± 0,01^{b}
	600	14 ± 0^{b}	1,66 ± 0,22^{bc}	23,61 ± 3,87^{ab}	0,53 ± 0,02^{d}
Valor de P					
	T o SG	0,1389	0,8913	0,4533	**0,0014**
	Resolución	**0,0007**	**0,0099**	**0,0010**	**0,0002**

* letras diferentes dentro de columnas indican diferencia estadística (LSD<0,05)

Impacto del algoritmo seleccionado para segmentar la imagen digital

Para comprobar la influencia del algoritmo seleccionado se utilizaron las imágenes de mayor resolución (600 ppp) al permitir mejorar la detección de alveolos de menor tamaño. El umbral aplicado por cada algoritmo (t) fue diferente dependiendo del color inicial de la imagen, es decir de la rebanada, pero se mantuvo constante en las réplicas dentro del mismo tipo de pan (Figura 2 y 3). En el caso de la muestra T, el umbral varió entre 192 y 200, sin embargo, en SG la variación fue mayor encontrando valores de umbral desde 186 hasta 208. Probablemente, estas diferencias son debidas a la menor uniformidad de la miga descrita en el anterior punto, que influye en el cálculo del umbral, y hace que las diferencias

sean más notables al aplicar uno u otro algoritmo. De hecho, a partir de la inspección visual de las imágenes se observó que en el caso de la muestra SG, a partir de t=200 (Mean, Huang y Percentile), las imágenes mostraron mayor fracción de aire (en negro) en la imagen binaria, lo que podría indicar que estos algoritmos sobreestimaron la fracción vacía (es decir de alveolado).

Una vez eliminada la variación en la cuantificación debida a la resolución, el ADI permitió discriminar de forma estadísticamente significativa ($P<0,05$) entre las dos estructuras de miga en todos los parámetros analizados (Tabla 2). Sin embargo, no se encontraron tendencias en cuanto al tipo de algoritmo, debido probablemente a las diferencias en el umbral establecido por cada uno de ellos dependiendo del tipo de muestra analizada. En el caso de la muestra de pan de molde de trigo a medida que se aumentó el valor de t en la aplicación del umbral (Figuras 2 y 3) se observó una tendencia a disminuir tanto el número de alveolos como la circularidad de estos. Por el contrario, las áreas y porosidad de la muestra aumentaron. En el caso de el pan de molde sin gluten, la tendencia fue inversa, el número de alveolos por cm^2 aumentó al aumentar el umbral, y el área disminuyó, no encontrando tendencias en la porosidad o la circularidad. Gonzales-Barron & Butler[29] observaron que algunos de los algoritmos estudiados, entre ellos IsoData, fueron sensibles a la presencia de grandes áreas oscuras, debido a que la forma de cálculo del umbral en este algoritmo se ve afectado por los cambios que se producen en el histograma al encontrarse con alveolos de gran tamaño, pudiendo ser la explicación a la diferencia en la cuantificación del número de alveolos en los panes sin gluten.

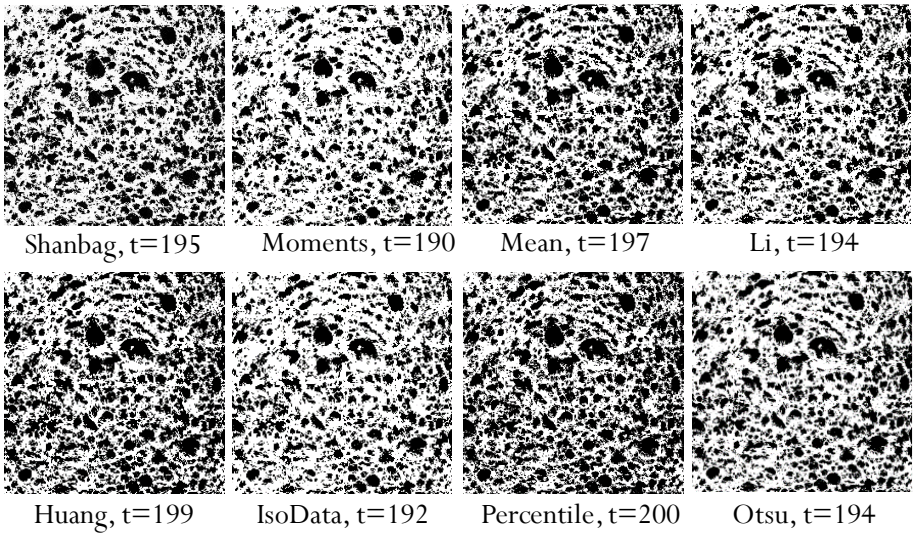

Figura 2. Imágenes binarias tras la aplicación de distintos algoritmos en la sección de miga de pan de molde de trigo.

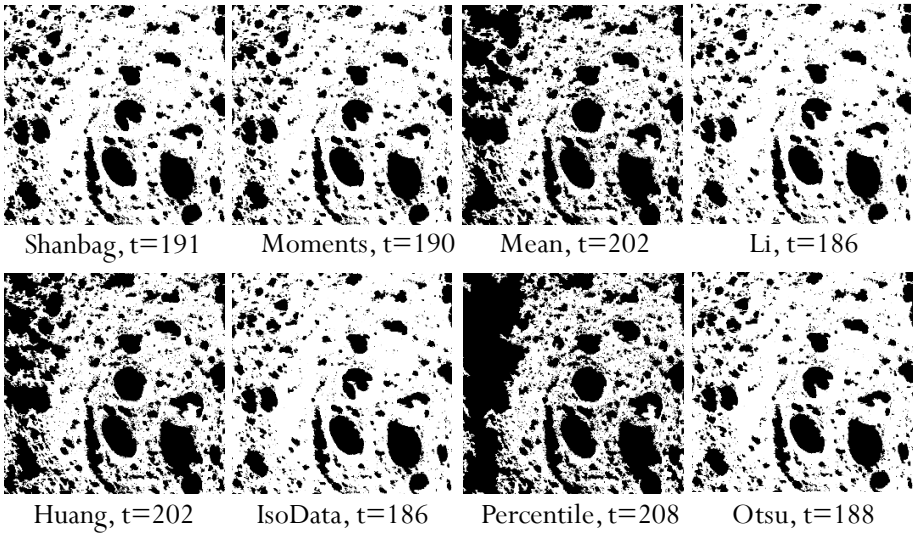

Figura 3. Imágenes binarias tras la aplicación de distintos algoritmos en la sección de miga de pan de molde sin gluten.

Tabla 2. ADI de pan de molde de trigo y pan de molde sin gluten utilizando diferentes algoritmos para crear la imagen binaria.

Pan	Algoritmo (t umbral)	Alveolos /cm2	Área media alveolo (mm2)	Porosidad (%)	Circularidad
T	Shanbhag (195)	30 ± 5	1,08 ± 0,13	32,00 ± 1,47	0,47 ± 0,02
	Moments (190)	32 ± 6	0,83 ± 0,05	26,41 ± 3,52	0,49 ± 0,02
	Mean (197)	29 ± 4	1,21 ± 0,16	34,22 ± 0,19	0,47 ± 0,02
	Li (194)	30 ± 5	1,05 ± 0,09	31,23 ± 2,57	0,47 ± 0,02
	Huang (199)	27 ± 3	1,31 ± 0,34	34,98 ± 4,79	0,46 ± 0,02
	IsoData (192)	29 ± 1	0,90 ± 0,15	27,77 ± 1,60	0,48 ± 0,01
	Percentile (200)	25 ± 4	1,47 ± 0,45	36,37 ± 5,62	0,46 ± 0,01
	Otsu (194)	28 ± 2	1,06 ± 0,07	29,22 ± 0,26	0,49 ± 0,00
SG	Shanbhag (191)	16 ± 1	1,44 ± 0,28	21,23 ± 0,57	0,42 ± 0,02
	Moments (190)	15 ± 2	1,58 ± 0,48	20,72 ± 0,23	0,45 ± 0,01
	Mean (202)	20 ± 3	1,07 ± 0,15	21,89 ± 5,66	0,40 ± 0,01
	Li (186)	12 ± 3	1,60 ± 0,36	17,43 ± 0,95	0,43 ± 0,01
	Huang (202)	20 ± 3	1,11 ± 0,12	21,14 ± 3,24	0,41 ± 0,02
	IsoData (186)	12 ± 3	1,60 ± 0,36	17,93 ± 0,24	0,43 ± 0,01
	Percentile (208)	20 ± 1	0,99 ± 0,04	19,88 ± 1,37	0,39 ± 0,02
	Otsu (188)	14 ± 0	1,66 ± 0,22	23,61 ± 3,87	0,53 ± 0,02
	Valor de *P*				
	T o SG	**0,0000**	**0,0349**	**0,0000**	**0,0000**
	Algoritmo	0,4665	0,9872	0,1530	**0,0021**

II. Validación del ADI para cuantificar diferencias en la distribución alveolar de las migas de pan (con y sin gluten) con distintos ingredientes/aditivos en su formulación.

El objetivo de la Sección II fue el validar la capacidad de discriminación entre estructuras de las migas de pan con diferentes formulaciones en panes con y sin gluten.

Materiales y métodos

Validación en panes con gluten

Se produjeron 14 panes variando los ingredientes o aditivos utilizados en su formulación (control, hidrocoloides, masas madre, emulgentes y enzimas). Los ingredientes/aditivos utilizados fueron:

- Hidrocoloides: hidroxypropilmetilcelulosa K4M (HPMC) y carboximetilcelulosa (CMC).
- Emulgentes: Ester diacetiltartárico de los monoglicéridos de los ácidos grasos (DATEM), estearoil lactilato de sodio (SSL), lecitina de girasol desgrasado (Lecitina), esteres de poliglicerol de los ácidos grasos (PGEF).
- Enzimas: dos lipasas (Lipopan XTRA BG y Lipopan 50 BG) y una lipasa con alfa amilasa (Sensea).
- Masas madre: de trigo (BÖKER W200), de centeno (BÖKER W350), de trigo y malta (BÖKER POLISH) y de germen de trigo (BÖKER GERME).

En la panificación se utilizó una receta básica: 0,7% de levadura seca, 1,5% de sal, 90% de agua en base harina, y la cantidad de aditivo o masa madre recomendada por el distribuidor (base harina): HPMC y CMC 1%, DATEM 2,75%, SSL y Lecitina 1%, PGEF 0,5%, Lipopan XTRA BG y 50 BG 0,003%, Sensea 0,006, BÖCKER W200 2,75%, BÖKER W350 3%, BÖKER POLISH y GERME 3,5%. Todos los ingredientes se mezclaron durante 8 minutos a 150 rpm en un agitador de laboratorio IKA 40. Con ayuda de una manga pastelera, se colocaron 500 g de masa en cada molde y se fermentaron durante 60 min a 35 °C. Finalmente, se

hornearon durante 40 min en un horno eléctrico precalentado a 185 ºC y se dejaron enfriar durante 60 min a temperatura ambiente.

Una vez fríos, los panes se rebanaron y la rebanada central se escaneó a 300 ppp de resolución. Las imágenes se analizaron con el programa ImageJ. En primer lugar, se aumentó el contraste de las imágenes y posteriormente se aplicó el algoritmo Otsu (t=192) para finalmente cuantificar la estructura de la miga (alveolos/cm^2, área media de los alveolos (mm^2) y circularidad).

Validación en panes sin gluten

Los materiales utilizados para la validación en pan sin gluten fueron: harina de arroz comercial (Harinera Belenguer, Valencia, España), almidón de maíz (Huici Leidan, Pamplona, Navarra), fibra de avena (Canadian Harvest® Oat Fiber-300, Sunopta Ingredients Group, Massachusetts, USA), almidón resistente HI-MAIZE® 260 (National Starch Food Innovation, New Jersey, USA). proteína de guisante (Roquette Frères, Lestrem, Francia), huevo en polvo (EPSA, Valencia, España), HPMC K4M (Dow Chemical, Pittsburg, EE. UU.), goma xantana (Jungbunzlauer, Ladenburg, Alemania), goma guar (Quimidroga, Barcelona, España) y UltraCel™ (Watson, West Haven, EE. UU.). Aceite vegetal de semillas, levadura prensada, azúcar y sal del supermercado local.

Para determinar el posible efecto de los hidrocoloides y las proteínas en la estructura de la miga de pan sin gluten, se seleccionaron seis formulaciones combinando proteínas de diferente origen y cada uno de los hidrocoloides elegidos (Tabla 3). La cantidad de agua utilizada fue del 201,5% en base harina.

Tabla 3. Combinaciones seleccionadas para el estudio.

Muestra	HPMC	Mezcla g.xantana y g.guar	Ultracel™	Proteína de guisante	Huevo en polvo
S1	x			x	
S2		x		x	
S3			x	x	
S4	x				x
S5		x			x
S6			x		x

El proceso de panificación consistió en amasar durante 10 min (4 min a baja velocidad y 6 min a velocidad rápida) en una amasadora Hobart N50. Posteriormente, 800 g de masa se colocaron en un molde (20 x 6,9 cm) y se fermentaron durante 50 min a 30 °C, con una humedad relativa del 90%. Los panes se hornearon en un horno eléctrico durante 90 min a 160 °C y se dejaron enfriar durante una hora hasta su caracterización. El análisis digital de imagen se llevó a cabo igual que en la validación de panes con gluten. Además, se utilizó el ADI para medir la morfogeometría de cada una de las muestras, registrando el ancho, alto, área 2D y perímetro.

Análisis de resultados

Los resultados se expresaron como los valores medios. Para cada parámetro, se realizó un análisis de la varianza (ANOVA) usando el programa estadístico Statgraphics Centurion XVII versión 17.2 (Maryland, EE. UU.). Se llevó a cabo el test LSD para establecer las diferencias significativas ($P<0,05$) entre las muestras y poder discriminar entre ellas.

Resultados y discusión

Impacto de la adición de ingredientes/aditivos en la estructura de la miga de pan de molde de trigo.

Las migas mostraron gran variabilidad dependiendo del ingrediente o aditivo utilizado (Figura 4 y Tabla 4). La adición de hidrocoloides (HPMC y CMC) mostraron migas con una estructura más cerrada, con mayor número de alveolos, pero reduciéndose hasta un 59% su área media. Los resultados concuerdan con lo descrito por Das, Raychaudhuri y Chakraborty[40] que atribuyeron estos cambios a la interacción de los hidrocoloides con la matriz almidón-gluten, que favorece una mejor distribución de la inclusión de aire durante el proceso. Las tres enzimas estudiadas aumentaron el número de alveolos y el tamaño de estos, a excepción de la enzima LIPOPAN EXTRA BG que mostró un tamaño de alveolo similar al control. No obstante, fueron las que mostraron mayor efecto en la circularidad, mostrando unos alveolos más redondos (0,647-0,689) que el control (0,512). El efecto esperado de la adición de emulgentes es la obtención de una miga más fina y cerrada,

incrementando la uniformidad de los alveolos [41]. Sin embargo, el análisis reveló efectos dispares dependiendo del emulgente adicionado. El emulgente DATEM y la lecitina de girasol produjeron alveolos de menor tamaño, originando migas más cerradas. El SSL no modificó la estructura alveolar, aunque si disminuyó la circularidad y el PGEF disminuyó el número de alveolos y aumentó su área media, además la estructura fue menos uniforme. A nivel visual, las masas madres originaron migas más cerradas y uniformes que el control. Este mismo efecto ha sido descrito por otros autores que cuantificaron el efecto de la utilización de masa madre en pan de trigo[11]. Sin embargo, al comprobar los datos del ADI (Tabla 4) el efecto observado no fue el esperado ya que presentaron un número de alveolos similar o ligeramente mayor que el control. Como se ha visto en la Sección 1, el umbral (valor de t) se modifica dependiendo del color inicial de la imagen, es decir, la miga analizada. En el presente estudio se estableció un umbral fijo para todas las muestras, lo que impidió la mejor cuantificación de la estructura de los panes con masa madre en su formulación, aunque si se pudieron cuantificar las diferencias en el resto de las estructuras.

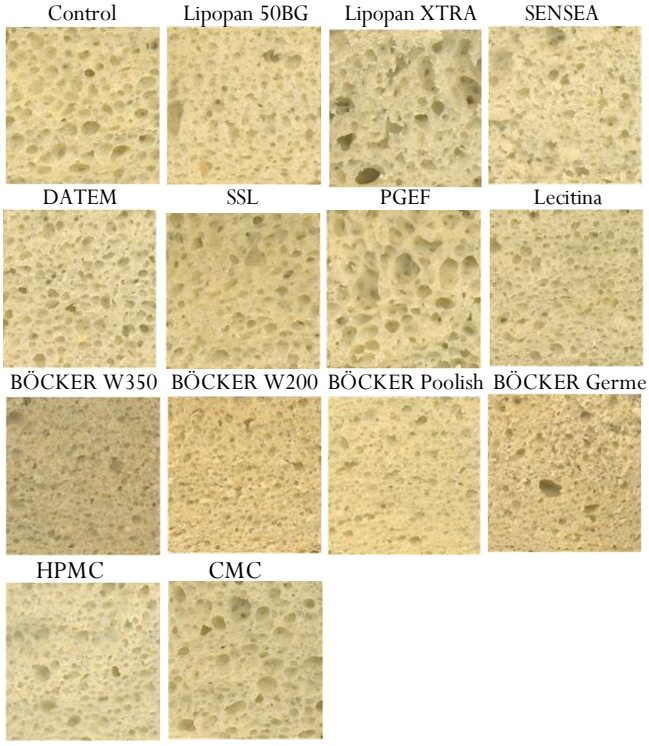

Figura 4. Imágenes escaneadas de las migas de las muestras de pan.

Tabla 4. ADI imágenes de las migas de las muestras con distintos ingredientes/aditivos.

Muestra	Alveolo/cm2	Área media (cm2)	Circularidad
Control	9^{gh}	$2,46^{c}$	$0,512^{d}$
DATEM	12^{ef}	$2,00^{de}$	$0,468^{ef}$
SSL	10^{fg}	$2,32^{cd}$	$0,428^{fg}$
PGEF	7^{i}	$3,93^{a}$	$0,417^{g}$
Lecitina	28^{a}	$0,77^{i}$	$0,559^{c}$
HPMC	23^{bc}	$1,01^{hi}$	$0,570^{c}$
CMC	19^{d}	$1,16^{gh}$	$0,528^{cd}$
LIPOPAN 50 BG	24^{b}	$1,14^{gh}$	$0,689^{a}$
LIPOPAN XTRA	13^{e}	$2,41^{c}$	$0,638^{b}$
SENSEA	21^{cd}	$1,39^{f}$	$0,647^{ab}$
BÖCKER W350	11^{efg}	$2,90^{b}$	$0,420^{g}$
BÖCKER W200	13^{e}	$2,19^{cde}$	$0,431^{fg}$
BÖCKER Poolish	11^{efg}	$1,87^{e}$	$0,502^{de}$
BÖCKER Germe	12^{ef}	$2,46^{c}$	$0,453^{fg}$

Impacto de la adición de los cambios en la formulación en la estructura de la miga de pan de molde sin gluten.

Las combinaciones propuestas mostraron diferentes morfologías y estructuras (Figura 5). La miga de la muestra S1 se desgarró en la parte superior de la rebanada, debido a que la estructura no pudo contener todo el gas liberado durante la fermentación. Además, las muestras S1, S2 y S3, que contenían proteína de origen vegetal en su formulación, mostraron una estructura de miga más abierta y menos uniforme. Por el contrario, se observó que a nivel visual las migas de las muestras S4, S5 y S6 presentaron una distribución alveolar más homogénea, más semejantes a las obtenidas en el pan de trigo. Esto pudo deberse a la presencia de proteína de huevo, que destaca por sus propiedades emulsionantes.

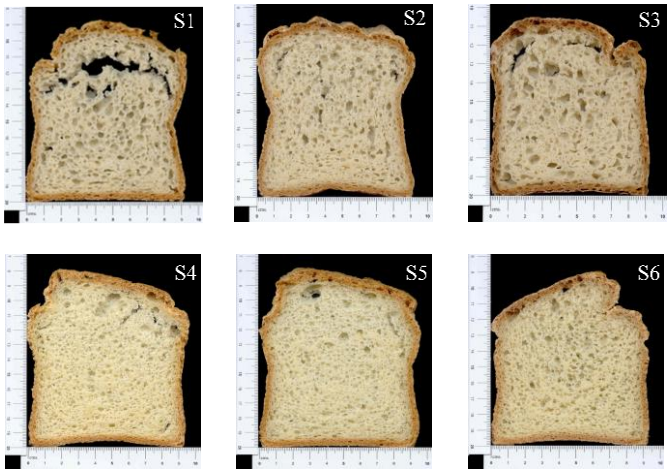

Figura 5. Imágenes de las rebanadas de pan libre de gluten obtenidas con las diferentes formulaciones.

Se utilizó el ADI para realizar el análisis morfogeométrico de las rebanadas (Tabla 5). Los resultados obtenidos reflejaron diferencias significativas principalmente en el ancho, alto y perímetro. Las muestras S3 y S6 mostraron valores de área más bajos, siendo ambas muestras las que contenían Ultracel™ como hidrocoloide. Sin embargo, las muestras S1 y S4 que contenían HPMC como hidrocoloide son las que originaron mayor área. Estos resultados concuerdan con los encontrado por Bárcenas y Rosell[42] que observaron un incremento en el volumen del pan cuando se incluía HPMC en panes de trigo. El efecto de HPMC puede ser atribuido a su habilidad para retener agua y su contribución en la formación de la red de gluten, que incrementa la viscosidad y permite expandir los núcleos de gas de la masa aumentando la retención de gas durante el horneado[40].

Tabla 5. Análisis morfogeométrico de las rebanadas de pan sin gluten.

Muestras	Ancho (cm)	Alto (cm)	Área 2D (cm^2)	Perímetro (cm)
S1	$9,26^{ab}$	$10,61^{c}$	$85,89^{c}$	$37,57^{b}$
S2	$9,33^{ab}$	$10,13^{ab}$	$81,84^{abc}$	$37,02^{ab}$
S3	$9,10^{a}$	$10,09^{ab}$	$80,05^{ab}$	$35,83^{a}$
S4	$9,65^{c}$	$10,31^{bc}$	$84,97^{bc}$	$37,54^{b}$
S5	$9,35^{b}$	$10,28^{abc}$	$83,57^{abc}$	$36,18^{ab}$
S6	$9,28^{ab}$	$9,92^{a}$	$79,61^{a}$	$35,76^{a}$

Los parámetros del ADI del pan sin gluten revelaron una gran variabilidad entre las estructuras de las rebanadas (Tabla 6). La muestra S4 y S5, presentaron mayor número de alveolos por cm^2, ambas muestras contenían proteína de origen animal que, como se ha descrito anteriormente, generaron migas con un estructura más uniforme y cerrada que las muestras con proteína de origen vegetal. Se observaron diferencias significativas para el área media de los alveolos, con valores comprendidos entre 0,60 y 2,19 mm^2. Los valores de circularidad variaron de 0,78 a 0,84, lo que indicó su divergencia hacia el círculo perfecto, aunque no se originaron diferencias estadísticamente significativas destacables entre las muestras.

Tabla 6. ADI de la estructura de la miga de los diferentes panes sin gluten.

Muestras	Alveolos/cm^2	Área media alveolo (mm2)	Circularidad
S1	9^{a}	$2,19^{d}$	$0,79^{ab}$
S2	14^{ab}	$1,48^{bc}$	$0,84^{b}$
S3	9^{a}	$1,66^{c}$	$0,83^{ab}$
S4	19^{bc}	$1,12^{ab}$	$0,82^{ab}$
S5	24^{c}	$0,98^{ab}$	$0,82^{ab}$
S6	13^{a}	$1,53^{bc}$	$0,78^{a}$

Para cada parámetro, valores seguidos de letras iguales indica que no hay diferencias estadísticamente significativas ($P<0,05$)

III. Puesta a punto y validación de la obtención de panes a pequeña escala (minipanes).

La panificación a pequeña escala puede ser una herramienta muy útil en investigación cuando se necesita constatar el efecto de un cambio en el proceso o la formulación y no se dispone de equipos o cantidades a gran escala. No obstante, es necesario optimizar el proceso y la caracterización para que los resultados de caracterización sean extrapolables independientemente del escalado utilizado. Por eso el objetivo de la Sección 3 fue la puesta a punto de la obtención y caracterización de panes a pequeña escala, así como comprobar su eficacia para poder identificar

las diferentes estructuras generadas en las migas al introducir cambios en el proceso.

Material y Métodos

Puesta a punto de la metodología y la caracterización

Se seleccionó una receta básica en base harina (harina de trigo, La Meta, Lleida, España) de pan: levadura seca (0,6%), sal (1,5%), azúcar (1%) y un 55% de agua. Se amasó durante 11 min (1 min lento y 10 min rápido) y se dividió la masa en tres porciones de distinto peso (500 g, 50 g y 4 g de masa). La masa se colocó en moldes ajustados a la cantidad de masa. Tanto la fermentación como la cocción fueron adaptadas para cada masa, con el fin de obtener panes con un nivel de fermentación y cocción similar. El seguimiento de la fermentación se realizó con ayuda de una probeta en la que se fue registrando el incremento de volumen de la masa. Para ello, se ajustó el diámetro de la probeta utilizada para el seguimiento en relación con la altura inicial de la masa, para que en todos los casos fuera el mismo volumen inicial.

Los panes se caracterizaron atendiendo a la textura y la estructura de la miga. Para ello, fueron cortados en rebanadas de 10 mm de grosor usando una rebanadora automática. La textura se analizó utilizando un texturómetro TA.XT.Plus (Stable Micro Systems, Surrey, Reino Unido) aplicando el test TPA con una doble compresión (hasta el 50%) a una velocidad de ensayo de 1 mm/s. El diámetro de la sonda utilizada se modificó dependiendo del área de la rebanada. Los parámetros seleccionados para caracterizar la textura fueron: dureza, elasticidad recuperable retardada, cohesividad, masticabilidad y velocidad de recuperación instantánea. El ADI de la estructura de la miga se realizó escaneando las rebanadas a 600 ppp de resolución. La sección capturada se adaptó atendiendo al tamaño de la rebanada, intentando capturar la mayor parte del área de la miga. Las imágenes se analizaron usando el programa ImageJ, utilizando el algoritmo predefinido “Otsu” para establecer el umbral. Se registró el tamaño de la sección cogida en cada muestra, el recuento total de alveolos, alveolos por cm^2, el área media y la circularidad de los alveolos. Las muestras están referidas a la cantidad inicial de masa.

Validación de la producción de panes a pequeña escala para identificar los cambios en la estructura de la miga de panes de trigo con distinta velocidad de amasado

Caracterización de la consistencia de las masas obtenidas con distinta velocidad de amasado

Se seleccionaron 6 velocidades de amasado: 50, 80, 100, 150, 200 y 240 rpm. Las condiciones de amasado se establecieron y registraron utilizando el equipo Mixolab (Chopin, París, Francia). El protocolo utilizado fue el Chopin + adaptado. Este protocolo consistió en un amasado (a la velocidad seleccionada), con una primera etapa a 30 ºC hasta que se produjo una caída del 10% de la máxima consistencia durante el amasado (C1) (Figura 6). Seguidamente se prosiguió con una etapa de calentamiento en la cual se incrementó la temperatura hasta 90 ºC. En esta etapa se registró la consistencia mínima (C2) asociada a la desnaturalización de las proteínas y la consistencia máxima producida tras la gelatinización del almidón (C3). Se mantuvo la temperatura a 90 ºC durante 7 min. Se prosiguió con la etapa de enfriamiento hasta los 50 ºC, donde se mantuvo el amasado durante 5 min. En esta última etapa se registraron C4 (estabilidad del gel de almidón formado) y C5 (retrogradación del almidón).

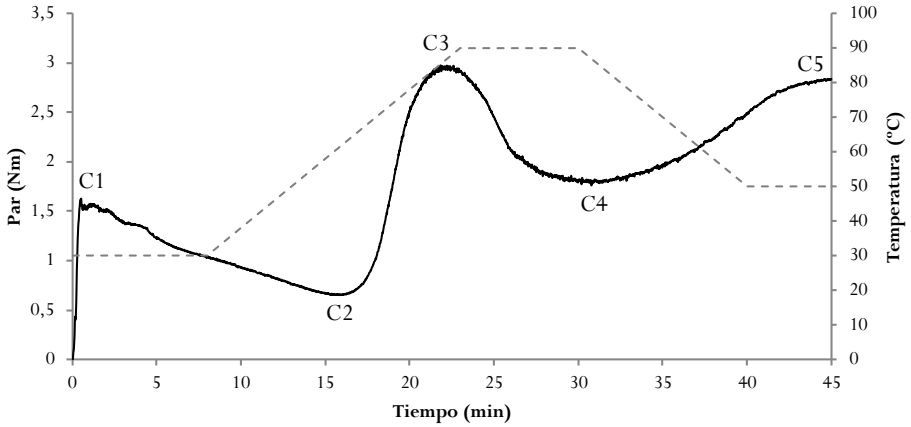

Figura 6. Perfil típico del registro durante el ciclo de calentamiento y enfriamiento con el Mixolab (Protocolo: Chopin+).

Panificación

La formulación utilizada para el estudio fue la descrita previamente en la puesta a punto de la metodología. Se amasó en el equipo Mixolab el tiempo requerido para alcanzar C1. Posteriormente se colocaron 4 g de masa en los moldes (2 cm de diámetro y 3,5 cm de alto) y se fermentaron durante 40 min a 30 °C. Finalmente se hornearon durante 11 min a 130 °C (condiciones establecidas en la puesta a punto de la metodología) y se enfriaron a temperatura ambiente fuera del molde durante 1 h. En los panes obtenidos se determinó la dureza y la estructura de la miga según la metodología previamente descrita.

Resultados

Puesta a punto de la obtención de panes a pequeña escala

El ajuste en la relación entre el diámetro de la probeta y la altura de la masa durante la etapa de fermentación permitió realizar igualar la evolución de la fermentación, independientemente de la cantidad de la masa (Figura 7). De forma similar se tuvo que ajustar los parámetros de cocción para obtener migas similares, reduciendo el tiempo y la temperatura al reducir la cantidad de masa (Tabla 7).

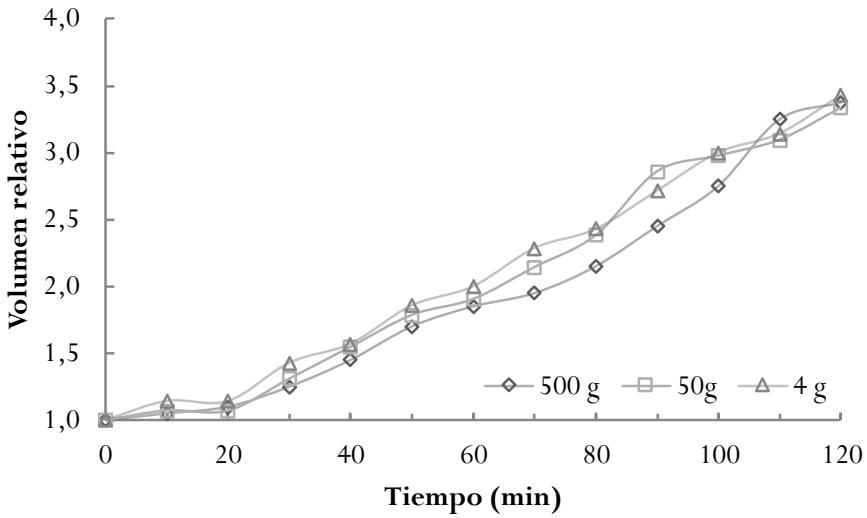

Figura 7. Incremento de volumen relativo de las diferentes cantidades de masa.

Tabla 7. Ajustes finales de la panificación atendiendo a la cantidad de masa.

Cantidad de masa (g)	Fermentación		Cocción	
	Tiempo (min)	Temperatura (ºC)	Tiempo (min)	Temperatura (ºC)
500	60	30	35	185
50	60	30	12	185
4	60	30	11	130

A simple vista, la estructura de la sección transversal de la miga fue similar (Figura 8). El ADI de la estructura de la miga (Tabla 8) reveló que las distintas migas mostraron valores similares en cuanto al c número de alveolos por cm^2 (25-30), el área media de estos (0,51-0,65 mm^2) y la circularidad (≈0.6). Dichos valores validaron las condiciones de fermentación y cocción empleadas para la obtención de panes de distinto peso y volumen.

Figura 8. Imágenes de las rebanadas de pan obtenidas a partir de distinta cantidad de masa.

La evaluación de la textura del pan también requirió la modificación del diámetro de la sonda para mantener una relación directa con el área de la rebanada. La sonda de 25 mm de diámetro se seleccionó para el pan de 500 g de masa, mientras que para los panes de 50 y 4 g de masa se utilizaron las sondas de 1,3 y 0,6 cm de diámetro, respectivamente. Los valores de elasticidad recuperable retardada, cohesividad y velocidad de recuperación instantánea fueron similares, debido a que son cocientes entre áreas o tiempos, por lo que pueden ser comparados

independientemente del tamaño de la muestra, siempre que la relación entre la sección de miga y el diámetro de la sonda se respete. Los datos experimentales de la dureza de la miga se ajustaron perfectamente a la ecuación logarítmica (Figura 9). Por lo tanto, es posible reducir el tamaño del pan a panes de 4 g de masa y los resultados de textura podrían utilizarse para estimar las características de panes con mayor cantidad de masa.

Tabla 8. Caracterización de la estructura y la textura de los panes con diferente cantidad de masa.

Cantidad de masa	500	50	4
Estructura de la miga			
Sección de la imagen (mm)	30,0x30,0	15,6x15,6	6,3x6,3
Número de alveolos total	258	64	10
Alveolos/ cm2	29	26	25
Área media (mm2)	0,654	0,510	0,517
Circularidad	0,649	0,668	0,677
Perfil de textura (TPA)			
Dureza (g)	471 ± 42	254 ± 24	48 ± 10
Elasticidad recuperable retardada	1,002 ± 0,014	1,008 ± 0,019	1,002 ± 0,050
Cohesividad	0,825 ± 0,005	0,825 ± 0,005	0,769 ± 0,037
Masticabilidad (g)	407 ± 48	213 ± 19	38 ± 7
Velocidad recuperación instantánea	0,446 ± 0,005	0,446 ± 0,009	0,282 ± 0,035

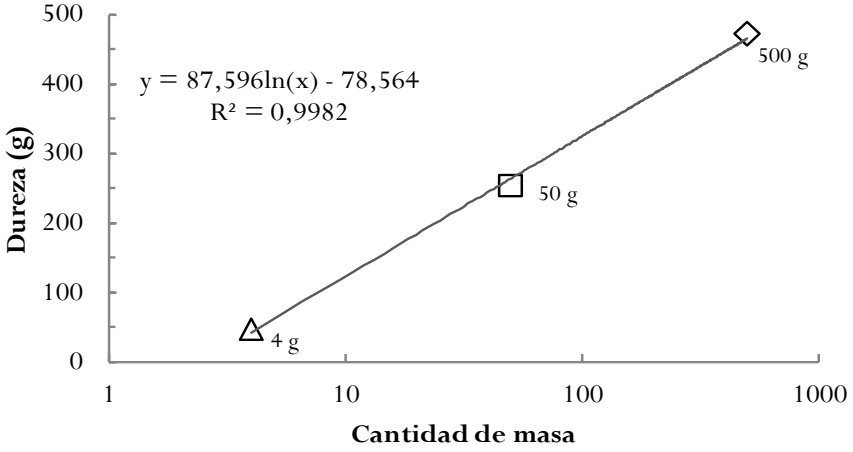

Figura 9. Relación entre la dureza de la miga y la cantidad de masa.

Comprender el impacto de la velocidad de amasado en la reología de la masa y la calidad del pan mediante panificaciones a pequeña escala.

Las curvas del mixolab mostraron que la consistencia de la masa aumentó mientras que la estabilidad disminuyó al aumentar la velocidad de amasado (Figura 10). Esto pudo ser debido al aumento de la energía aplicada al aumentar la velocidad[43], como puede observarse en la Tabla 9, la cantidad de energía aportada a la masa (PA) fue mayor al aumentar la velocidad. Esto originó una reducción en el tiempo requerido para desarrollar la masa, es decir se requirió menor tiempo de amasado a medida que se incrementó la velocidad (1,75 a 0,60 min). La red formada durante el amasado sufrió mayor debilitamiento a medida que se aumentó el aporte de energía mecánica, como se puede observar en la diferencia C2-C1. Los resultados concuerdan con lo descrito en el estudio de Wilson et al.[20]. Estos autores estudiaron el impacto de la velocidad (75-400 rpm) y describieron que la consistencia de la masa aumentó y disminuyó el tiempo de amasado a medida que aumentaron la velocidad.

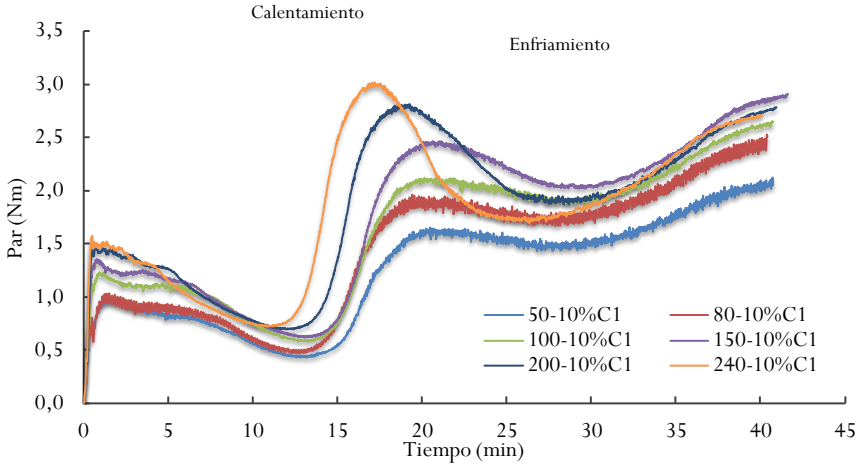

Figura 10. Perfil de consistencia de la masa, con distinta velocidad de amasado.

Tabla 9. Energía aportada durante el análisis completo del Mixolab a cada una de las velocidades estudiadas.

Velocidad de amasado (rpm)	50	80	100	150	200	240
PA (Wh/ Kg)	58,5	107,9	151,6	256,5	346,3	403,8

Los panes obtenidos a pequeña escala mostraron diferente textura y estructura de miga, dependiendo de la velocidad de amasado (Figura 11 y Tabla 10). Además, la velocidad de amasado afectó significativamente a la textura de la miga de pan, mostrando migas más duras, excepto a 240 rpm de velocidad. Este efecto se ha atribuido a la reducción significativa del volumen de los panes al aumentar la velocidad de amasado, lo que puede influir en la dureza de la miga[20,44]. Las muestras de pan de 200 y 240 rpm de velocidad de amasado mostraron menor número de alveolos/cm^2 que los panes obtenidos a 50 y 80 rpm. Siendo las muestras amasadas a velocidades intermedias (100 y 150) las que originaron mayor número de alveolos. Este efecto podría atribuirse a la necesidad de alcanzar un umbral a partir del cual el esfuerzo ejercido sobre la red es demasiado fuerte, produciendo la coalescencia posterior de los núcleos formados.

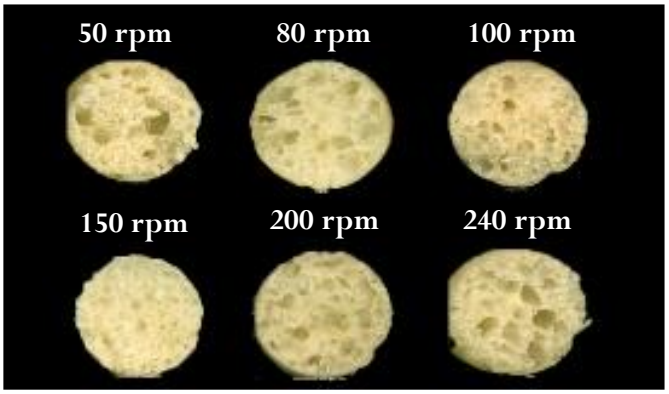

Figura 11. Imágenes de la miga de los panes obtenidos con distinta velocidad de amasado.

Tabla 10. Textura y estructura de los panes obtenidos con distinta velocidad de amasado.

Velocidad	Dureza	Alveolos /cm2	Área media (mm^2)	Circularidad
50	53 ± 4^{c}	26 ± 5^{b}	$0,50 \pm 0,09^{b}$	$0,75 \pm 0,02^{bc}$
80	78 ± 7^{b}	25 ± 4^{b}	$0,52 \pm 0,10^{b}$	$0,78 \pm 0,03^{a}$
100	103 ± 12^{a}	32 ± 5^{a}	$0,45 \pm 0,06^{b}$	$0,75 \pm 0,02^{bc}$
150	105 ± 17^{a}	31 ± 5^{a}	$0,47 \pm 0,05^{b}$	$0,76 \pm 0,03^{b}$
200	111 ± 14^{a}	17 ± 5^{cd}	$0,91 \pm 0,54^{a}$	$0,76 \pm 0,04^{bc}$
240	72 ± 6^{b}	15 ± 4^{d}	$0,89 \pm 0,45^{a}$	$0,73 \pm 0,03^{c}$

Conclusiones

El análisis de imagen se confirma como método no destructivo para discriminar la estructura de los panes. Esto se ha confirmado con pan de molde con y sin gluten. Sin embargo, se debe prestar especial atención a los ajustes en la captura de la imagen, ya que dependiendo de la resolución utilizada se aumenta o disminuye la capacidad del análisis para detectar objetos pequeños. Además, es importante conocer el valor de umbral que se aplica para generar la imagen binaria y conocer el algoritmo utilizado para su cálculo ya que los resultados pueden variar, sobre todo si se pretende comparar entre muestras.

El análisis digital de imagen de las migas permitió analizar la calidad de los panes con y sin gluten distinguiendo la estructura obtenida al realizar

pequeñas modificaciones en la formulación. Mediante la utilización de distintos ingredientes/aditivos se puede mejorar el aspecto de la rebanada y la estructura de los panes.

La panificación a pequeña escala es un buen modelo para estudiar el impacto de la panificación en la calidad del pan, siempre que se adapte el proceso de panificación a la cantidad de masa utilizada. El análisis de imagen permitió confirmar la similitud en la estructura independientemente del peso y volumen de los panes. Del mismo modo, cambios en las condiciones del proceso, como la velocidad de amasado, pudieron detectarse al analizar los parámetros definidos con el análisis de imagen de las migas.

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Capítulo 2

Estudio de los cambios estructurales del almidón durante los procesos de gelatinización y gelificación mediante un analizador rápido de fuerza.

I. Rapid assessment of starch pasting using a rapid force analyzer[1]

Raquel Garzon, Cristina M. Rosell

Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain
**Corresponding author:* crosell@iata.csic.es

Abstract:
Background and objectives: The falling number is a rapid method developed for assessing amylase level on flours, based on viscosity changes. This measurement records the time required for a stirring rod to travel through a flour paste. The Chopin Amylab FN, developed for assessing the FN, allows working as a Rapid Force Analyzer (RFA), recording the force changes of a starch/flour slurry under controlled mixing/heating conditions. The objective of this research was to underline the starch changes occurring along 90 s under continuous mixing and heating.
Findings: Four different starches (corn, rice, wheat and potato) were analyzed using the RFA, evaluating step-by-step the structural and textural modifications. Scanning electron micrographs revealed the progressive gelatinization process, that was specific for each type of starch. Nevertheless, the 90 s procedure was sufficient to ensure complete gelatinization of all starches. Parameters recorded from the RFA showed strong significant correlations with onset and peak gelatinization temperature, besides gelatinization enthalpy.
Conclusion: RFA could be used as a rapid method for starch pasting assessment, being valid for discriminating among different types of starches.
Significance and novelty: Study shows the potential of Amylab as RFA that records starches pasting performance in 90 s.

Key words: crust, crumb, bakery, gluten-free, confectionary, microstructure, texture, color

Raquel Garzon: Investigation, Formal analysis, Data curation, Visualization, Writing-Original Draft
Cristina M. Rosell: Conceptualization, Funding acquisition, Supervision, Writing-Review & Editing

[1] in *Cereal Chemistry*,2021, *98(2), 305-314.*

Introduction

Starches are common ingredients for numerous industrial food applications, which explains why their quality control have been the objective of much researches. Gelatinization is a primary physicochemical property of the starches, because of that viscosity measurements are widely applied for assessing the starch quality, particularly consisting of heating-cooling cycles to follow gelatinization and gelling phenomena[1]. Different systems (Brabender amylograph and viscograph, Rapid Viscoanalyzer) have been developed for assessing the starch viscosity. The majority of them are empirical methods based on recording the relative or apparent viscosity, derived from a torque, of a starch paste subjected to thermal and mechanical constraints[2]. Those systems record the starch gelatinization in an excess of water, going from granular state to pastes, because of that it is widely referred as pasting performance. Differences on those systems rely on the amount of sample (2-80 g), the rotational speed, temperature gradient and so on. As consequence, differences in the pasting parameters obtained from each instrument have been reported, which have been attributed to differences in the spindle structure[2]. One of the most successful devices for assessing apparent viscosity of starches has been the rapid viscoanalyzer (RVA) designed back in 1987 [3], owing to the lower amount of sample required and the short-time assay (within minutes), besides the versatility of the operational conditions [4,5]. Nowadays, similar versatility and benefits can be obtained with the micro-visco-amylograph [6]. In addition, the change of operational conditions is continuously extending those instruments' applications, for instance to assess ingredients impact [7], batter characteristics [8], breeders selection [9], or even more fundamental information like proteins impact on starch pasting properties [10], among others. All those applications reveal the endless interest for assessing pasting performance of starches using rapid methods.

Apart from starch/flour quality assessment, viscosity has been the basis for predicting α-amylase activity and cereal sprouting in a rapid test (measurement in seconds), widely known as falling number (FN) parameter [11]. The Hagberg falling number method approved by AACC International (AACCI) consists in a rapid heating of a starch suspension, with simultaneous stirring during the first 60 s, till reaching a point where

the stirrer falls following the gravity force and it is recorded the time required for the rod to cross the viscous suspension [12]. This methodology is extensively used and the FN is one of the most extended parameters in wheat quality surveys, although its repeatability and precision have been lately investigated [13]. Therefore, there is extensive use of instruments for assessing the pasting properties of starches. Likewise, the falling number methodology is also based on apparent viscosity, recording the travelling time of the stirring rod through a starchy paste.

Recently, Chopin Technologies launched the Amylab FN to measure the Hagberg FN. Although this device was originally design to determine the falling number index of the flours, which is related to the α-amylase activity, such a rapid test might be very helpful for assessing starch performances if provided enough comprehensive information for understanding the process. This research aims to study the starch changes underlining during a short (90 seconds) heating cycle with simultaneous stirring. The rheological properties of different starches during their Rapid Force Analysis (RFA) carried out in the Chopin Amylab have been related with the changes undergone on their microstructure and textural features.

Materials and methods

Materials

Commercial native starches of food grade for wheat starch (ADM, Chicago, US), corn starch (Tate & Lyle, London, UK) and potato starch (Tereos, Zaragoza, Spain) were directly used in the study. Rice starch was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Other reagents were of analytical grade.

Starches characterization

Water (WBC) and oil (OBC) binding capacities were determined to evaluate the hydration properties of the different starches. Those properties were assessed as previously described [14]. Briefly, starch (100 mg) was suspended into one milliliter of water, in the case of WBC, or vegetable oil for the OBC. Suspensions were vortexed for 5 min and then centrifuge at 5000 x *g* for 5 min. The sediment was weighed after draining

the tubes. Results were expressed as grams of water or oil adsorbed by gram of starch. Five replicates were made for each experimental result.

Thermal properties were determined using a Differential Scanning Calorimeter (DSC-TAQ2000, TA Instruments Ltd., New Castle, DE, US). Starch samples (8 mg) were weight in stainless steel pans and distilled water was added in the proportion 1:4 (starch: water, w/w). Pans were heated at 10 °C/min from 30 to 120 °C, an empty pan was used as reference. Onset temperature (To), heat of transition (ΔH, in joules per gram of starch) and peak temperature (Tp) were determined. All measurements were done at least in triplicate.

Rapid force analyzer of different starches

The Chopin Amylab® (Chopin Technologies, Villeneuve-la-Garenne, Cedex, France) in its testogram mode was used as a rapid force analyzer (RFA) to record the gelatinization of the different starches. This device operates with a continuous up and down motion of the stirrer rod during 90 s at a constant temperature of 100 °C. A slurry containing 7 g (14% mb) of starch and 25 ml of distilled water were placed into the precision test tubes of the device and manually shaken vigorously for 30 s. After immersing the stirring rod into the slurry, the tube was capped with a plunger and placed into the holder of the device. An insulated thermocouple (type K) was inserted and the wire leads attached to the bottom of the rod to record the temperature changes during measurements with a Comark N2014 multi sensor temperature data logger (Comark Instruments, Norwich, Norfolk, UK). Temperature readings were recorded every second. Plot recorded shows the force, expressed in Newtons, of the slurry/gel under continuous heating/shearing (Figure 1). Parameters defined include: onset force (F0) before force increase due to gelatinization, onset time (s) at which F0 is detected, 50% (t1) and 75% (t2) of time to onset force, maximum force (F1) and final force at 90 s (F2). Other parameters calculated from the previously identified forces included: α (slope between F0 and F1), gel stability as the elapsed time in which force was kept ± 10% of the maximum force (F1) and the force difference between F1 and F2 was associated to starch breakdown. To understand starch changes along the gelatinization carried out with this device, the equipment was stopped to

allow sampling at different times (t1, t2, onset, time to reach F1 and F2) as indicated in Figure 1. Three replicates were carried out for each sampling point and type of starch and results showed the average of experimental data.

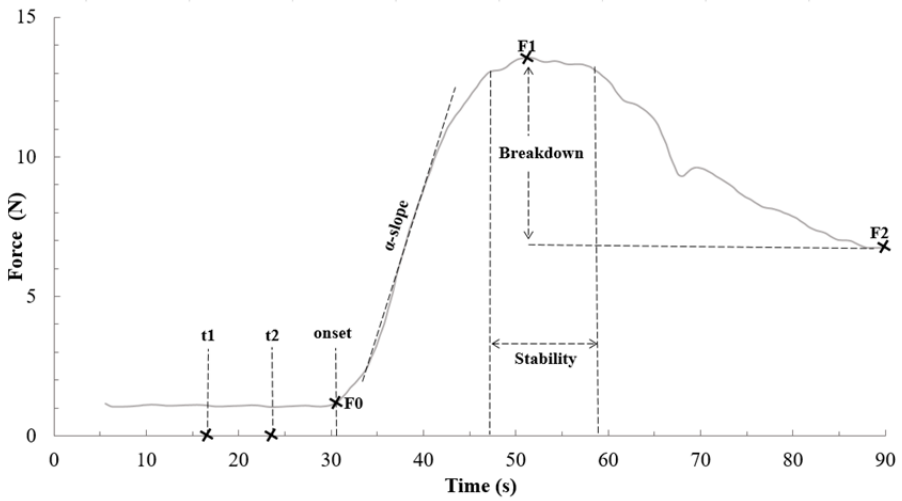

Figure 1. Typical plot recording force vs time along starch gelatinization using a Rapid Force Analyzer (Amylab). The main parameters used to evaluate slurries/gels are detailed in the drawing. Parameters defined include: onset force (F0) or starting force before gelatinization, onset time (s) at which F0 is detected, 50% (t1) and 75% (t2) of time to onset force, maximum force (F1), final force at 90 s (F2), α (slope between F0 and F1), gel stability as the elapsed time in which force was kept ±10% of the maximum force (F1) and breakdown (force difference between F1 and F2).

At each point, samples obtained from the RFA were immediately poured into cylindrical containers of 25 mm internal diameter, covered with lids and kept at room temperature to cool down up to 25 °C in the center of the gel. Simultaneously, the slurry/gel samples obtained from each stage were immediately frozen in liquid nitrogen and then freeze-dried to avoid microstructure alteration during freezing.

Gel texture and degree of gelatinization

In each stage, gel firmness analysis was conducted in a TA-XT2 Texturometer fitted with a 5 kg load cell (Stable Microsystems, Surrey,

UK). Then, gels were measured through a single compression test using a 10 mm diameter aluminum round probe. Measurements were carried out at 1 mm/s crosshead speed and 50% of strain. Firmness was considered as the maximum penetration force and adhesiveness defined as the area required to remove the probe from the gel. All textural analysis in each stage were done at least four times.

The degree of gelatinization (DG %) for samples from the different stages was assessed by running DSC test as previously described for native starches, but using the freeze-dried powder of the samples taken from rapid force analyzer. DG was calculated using the equation suggested by Sakiyan, et al. [15]:

$$\text{DG}\ (\%) = \left(1 - \frac{\Delta\text{H sample}}{\Delta\text{H native}}\right) \cdot 100$$

Scanning electron microscopy (SEM)

Native starches and freeze-dried samples, from slurries/gels taken at different times from the rapid force analyzer, were observed using SEM (Hitachi S-4800, Tokyo, Japan). All samples (native and gels) were coated with gold using a vacuum evaporator (JEE 400; JEOL, Tokyo, Japan) for 5 min. The images taken at 10 kV acceleration voltage were captured using 900x magnification. Four micrographs captured at each stage were analyzed by digital image analysis using ImageJ software (ImajeJ 1.52p, National Institutes of Health, Bethesda, Maryland, US) to characterize microstructure. All micrographs were modified as 8-bit color and improved in contrast and brightness as reported by Espinosa-Ramírez, et al. [16]. Threshold was assessed by adapting the software algorithm to each micrograph. Finally, sample analysis was carried out and mean cell area (μm^2) and gel porosity (%) were calculated.

Statistical analysis

Each quality parameter was subjected to a one-way analysis of variance (ANOVA) using Statgraphics Centurion XVII V 17.2 (Statgraphiscs Technologies, Inc., The Plains, Virginia, US). Fisher least significant difference test was used to assess significant differences ($P < 0.05$) among samples that might allow discrimination among them. Additionally,

Pearson correlation analysis was applied to establish possible relationships among experimental variables extracted from the different analysis.

Results and Discussion

Starches characterization

To understand the starch changes when subjected to a rapid pasting procedure (90 s), different starches were selected, three from cereals (corn, rice and wheat) and one tuber starch from potato. Hydration properties and thermal parameters of those starches were determined because they might be potentially related to the pasting performance in this rapid procedure. Specifically, water and oil binding capacities reflect the hydrophilic and hydrophobic surface of the starch granules, which might affect the water uptake during granule swelling. Likewise, thermal properties have been related to changes in granular structure and endothermic gelatinization. Cereal starches showed much higher values for OBC than for WBC (Table 1), indicating greater superficial hydrophobicity, likely their A-type polymorphs with the double helixes closely packed are responsible of that behavior [17]. Wheat starch exhibited the lowest WBC compared to the other starches. Conversely, potato starch showed similar values for WBC and OBC, likely its B-type polymorphs that allow more water within the loosely packed helices could be responsible of that result.

The DSC parameters confirmed the significantly lower onset temperature of wheat starch, whereas the highest one was exhibited by corn starch. The peak temperature of the endothermic peak showed the same tendency described for the onset temperature. Nevertheless, potato starch required greater enthalpy for gelatinization, and the opposite behavior was observed in the rice starch. Results are within the range of gelatinization properties previously reported for those starches [1].

Table 1. Characterization of raw starches regarding hydration properties, calorimetric parameters and performance during rapid force analysis assessed with the Chopin Amylab.

	Corn	Potato	Rice	Wheat
Hydration properties				
WBC	2.09±0.23^{a}	2.05±0.17^{ab}	2.16±0.18^{a}	1.85±0.05^{b}
OBC	2.56±0.09^{a}	2.17±0.06^{c}	2.60±0.06^{a}	2.44±0.08^{b}
Calorimetric properties				
To (ºC)	69.90±0.11^{a}	62.90±0.54^{c}	65.62±0.23^{b}	60.66±0.23^{d}
ΔH (J/g)	12.76±0^{b}	14.46±0.25^{a}	6.56±0.04^{d}	11.42±0.11^{c}
Tp (ºC)	74.37±0.21^{a}	67.77±0.60^{c}	72.13±0^{b}	65.29±0.04^{d}
RFA-Amylab				
t1 (s)	19±0^{a}	15±0^{b}	20±0^{a}	15±0^{a}
t2 (s)	28±1^{a}	23±1^{b}	30±0^{a}	23±0^{a}
F0 (N)	2.45±0.07^{b}	3.76±0.16^{a}	3.76±0.01^{a}	2.92±0.36^{b}
Onset (s)	36±0^{c}	30±0^{b}	36±0^{c}	29±0^{a}
α-slope	1.83±0.01^{b}	2.07±0.04^{a}	0.38±0.02^{d}	0.65±0.01^{c}
F1 (N)	11.51±0.07^{c}	21.04±0.30^{a}	11.34±0.29^{c}	15.33±0.20^{b}
Time F1 (s)	47±0^{c}	38±0^{d}	67±0^{a}	57±3^{b}
Stability (s)	12±2^{b}	4±1^{c}	22±1^{a}	22±1^{a}
F2 (N)	7.70±0.04^{c}	12.78±0.34^{a}	9.56±0.05^{b}	12.21±0.47^{a}
Breakdown (N)	3.81±0.10^{b}	8.26±0.64^{a}	1.78±0.24^{c}	3.12±0.28^{b}

Means within the same row followed by different letters indicate significant differences by LSD multiple range test $P<0.05$.

Starch gelatinization recorded with a Rapid Force Analyzer

The Chopin Amylab has been designed for inducing starch gelatinization in a rapid test (90 s) applying heating to a starch slurry subjected to continuous stirring (Figure 1). The device records the force changes during the starch gelatinization that occurs within the 90 s, acting as a Rapid Force Analyzer (RFA), although there is no previous information about changes occurring during the assay. A brief explanation of the plot recorded is following. In the present study 7 g of starch (adapted to 14% moisture content) suspended on 25 ml water was used, which corresponds to the best precision for measuring the FN [12]. Firstly, the starch or flour slurry is in a liquid form that does not require any force for stirring. As the heating progresses, swelling of starch granules increases viscosity and in consequence the force require for keeping

homogenous shearing increases significantly. The initial force (F0) of the slurry, before granules swelling, indicates the slurry consistency and might be related to the rapid starch water uptake on the surface besides the potential impact of granules size. The time at which F0 is detected, is referred as the gelatinization onset and it could be related to the gelatinization temperature. The slope (α -slope) was also quantified to evaluate if the rate of starch swelling could be related to granules morphology. It is well known that granules swelling continues till their disintegration or breaking down that leads to a force decrease, which lasted until an even gel is formed (Figure 1). The maximum force detected was referred to F1, and the time of holding force was defined as stability. To understand morphological and textural changes during the gelatinization process, the assay was stopped at the points (t1, t2, onset, time at F1 and at 90 s) where major changes were expected (Figure 1). Microstructure on those points was compared with the native granular structure of each starch (Figure 2).

Micrographs of corn and rice starches at t1 were rather similar to the native starches, showing intact granules that kept their polyhedral shape forming agglomerates (Figure 2). Therefore, t1 was not enough to alter those starches structure and initiate the gelatinization, which agrees with their higher onset temperature (Table 1). Conversely, potato and wheat starches even at t1 exhibited signals of either distortion (wheat) or leaching (potato). In wheat starch, the two granules populations were detected but the bigger A-type displayed flat morphologies and some small B-type granules showed a deep groove in the center. In the case of potato starch, swollen granules together with some amylose leaching were observed, although it seems that those changes were not sufficient to modify the stirring force (Figure 3). Despite potato has a B-type starch its early gelatinization has been attributed to the negative charges of the phosphate-monoester derivatives that destabilize double helical structure [1]. Considering the different thermal properties of the tested starches (Table 1), significant correlations were detected between t1 recorded in the RFA with DSC parameters, To ($r= 0.78$) and Tp ($r= 0.89$). In t2, which corresponded to 75% of the total time required for the onset, potato starch was completely gelatinized and a network was already observed, although it was not completely homogenous, showing irregular cavities sizes. At that point wheat starch was even more distorted, with

flat structures that were held together by the leached molecules. Wheat granules perimeter was still defined in some granules and granules were interacting one to another. Similarly, corn and rice starches started their gelatinization with the deformation of the structures; thus, deformed granules were surrounded by leaching material and granules fragments resulting in an irregular mass. At the onset force (F0) the potato gel was completely formed, exhibiting an uniform network structure, whereas granules deformation progressed in cereal starches. At this point, some gel network was envisaged in corn and rice starches, but wheat granules were even thinner adopting flakes-like structures, with still defined perimeters.

Micrographs captured at the maximum force (F1) confirmed the gels formation in all the starches although some irregularities were observed in wheat gel. Considering that the maximum force is the inflection force point corresponding to the balance between swollen granules and fragmented ones, all starches lost their integrity and the granules interaction led to the gel mass. Further heating and gel shearing (till 90 s) did not provoke additional changes in the gel mass that could be visually detected, with the exception of the rice starch that exhibited more packed network at the end of the assay (90 s), likely better organization was achieved with the continuous stirring motion that allowed air bubbles removal. Similar structural changes were described for corn starch when gelatinization was sufficiently extended with the RVA and sampling was carried out at different times[18]. Therefore, considering the micrographs information, the force plot recorded with the RFA reproduced the rheological changes that has been reported to describe starch gelatinization [19].

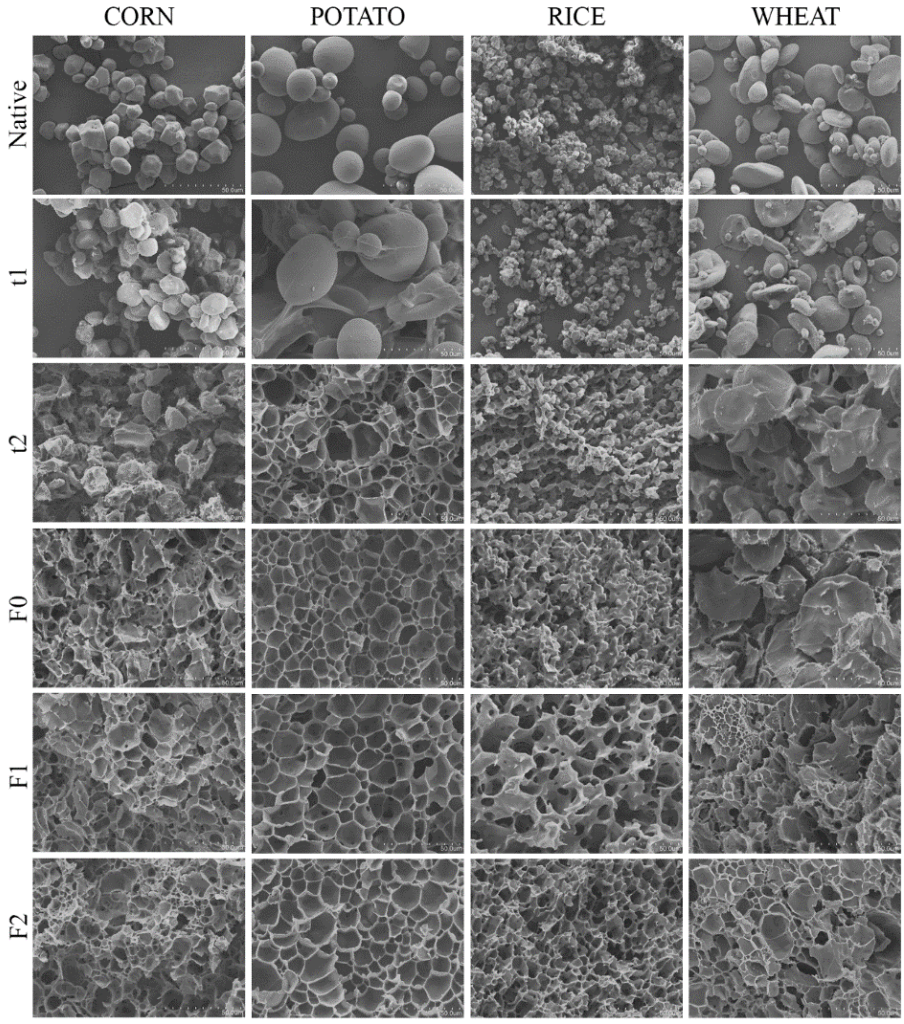

Figure 2. SEM micrographs captured along the Rapid Force Analyzer (RFA) cycle showing the starch changes along heating and stirring. Micrographs were taken at 900x magnification. Micrographs were taken at the different RFA stages described in Figure 1.

The plots recorded by the rapid force analyzer (Figure 3) confirmed differences among the different starches. The temperature profile shows that 40±3 s was required to reach 95 °C, which is much faster than the over 150 s reported for the Perten FN [12]. Initially, all slurries required low force for stirring, but after variable heating time (30-40 s) a fast increase of the recorded force was detected due to starch gelatinization.

At that moment, the rapid heating rate underwent a decrease due to the energy required for the endothermic gelatinization, which agrees with reported results for the Perten FN[12]. Cereal starches showed some minor decay after reaching the maximum force. In opposition, potato starch showed a well-defined peak of gel force with a large force decrease when heating-stirring progressed.

Parameters defined from the plots are included in Table 1. Potato and rice starches required significantly ($P< 0.05$) higher force (F0) for keeping homogenous slurries, which might be related to their WBC. In fact, a significant correlation ($r= 0.89$) was identified between F0 and WBC, in accordance to the relationship reported among the capacity to hydrate and swell and the starch viscosity [20]. The lower onset observed for potato and wheat starches indicated that their gelatinization started at lower temperature, which agrees with data from DSC. In fact, a very strong positive correlation was encountered between the RFA onset time and the DSC To ($r= 0.90$) and Tp ($r= 0.97$). Potato starch displayed the faster gelatinization (α-slope) and rice starch had the lowest rate of gelatinization, with higher time (Time F1) to reach the maximum gel force (F1). Interestingly, a significant positive correlation ($r= 0.87$) was detected between the α-slope and the gelatinization enthalpy (ΔH).

As expected, potato starch exhibited the highest force (F1) followed by wheat starch, whereas corn and rice starches did not differ significantly ($P< 0.05$) on their maximum force. A negative correlation ($r= 0.88$) was observed between the OBC and the F1. To assess behavior of gels after complete granule disintegration, gels stability was defined as the time holding the maximum force, which confirmed the short stability of the potato gel, and the longer stability of rice and wheat gels. Potato and wheat gels required higher forces indicating their higher viscosity. The force decay indicated by the breakdown reflected the granules resistance to physical rupture, which has significantly higher for potato starch, supporting that starches exhibiting higher swelling are less resistant to breakdown on cooking[19]. In fact, a highly significant correlation ($r= 0.90$) was observed between F1 and breakdown.

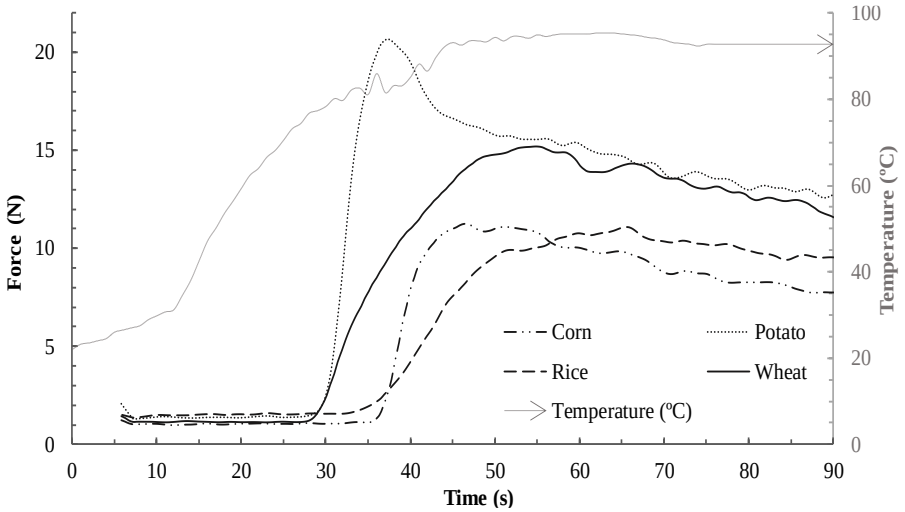

Figure 3. Plots from the Rapid Force Analyzer for corn, potato, rice and wheat starches assessed with the Amylab. Temperature was simultaneously recorded with a multi sensor temperature data logger (plot with secondary y-axis).

Starch gels properties along gelatinization in RFA

The texture, RFA parameters, gelatinization degree (GD) and microstructure of the samples taken along the gelatinization carried out in the RFA were evaluated (Table 2). The statistical analysis of the variance showed that the starch factor significantly ($P< 0.05$) affected all the parameters tested, with exception of the degree of gelatinization. Likewise, samples taken at the different stages of the gelatinization (different times along RFA analysis) showed significantly ($P< 0.05$) different properties.

The RFA provokes a rapid gelatinization of the different starches, which was confirmed with the 100% gelatinization degree determined with the DSC. As it was previously described for the microstructure changes, the GD was reached at different times depending on the type the starch. Corn and wheat starches required 28 s and 23 s (Force time column in Table 2), respectively, to reach 100% GD. Conversely, potato and rice starches reached 100% GD at 30 s and 36 s, respectively. Surprisingly, according to the GD, corn and wheat starches were rapidly gelatinized, when the

SEM micrographs still revealed granular structures at those times. Considering that the gelatinization degree was calculated based on the transition enthalpy, it might be that the changes required to obtain a complete lattice structure do not require additional energy, and in consequence no enthalpy was detected when evaluated those samples.

Potato gel had the highest firmness (5.5 N), which agree with the highest force value recorded with the RFA. But those maxima values were reached at different times, that is the firmest potato gel was obtained after 30 s, whereas its maximum force recorded with the RFA was observed at 38 s. This result agrees with the SEM observation that indicated smaller air voids in the potato gel at F0 than at F1 (Figure 3), leading to firmer gels. On the contrary, for cereal based gels the highest firmness was in accordance to the maximum force. Rice gel was the softer one with the lowest firmness. Nonetheless, in cereal based gels, there was not a direct trend between the firmness of the gels and the maximum force (F1) detected with the RFA. Previous studies carried out with potato and wheat starches stated the linear relationship between macroscopic and microscopic viscosity determined with creep and rotational measurements, respectively, within the temperature range 30-50 ºC [21]. Nevertheless, with this rapid analysis carried out at higher temperatures, no linearity was observed between F1 and firmness. The firmness of the gels obtained after the 90 s (at F2) tended to decrease, although no significant differences were observed. Presumably, the molecular order of the gels was kept till a point where the thermal and mechanical constraints caused their weakening, and that effect was more noticeable in firmer gels like potato. Similarly, a significant ($P< 0.05$) decrease in the force values was observed in all starches as gelatinization progresses, which might be related to the thixotropic (shear thinning) behavior of the starch pastes with respect to time [22].

Table 2. Characterization of the starchy gels obtained along the gelatinization process including texture, RFA parameters, gelatinization degree (GD) and microstructure. RFA: Rapid force analyzer (Chopin Amylab working in its testogram mode).

Starch	RFA-Stage	Force time (s)[a]	Force (N)	Firmness (N)	Adhesiveness (N·s)	GD (%)	Porosity (%)	Median cell area (μm²)
Corn	t1	19 ± 0[e]	1.1 ± 0[d]	n.d.*	n.d.	40 ± 4	n.d.	n.d.
	t2	28 ± 1[d]	1.1 ± 0[d]	2.3 ± 0.9[b]	1.4 ± 0.9[b]	100 ± 0	n.d.	n.d.
	F0	36 ± 0[c]	2.5 ± 0.1[c]	4.7 ± 0.5[a]	3.0 ± 0.7[a]	100 ± 0	37.8 ± 0.3[b]	24 ± 8[a]
	F1	47 ± 0[b]	11.5 ± 0.1[a]	4.8 ± 0.6[a]	2.6 ± 0.7[ab]	100 ± 0	45.5 ± 2.8[ab]	18 ± 4[ab]
	F2	90 ± 0[a]	7.7 ± 0.0[b]	4.4 ± 0.4[a]	3.4 ± 0.3[a]	100 ± 0	52.2 ± 3.7[a]	6 ± 1[b]
Potato	t1	15 ± 0[e]	1.3 ± 0.1[d]	n.d.	n.d.	49 ± 4	n.d.	n.d.
	t2	23 ± 1[d]	1.4 ± 0.2[d]	4.8 ± 0.8[b]	1.4 ± 0.3[a]	84 ± 3	49.3 ± 3.5[b]	27 ± 9[b]
	F0	30 ± 0[c]	3.8 ± 0.2[c]	5.5 ± 0.5[a]	1.3 ± 0.3[a]	100 ± 0	52.4 ± 3.0[b]	28 ± 2[b]
	F1	38 ± 0[b]	21.0 ± 0.3[a]	2.5 ± 0.3[c]	0.9 ± 0.2[b]	100 ± 0	67.8 ± 2.0[a]	62 ± 5[a]
	F2	90 ± 0[a]	12.8 ± 0.3[b]	2.1 ± 0.1[c]	0.7 ± 0.1[b]	100 ± 0	64.6 ± 3.8[a]	53 ± 3[a]
Rice	t1	20 ± 0[e]	1.5 ± 0.1[d]	n.d.	n.d.	22 ± 2	n.d.	n.d.
	t2	30 ± 0[d]	1.5 ± 0.1[d]	0.5 ± 0.1[b]	0.5 ± 0.2[b]	96 ± 1	n.d.	n.d.
	F0	36 ± 0[c]	3.8 ± 0.0[c]	0.9 ± 0.1[a]	1.0 ± 0.1[a]	100 ± 0	15.0 ± 2.5[c]	8 ± 1[b]
	F1	67 ± 0[b]	11.1 ± 0.3[a]	1.0 ± 0.1[a]	1.1 ± 0.1[a]	100 ± 0	35.0 ± 2.5[b]	29 ± 5[a]
	F2	90 ± 0[a]	9.5 ± 0.1[b]	1.0 ± 0.1[a]	0.7 ± 0.2[b]	100 ± 0	51.0 ± 2.8[a]	18 ± 3[b]
Wheat	t1	15 ± 0[e]	1.1 ± 0.1[d]	n.d.	n.d.	73 ± 1	n.d.	n.d.
	t2	23 ± 0[d]	1.1 ± 0.2[d]	1.4 ± 0.1[c]	1.5 ± 0.2[b]	100 ± 0	n.d.	n.d.
	F0	28 ± 0[c]	2.9 ± 0.4[c]	2.7 ± 0.6[b]	1.9 ± 0.9[b]	100 ± 0	n.d.	n.d.
	F1	57 ± 3[b]	15.3 ± 0.2[a]	4.4 ± 0.4[a]	3.5 ± 0.8[a]	100 ± 0	16.7 ± 2.1[b]	12 ± 2[a]
	F2	90 ± 0[a]	12.2 ± 0.5[b]	4.2 ± 0.2[a]	3.2 ± 0.5[a]	100 ± 0	64.9 ± 0.5[a]	16 ± 1[a]
P-value								
Starch		**0.0006**	**0.0016**	**0.0000**	**0.0000**	0.0506	**0.0000**	**0.0000**
RFA-stage		**0.0000**	**0.0000**	**0.0000**	**0.0006**	**0.0000**	**0.0000**	**0.0157**

a Force time: time to reach sampling point as displayed in Figure 1.

*n.d. (not detected)

Means within the same column in each starch followed by different letters indicate significant differences by LSD multiple range test P<0.05

From the image analysis of the gels structure it was calculated the porosity of the network and the median cell area, the former to avoid the misrepresentation that using the average value could create. Porosity observed in the micrographs corresponded to the regions initially occupied by water that was removed by freeze-drying. Those parameters were identified as soon as a gel was detected, which was reached in the early stages in the case of potato starch. From the porosity results it was evident in all the gels, the progressive increase in the porosity as the RFA progresses, indicating the gradual formation of an even gel structure, as was observed in the micrographs. The continuous stirring might help to obtain a more uniform structure since the shear force favors the alignment of the molecules within the lattice structure of the gels [18]. The higher median area of the lattice voids of potato gel indicated a more open structure of this gel with bigger cavities than those obtained for cereal based gels. Moreover, in the last stages it could envisage a decrease in the median area of the holes, with exception of wheat gel. Although the network structure visualized in the micrographs resulted from the removal of water leading to voids, changes in the last stages after complete gelatinization might be associated to the removal of air bubbles entrapped within the gel forced by the stirring motion.

A correlation matrix within the measured parameters confirmed the significant relationship among parameter recorded from the RFA and resulting gel features (Table 3), allowing better understanding of the changes undergone in the RFA along gelatinization. Only a very strong positive correlation ($r \geq 0.7$) was observed between the force measured in the RFA and the porosity of the gels, indicating that higher force gels would lead to more porous gels. Positive moderate ($0.4 < r < 0.7$) correlations were observed between force and the gelatinization degree and median cell area, confirming the total gelatinization of the starches and higher force resulted from gels with thicker walls and big holes. There was a highly significant ($P < 0.0000$) correlation ($r = 0.66$) between force and time to reach those forces, which suggested longer time in the RFA was required for the stronger gels, like it was observed in the case of potato. The correlation between force and gel firmness, although statistically significant, was rather weak ($r < 0.3$). In the same sense, no correlation was found by Gaines, et al. [23] between pasting properties and gel hardness. Force time was also positively correlated with the GD and

gel porosity. Other important correlations were detected among the gel firmness with adhesiveness (r = 0.66; P= 0.0000), GD (r = 0.52; P= 0.0005) and porosity (r = 0.62; P= 0.0000). The moderate negative correlation observed between the adhesiveness and median cell area suggested that gels with smaller voids were more adhesive.

Table 4. Correlation matrix among texture properties, RFA parameters, gelatinization degree (GD) and microstructure obtained from the different starches.

	Firmness (N)	Adhesiveness (g·s)	Force (N)	Force time (s)	GD (%)	Porosity (%)
Adhesiveness (g·s)	**0.66** (0.0000)					
Force (N)	**0.33** (0.0371)	0.13 (0.4885)				
Force time (s)	0.27 (0.0869)	0.20 (0.3041)	**0.66** (0.0000)			
GD (%)	**0.52** (0.0005)	0.16 (0.3947)	**0.47** (0.0022)	**0.50** (0.0010)		
Porosity (%)	**0.62** (0.0000)	0.08 (0.6656)	**0.70** (0.0000)	**0.68** (0.0000)	**0.48** (0.0016)	
Median cell area (µm2)	-0.14 (0.5089)	**-0.49** (0.0248)	**0.44** (0.0298)	-0.15 (0.5587)	-0.05 (0.8312)	**0.55** (0.0052)

Upper row= *Pearson* correlations, bold values indicate significant correlations.
In parenthesis *P*-values for statistical significance of estimated correlation.

Conclusions

The underlying mechanism occurring during a rapid starch gelatinization carried out with a Rapid Force Analyzer was investigated by stopping the measurement at different stages. Different parameters have been defined from the RFA plots to characterize starch performance during pasting. The microstructural changes observed with four different starches (corn, potato, rice and wheat) confirmed the complete starch gelatinization within the 90 s test, although time to reach gelatinization was dependent on the starch source. The force plots obtained from the RFA allowed the discrimination among the different starches. Significant correlations were detected between the maximum force (F2) recorded by RFA with the gelatinization degree and gel microstructure (porosity and median cell area).

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Conflict of Interest. The authors declare that they have no conflict of interest.

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Capítulo 3

Estudio del impacto distintos ingredientes y aditivos panarios en la formación de la estructura de los productos resultantes.

I. Interaction of dough acidity and microalga level on bread quality and antioxidant properties.

II. Functional and nutritional replacement of gluten in gluten-free yeast-leavened breads by using β-conglycinin concentrate extracted from soybean flour.

III. Understanding emulsifiers effect on bread aeration during breadmaking

I. Interaction of dough acidity and microalga level on bread quality and antioxidant properties[1]

*Raquel Garzon[a], Adriana Skendi[a,b], Marco Antonio Lazo-Velez[a,c], Maria Papageorgiou[b], Cristina M. Rosell[a]**

*[a]Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain. [b]Department of Food Science and Technology, International Hellenic University, POB 141, GR-57400, Thessaloniki, Greece. [c]Universidad del Azuay, Grupos Estratégicos de investigación en Ciencia y Tecnología de Alimentos y Nutrición Industrial (GEICA-UDA), Av. 24 de mayo 7-77, Apartado01.01.981, Cuenca, Ecuador. *Corresponding author:* crosell@iata.csic.es

Abstract:

Microalgae nutritional and healthy dietary pattern might be affected by processes like breadmaking when used as bread ingredients. The objective of this study was to determine the role of dough acidification on the nutritional pattern of Chlorella vulgaris enriched breads. Different levels of the microalga (1%, 2% and 3%) were incorporated in the recipe in the presence of either 10% sourdough or chemically acidified doughs. Dough and bread characteristics, besides the total phenolic content and antioxidant activities were evaluated. The addition of microalga reduced the slice area and increased the crumb hardness, but it could be counteracted by increasing dough hydration and optimizing proofing time. Doughs and breads enriched with C. vulgaris displayed a characteristic green color. Dough acidification led to softer breads and enhanced the antioxidant activity of C. vulgaris enriched breads. Microalgae incorporation increased the protein and ash content of the breads. Microalgae enriched breads made with chemically acidified doughs or sourdoughs had higher Total Phenolic Content and antioxidant activity as assessed by FRAP and ABTS methods.

Key words: bread; Chlorella vulgaris; microalgae; sourdough; bread quality; antioxidant activity

Raquel Garzon: Investigation, Formal analysis, Data curation, Writing- Original Draft
Adriana Skendi: Validation, Formal analysis, Writing- Original Draft
Marco Antonio Lazo-Velez: Investigation, Writing- Review & Editing
Maria Papageorgiou: Writing- Review & Editing
Cristina M. Rosell: Conceptualization, Supervision; Funding acquisition, Writing- Review & Editing

[1] In *Food Chemistry*,2020, 128710.

Introduction

Bread, as one of the most consumed bakery products worldwide (with average per capita consumption 24.5 kg in 2020 and an expected market growth annually by 2.8% (CAGR (Compound Annual Growth Rate) 2020-2025)) has gained the researchers' interest to be used as a vehicle for delivering bioactive compounds to the consumers [1]. To this end, a great number of plant materials rich in bioactive compounds have been incorporated in a bread recipe such as aromatic plants [2], fruits and vegetables [3], microalgae [4], etc., returning breads with an enhanced nutritional profile. Microalgae have received increased consideration due to the high content of pigments, favourable protein and polyunsaturated fatty acid profiles, phenolics, vitamins, and minerals [5,6,7]. The microalgae *Chlorella luteoviridis*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris*, used prior to May 1997 in Europe, are authorized as food (European Union, Novel Food catalogue). Among the genus *Chlorella*, in the USA, only *Chlorella protothecoides* is included in the GRAS list (FDA, GRAS Notices) and in Australia, all *Chlorella* species (including *Chlorella sorokiniana*) and derivatives for which a novel food application was submitted have been so far considered as traditional food [8]. *Chlorella* sp. is a rich source of proteins (51-58%), lipids (14-22%) and carbohydrates (2-17%) [9]. Those facts have prompted the research of its incorporation in different bakery products. Incorporation of 2% *C. vulgaris* resulted in "high in selenium" cookies [10] whereas the addition level of both 2% and 6% resulted in higher protein and total phenolic contents as well as in higher *in vitro* antioxidant capacity while maintaining texture stability and sensory quality of cookies [11]. According to Batista, et al. [12] crackers with 6% *C. vulgaris* could be claimed to be a "source of protein". Fradique, et al. [13] added *C. vulgaris* (up to 2% w/w) in a pasta recipe rendering pasta with high levels of protein and minerals, without interfering with sensorial quality, cooking and textural properties of the final product. Graça, et al. [4] studied the effect of adding *C. vulgaris* (up to 5% in wheat flour base) on dough and bread quality. Up to 3% addition level they observed a strengthening of the gluten network and thus a positive impact on dough rheology and viscoelastic characteristics. Nevertheless, to fully exploit their potential derived from the pigments and phenolic compounds, the impact of process conditions might be considered, since pH and temperature affect antioxidant activity [14].

It is already stated the importance of food processing on the bioavailability of functional compounds owing to the impact of process conditions on their physicochemical structure [15]. In the particular case of phenolic compounds and their antioxidant capacity, the processing conditions might play a crucial role modulating bioavailability through chemical modifications or cleavage of phenolic compounds-matrixes bonds [16]. Breadmaking is a very complicated process where mechanical constraints are entwined with the microbial action during fermentation encompassing dough acidification and activation of endogenous enzymes [17]. Both endogenous and bacterial enzymes are responsible for the solubilisation of dough components thus increasing their bioavailability and digestibility and/or produce through specific synthetic pathways new bioactive compounds thus improving antioxidative activity [18]. Actually, an increase in the amount of free and bound polyphenols was obtained by fermenting KAMUT® khorasan and durum wheat flours with sourdoughs [19]. The same trend was observed by Ferri, et al. [20] for fermented doughs with sourdoughs containing different lactic acid bacteria strains. Those authors confirmed the positive correlation between short fatty acids with the polyphenol content and antioxidant activity in wheat breads.

The beneficial effect of *C. vulgaris* addition has been related with its nutritional content and antioxidant properties, but when used as ingredient, those could be affected during the process. The aim of this research was to evaluate the impact of breadmaking (dough acidification) on microalga enriched dough and bread. To this end, three different levels of *C. vulgaris* were used (1%, 2% and 3%) aiming to establish the maximum acceptable content of *C. vulgaris* that could be used in industrial applications while maintaining acceptable bread quality. To unravel the effect of simultaneous addition of microalgae and dough acidification on dough properties and bread quality, two sets of the same experiment were performed. The first set contained 10% sourdough and in the second set chemically acidified dough was prepared by adjusting the pH with organic acids so as to reach the pH of the sourdough.

Materials and Methods

Materials

Wheat flour for breadmaking (12.07% of protein (N × 5.7), 12.9% moisture) was purchased from Harinera la Meta S.A. (Valencia, Spain). Commercial *Chlorella vulgaris* microalga flour was purchased from Allmicroalgae (Lisbon, Portugal). The DPPH (2,2-diphenyl-1-picrylhydrazyl) was from Sigma Aldrich, USA, whereas TPTZ (2,4,6-tripyridyl-s-triazine) was obtained from Alfa Aesar, GmbH & Go KG, Karlsruhe, Germany. ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and gallic acid were from J&K Scientific GmbH, Pforzheim, Germany. Folin-Ciocalteu reagent, and methanol (HPLC grade) were obtained from Chem-Lab NV, Zedelgem, Belgium. Iron (III) chloride hexahydrate was from Merk, KGaA, Darmstadt, Germany. All the other chemicals used were of analytical grade.

Chemical composition

The proximate composition of raw materials and breads was determined using the standard methods of the International Organization for Standardization (ISO): moisture (712:2009), protein (16634-2:2016), ash (2171:2007) and total fat (11085:2015). Carbohydrates content was calculated by difference.

Sourdough preparation and cell count

Sourdough of type IV [21] was prepared without the use of baker's yeast. Wheat flours (200 g) were mixed with tap water (126 mL) to obtain a final dough yield (DY) [dough weight ×100 / flour weight] of 163. Fermentation and daily back-slopping (refreshments) were conducted according to Barber, et al. [22]. To initiate fermentation, 60% of water was substituted with previously macerated wheat bran (that contains endogenous microorganisms) with tap water at a ratio of 20:80 for at least 20 min. Daily refreshments were carried out for the 3 successive days by mixing 50% of the previously fermented dough with flour and water (final dough yield of 163). Dough was incubated at 30 °C and RH 80%

under anaerobic conditions. The resulting sourdoughs were freeze-dried, pulverized, packed in plastic bags and stored at 4 °C for further analysis.

Microbial plate counts were determined by plating successive dilutions of sourdough and dough samples onto selective media. Man, Rogosa and Sharpe agar (MRS) and Potato Dextrose Agar (PDA) and standard medium glucose, yeast extract and calcium carbonate (GYC) were used to determined lactic acid bacteria (LAB) and yeasts and acetic bacteria, respectively. MRS plates were anaerobically incubated at 32 °C for 48-72hours, while PDA plates were incubated at 37 °C for 24-48 h and GYC at 30 °C for 72 h, both under aerobic conditions.

Breadmaking procedure

Reference bread doughs (W) conventionally prepared (by using compressed baker's yeast), bread doughs containing sourdough (SW) and bread doughs chemically acidified (AW) containing different levels of microalgae powder (0, 1, 2 or 3%) were prepared. A basic recipe, based on white wheat flour (f.b.) was used: 2% compressed baker's yeast, 1.5% salt (corrected based on the salt present in the microalgae powder added), and the amount of water that dough needs to reach optimum consistency (1.1 Nm) in Mixolab (Chopin, Paris, France). Sourdough containing doughs were prepared using the basic recipe but replacing 10% wheat flour by freeze-dried sourdough. After preliminary assays for checking the final pHs of the sourdough containing doughs, chemically acidified doughs with pH value of 4.90 ± 0.05 were prepared with the basic recipe and adding 0.15% of a mixture 1:4 of acetic acid (100% w/w) and lactic acid (85% w/v) [23]. Ingredients were mixed in a Farinograph 300 g-bowl (Brabender, Germany) at 30 °C for 10 minutes. The dough was allowed to rest for 10 minutes after which, it was divided in portions of 40 g, shaped manually, placed into silicon pans (100 mm length × 50 mm width × 50 mm height) and allowed to ferment at 30 °C till the volume doubles itself. Finally, fermented doughs were baked at 180 °C for 15 minutes and then cooled down at room temperature for 45 minutes. Samples codes indicated the type of bread followed by a number that described the level of microalgae, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0,

SW1, SW2, SW3). Two batches in different days were made for each sample.

Dough characterization during breadmaking

The water absorption (WA) or the amount of necessary water to reach dough optimum consistency (1.1. Nm) for each formulation (without added yeast) was calculated using a Mixolab (Chopin, Paris, France) equipment following the method S defined by the supplier. Consistency of doughs was registered during 30 minutes at 30 °C in the Mixolab. Proofing performance of the doughs was monitored by recording the increment of dough volume (ΔV) every 10 min during 100 minutes. To evaluate the fermentation kinetics, the slope (mL/min) of the volume increase, the maximum volume (mL) and the time to duplicate initial dough volume (2V (min)) were calculated. Samples were analyzed in duplicate.

pH, Total Titratable Acidity (TTA) and acid content in doughs

The pH of fermented doughs was potentiometrically determined using a penetration electrode. The pH of breads was measured after vortexing one gram of bread diluted with 9 mL deionized water. Total Titratable Acidity (TTA) was measured in 10 g of doughs or breads blended with 100 mL distilled water that were titrated with 0.01N NaOH and expressed as mL NaOH/10 g.

Concentrations of D-lactic acid and acetic acid were analyzed using a specific kit Acetic Acid Assay Kit (Acetate Kinase) and D-Lactic Acid Assay Kit (D-Lactate) (Megazyme International, Ireland), respectively, according to the manufacturer's instructions. The results were expressed as g of D-lactic or acetic acid per kilogram of sourdough, fermented dough or bread. Three replicates were averaged for each sample.

Technological characterization of breads

Breads were automatically cut it in 15 mm slice thickness and then characterized. Color of bread crumb and crust was measured using a chroma meter CR-400 (Konica Minolta, Japan). The color was recorded using CIE-L^* a^* b^* scale, where L^* indicates lightness, a^* indicates hue

on a green (-) to red (+) axis, and *b** indicated hue on a blue (-) to yellow (+) axis. Total color difference *(DE**) was calculated with the following equation:

$$\Delta E^* = ((L_r^* - L_i^*)^2 + (a_r^* - a_i^*)^2 + (b_r^* - b_i^*)^2)^{1/2}$$

Where L_r^*, a_r^*, b_r^* are color parameters of reference bread without microalgae, and L_i^*, a_i^*, b_i^* are color parameters of breads containing microalgaes and/or acidified doughs. Crumb hardness (N) was determined using a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) applying a TPA test with a double compression. A piece of crumb (20 mm diameter) was taken from the center of each slice and was compressed up to 50% using a 36 mm aluminum probe. The following parameters were calculated from the compression curve: hardness, springiness, cohesiveness, chewiness, and resilience.

The slice 2D area and crumb structure were evaluated by digital image analysis using the ImageJ software (ImageJ 1.52p, National Institutes of Health, USA) following the method described by Espinosa-Ramírez, et al. [24]. Briefly, images of bread slices were captured using a flatbed scanner (Epson V550, Japan) at 600 dpi of resolution. Then images were improved by splitting channels, taking the green channel for the analysis and enhancing the contrast; for segmentation method a predefined algorithm ("Otsu") was applied. Finally, the 2D slice area (cm^2) was measured in order to analyze the crumb cell distribution, mean cell area (mm^2) and porosity (%). At least four bread loaves for each recipe were used for the above measurements.

Extraction of bioactive compounds

Bioactive compounds from the freeze-dried breads were extracted in 70% aqueous methanol as reported in Skendi, et al. [2] with some modifications. Freeze-dried bread (1.5 g) was first homogenized in 4 mL of 70% methanol on a vortex for 30 seconds and then the extraction was performed in an ultrasonic bath for 15 min. Following, the sample was centrifuged at 2264 x *g* for 15 min and the supernatant was collected. The precipitate was re-dissolved into 3 mL of 70% methanol and extracted for a second time as reported previously. The supernatants were pooled

together and centrifuged once more at 8400 x *g* for 10 min and finally frozen stored until analysis. Extraction of the bioactive compounds from the bread samples was carried out at least in triplicate.

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method reported by Singleton, et al. [25]. For the determination the extract (200 mL) was first oxidized with 800 mL of the Folin-Ciocalteu reagent diluted with water (1:10 v/v) for 2 min. Following, 2mL sodium carbonate 7.5% w/v was added to the mixture and allowed to react for 2 minutes before adding 8 mL of distilled water. Finally, the mixture was incubated in the dark for one hour at room temperature. The absorption of the mixtures was measured at 725 nm and the results were expressed as milligrams of gallic acid equivalents per gram of dry weight basis (mg GAE/gdw).

Determination of antioxidant activity

Three assays were utilized to determine the antioxidant activity of breads based on antiradical activities: a) radical scavenging capacity activity (DPPH), b) radical cation scavenging activity (ABTS) and c) ferric reducing antioxidant power (FRAP). Radical scavenging activity (DPPH) was measured according to Yen and Chen [26]. The extract (150 µl) was mixed with 2.85 mL of 100 µM2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent and allowed to rest for 5 minutes before reading the absorbance at 516 nm.

The radical cation scavenging activity (ABTS) was determined by mixing 100 µL extract with 3.9 mL ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) reagent with an absorbance of 0.70prepared according to Re, et al. [27]. The absorbance of the mixture was measured at 734 nm after reacting for 4 min at room temperature.

The ferric reducing antioxidant power (FRAP) assay was carried out by mixing 100 µl of extract with 3 ml of FRAP reagent for 4 min at 37°C under dark conditions as reported from Benzie and Strain [28]. The FRAP assay consisted of a mixture of three solutions: 20 mM ferric chloride

solution, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 0.3 mM (pH 3.6) acetate buffer in a proportion of 1:1:10, respectively. The absorbance of the mixture was measured at 593 nm against a blank.

For all the three assays the results were expressed as mg Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)equivalents per gram of sample on a dry weight basis (mg TE/g dw). All the measurements for determination of TPC and antioxidant activity were performed at least in duplicate.

Statistical analysis

The results are reported as the average and standard deviation of measurements. Analysis of variance (ANOVA) was applied using Statgraphics Centurion XVII (Statistical Graphics Corporation, UK) and SPSS Statistics 25.0 software (SPSS Inc., Chicago, USA). The Duncan test was used to assess significant differences ($P < 0.05$) among samples. The statistical analysis of TPC and antioxidant activity includes the application of general linear model for multiple variables as well as correlation analysis among the variables. Additionally, a principal component analysis (PCA) was carried out to detect possible discrimination among samples.

Results and Discussion

Different levels of *C. vulgaris* powder (0, 1, 2 or 3%) were incorporated into breads. Based on the optimum amount reported by Graça, et al. [4], 3% was selected as the maximum incorporation level. To evaluate the potential impact of acidification, doughs fermented with a spontaneous sourdough and doughs chemically acidified were carried out and the resulting breads evaluated. The final yeast, LAB and acetic acids counts (CFU/g) in the lyophilized sourdough were 2×10^3, 3×10^8; and $1x10^5$, respectively.

Dough characteristics

Dough rheological properties during mixing were determined using the Mixolab, which also allowed defining the water absorption to be used in

the breadmaking for obtaining doughs with constant consistency. The addition of microalgae powder resulted in a steady increase of the water absorption required to reach constant consistency of 1.1 Nm (Table 1). This increase might be expected considering that microalgae are a source of hydrocolloids, which are generally recognized for their ability to enhance water absorption in breadmaking [29]. Specifically, in *C. vulgaris*-based crackers, Batista, et al. [12] reported that the addition of high concentrations of microalgae biomass could lead to a weaker gluten network, unable to efficiently trap gas bubbles and water molecules. Batista, et al. [30] also reported that *A. platensis* biomass (commercially known as spirulina) impairs starch gelatinization in model gel systems, namely by increasing the gelatinization temperature, likely due to competition for water binding zones during the hydration of starch granules. In fact, during dough mixing, the available free water is partitioned between microalgae and wheat flour hydrophilic sites, which might also impair gluten protein network development. It is possible that the formation of a weaker gluten network might result in the collapse of small gas cells into larger cavities, which might influence gas and water trapping during baking [12]. Likewise, sourdough containing doughs required significantly higher water absorption compared to their counterparts (reference and chemically acidified dough). The presence of microalgae at different levels did not significantly modify the doughs' stability during mixing (Figure S1). All doughs showed a good performance during mixing for 30 min, although those containing sourdough showed some decay after 20 min of mixing. The effect was more pronounced in samples with added microalgae powder, showing that the interaction between microalgae and sourdough resulted in a slight weakening of the dough when overmixed. *C. vulgaris* effect on dough mixing agrees with results reported by [4] within the level range used in the present study, showing the increase in water absorption but without affecting dough stability, effects that these authors ascribed to the supplemented proteins from the microalga. Same absorption trend has been reported with red and brown seaweeds powder when added up to 8% to wheat flour [31,32], due to their high water retention capacity.

The fermentation of the doughs was monitored to compare breads obtained with the same level of fermentation. Doughs were baked when dough volume doubled itself and to reach that level, doughs containing

sourdough (SW0) showed the faster fermentation (50 min) compared to reference dough (W0) (60 min) and chemically acidified dough (AW0) (60 min) (Figure S2 and Table S1). Addition of the microalga in the recipe accelerated the fermentation of the chemically acidified doughs (40 min) but not of the reference dough and sourdough. Therefore, *C. vulgaris* addition up to 3% did not affect the fermentation kinetics by bakers' yeast as reported by Graça, et al. [4], but in the presence of acids an underlying more complex mechanism than the obvious "lowering of pH" might affect fermentation, thus speeding the process. The compounds comprising *C. vulgaris* could represent an attractive media for yeast growth. It is recognized that it is rich in amino acids, fatty acids and minerals [9]. The presence of these compounds in certain amounts could represent the right combination that possibly boosts the yeast metabolism and increase the fermentation rate. Nevertheless, that effect was not observed in the presence of sourdough due to the LAB competition with yeast for nutrients.

As expected, the presence of the sourdough significantly ($P<0.05$) decreased the dough pH and increased the TTA, which was similar to that of chemically acidified doughs (Table 1). Addition of the microalga decreased only the pH of the reference dough without affecting significantly that of other types of dough that already had lower pH due to the presence of the sourdough or acidification process. The TTA values in the reference dough were affected by the presence of the microalga but not by the addition level, whereas a progressive increase with the fortification level was observed for the other two types of dough.

Table 1. Impact of the microalgae level and type of dough on water absorption (WA), pH, TTA and acids content in doughs and breads. Samples codes indicated the type of bread followed by a number that described the level of microalgae, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Sample	WA (g/100g)	pH		TTA (ml NaOH/10 g)		D-Lactic acid (g/kg)		Acetic acid (g/kg)	
		Dough	Bread	Dough	Bread	Dough	Bread	Dough	Bread
W0	56.6	5.48 ± 0.00^{e}	5.82 ± 0.02^{f}	4.2 ± 0.1^{a}	0.4 ± 0.0^{a}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	1.86 ± 0.17^{a}	0.30 ± 0.07^{abcd}
W1	56.8	5.38 ± 0.01^{d}	5.77 ± 0.01^{e}	5.3 ± 0.2^{bc}	0.6 ± 0.1^{b}	0.07 ± 0.02^{a}	0.04 ± 0.00^{a}	2.06 ± 0.16^{abcd}	0.27 ± 0.03^{abc}
W2	58.5	5.39 ± 0.01^{d}	5.81 ± 0.01^{ef}	5.6 ± 0.2^{cd}	0.6 ± 0^{c}	0.03 ± 0.01^{a}	0.06 ± 0.01^{ab}	1.98 ± 0.13^{abcd}	0.26 ± 0.07^{ab}
W3	60.4	5.40 ± 0.01^{d}	5.83 ± 0.04^{f}	5.3 ± 0.9^{bc}	0.7 ± 0^{d}	0.06 ± 0.00^{a}	0.06 ± 0.02^{ab}	1.93 ± 0.19^{ab}	0.20 ± 0.06^{a}
AW0	56.6	4.88 ± 0.02^{ab}	5.38 ± 0.01^{c}	4.8 ± 0^{ab}	0.7 ± 0^{d}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}	2.30 ± 0.09^{e}	0.52 ± 0.07^{d}
AW1	56.8	4.86 ± 0.01^{a}	5.34 ± 0.01^{b}	6.3 ± 0.2^{de}	0.8 ± 0^{f}	0.06 ± 0.01^{a}	0.09 ± 0.04^{ab}	1.97 ± 0.04^{abc}	0.49 ± 0.14^{cd}
AW2	58.5	4.88 ± 0.03^{abc}	5.44 ± 0.01^{d}	6.9 ± 0.1^{e}	0.8 ± 0^{ef}	0.06 ± 0.01^{a}	0.07 ± 0.03^{ab}	2.01 ± 0.05^{abcd}	0.44 ± 0.13^{bcd}
AW3	60.4	4.88 ± 0.01^{ab}	5.45 ± 0^{d}	8.2 ± 0.0^{f}	0.9 ± 0^{g}	0.04 ± 0.00^{a}	0.12 ± 0.03^{b}	1.89 ± 0.06^{a}	0.37 ± 0.11^{abcd}
SW0	61.5	4.90 ± 0.01^{bc}	5.27 ± 0.03^{a}	4.9 ± 0.1^{ab}	0.6 ± 0^{b}	0.48 ± 0.06^{b}	0.51 ± 0.04^{c}	2.15 ± 0.04^{bcde}	0.27 ± 0.08^{abc}
SW1	63.9	4.91 ± 0.01^{c}	5.32 ± 0.02^{b}	6.3 ± 0.4^{e}	0.7 ± 0^{d}	0.49 ± 0.05^{b}	0.54 ± 0.09^{c}	2.17 ± 0.21^{cde}	0.51 ± 0.18^{d}
SW2	64.1	4.89 ± 0.01^{abc}	5.38 ± 0.01^{c}	7.0 ± 0.4^{e}	0.8 ± 0de	0.44 ± 0.06^{b}	0.56 ± 0.03^{c}	2.13 ± 0.01^{bcde}	0.30 ± 0.09^{abcd}
SW3	64.6	4.88 ± 0.01^{ab}	5.35 ± 0.01^{bc}	9.0 ± 0.4^{g}	0.7 ± 0de	0.49 ± 0.04^{b}	0.68 ± 0.03^{d}	2.20 ± 0.03^{de}	0.31 ± 0.02^{abcd}
P-value									
Type of dough		**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0072**	**0.0010**
Chlorella vulgaris level		0.0562	**0.0008**	**0.0000**	**0.0000**	0.4078	**0.0008**	0.5900	0.1169

Means with different letters within the same column denote significant differences ($P \leq 0.05$) among the means, as determined by the Duncan test.

The type of dough significantly ($P<0.05$) affected the content of lactic and acetic acids in fermented doughs (Table 1). Sourdough containing doughs showed higher content of lactic acid than their counterparts without sourdough, whereas, chemically acidified dough exhibited the highest concentration of acetic acid (Table 1). The ANOVA statistical analysis indicated that microalga addition did not affect significantly the amount of lactic and acetic acids of the doughs. Lactic and acetic acids are the main metabolites of the sourdough processes; lactic acid bacteria being a part of the stable biota of the mature sourdoughs [17]. In consequence, compared to conventionally produced breads with baker's yeast, sourdough fermentation increased lactic and acetic acid values of doughs, increasing acidity and reducing pH, whereas the presence of microalga, no related to the level of incorporation, increased the acidity and reduced the pH in minor extent possibly due to the presence of short fatty acids diverse than lactic and acetic acids.

Bread technological characteristics and composition

The presence of microalga to doughs and respective breads was readily visible from the change in their color (Figure 1), showing a greenish color that was intensified with the amount of C. *vulgaris* powder. Nevertheless, a nice crumb was obtained from all the recipes. At first glance it was observed that acidified breads and sourdough breads had lighter crumb than their reference counterparts. As it was visually detected, microalga powder significantly ($P<0.05$) altered the color of both the crust and crumb of the breads (Table S2). The crust showed a steady reduction of the luminosity when increasing the amount of microalga, independently of the type of dough. Likewise, b^* parameter decreased with the amount of microalga in all the breads, thus reducing the yellowish tone of the crust. The total color difference (ΔE^*) confirmed the drastic color change, since values were higher than 3.0 [31]. Similarly, the luminosity of the crumb was reduced when augmenting the level of microalga powder. Conversely to the trend observed in the crust, b^*values of the crumbs increased in the presence of microalga, with a steady decrease when augmenting the level of microalga powder, although in sourdough breads, no trend was detected. Again, the total color difference (ΔE^*) of the crumb confirmed the substantial change in the color of the crumbs

containing the microalga but also the very different color induced by the type of dough (AW0, SW0).

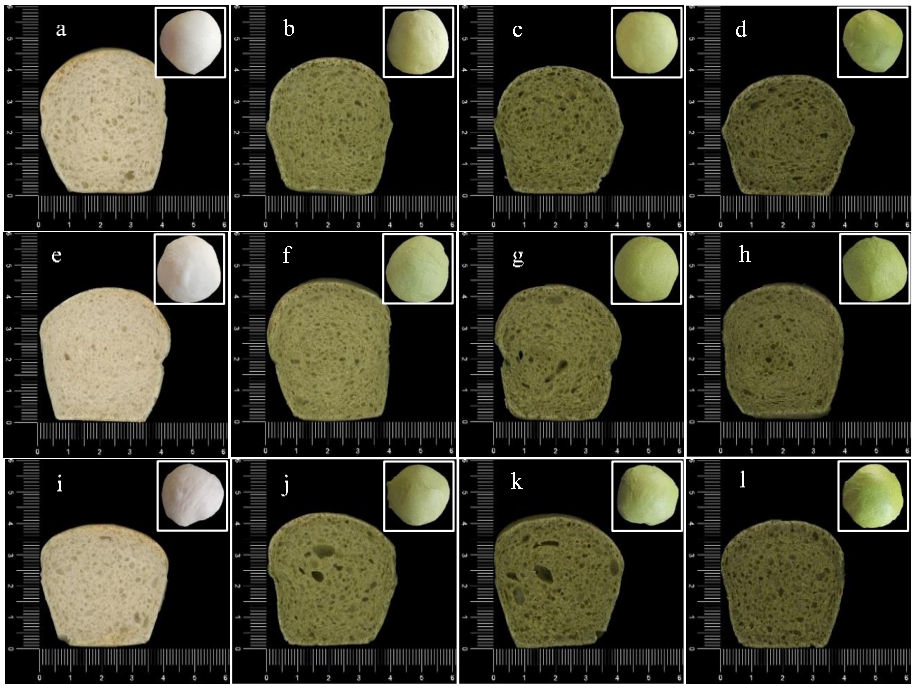


Figure 1. Captured images of doughs and bread slices. Reference (W): a - d, Chemically acidified (AW): e - h and Sourdough (SW): i - l, with 0, 1 ,2 or 3% of C. vulgaris powder from left to right.

Breads prepared with sourdough had significantly ($P<0.05$) lower pH followed by breads obtained from chemically acidified doughs. Breads containing *C. vulgaris* had significantly higher pH than the respective controls, with exception of the reference recipe, in which no trend was observed when increasing the level of the microalga. In regard to concentration of organic acids in breads, *D*-lactic acid enhanced its concentration compared to its dough counterparts, either by type of dough and microalgae levels, with exception of the reference recipe (Table 1). Meanwhile, acetic acid was significantly lower in breads than in doughs (Table 1). In a similar way, Brandt [33] noticed that, during baking or drying, acetic acid is volatilized, while lactic acid is more stable, due to the non-volatility of this compound.

When the bread technological characteristics were statistically analyzed considering the type of dough and the microalgae level, it was observed that the type of dough significantly affected the bread hardness and the mean cells area of the crumb, whereas the level of microalgae significantly affected the bread 2D slice area (related to bread volume) and the porosity of the crumb, as well (Table 2). The textural properties of the breads are included in Table S1. The 2D crumb area decreased when increasing microalgae level, with exception of sourdough-bread, where no significant direct trend was observed. In spite of reference and acidified doughs were fermented to the same level, the oven rise during baking seems to be affected by microalgae incorporation, leading to a reduction of the bread expansion and in consequence the bread volume. Arufe, et al. [31] described a decrease in the dough porosity during fermentation, in the presence of brown seaweeds, which was associated to the high elongational viscosity provided by the seaweeds, but that effect was more ascribed to proofing operation than baking. Similar bread volume reduction was reported by Graça, et al. [4], who described noticeable changes in the crumb structure, with bigger gas cells due to dough weakening when adding *C. vulgaris*. The same trend was observed in the present study, microalgae addition led to an increase in porosity and the size of gas cells showed a steady increase, but the effect was only significant ($P<0.05$) at the higher microalgae level tested. Probably, the adapted fermentation time limited the bubbles coalescence during fermentation, minimizing the microalgae impact on crumb structure, and only the highest level tested impacted on the dough viscoelasticity.

Breads containing sourdough showed softer crumbs, although slight increase was observed when increasing the microalgae level. Taking into account the crumb image analysis, in general those breads displayed higher porosity and bigger gas cells that suggests a more open crumb structure due to dough bubble coalescence, likely derived from the weakening of gluten matrix due to both microalgae addition and lactic acid bacteria metabolites.

Table 2. Technological characteristics of breads supplemented with different levels (0, 1, 2, 3%) of C. vulgaris powder. Reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3) and sourdough breads (SW0, SW1, SW2, SW3) were evaluated.

Sample	2D Slice Area (cm^2)	Hardness (N)	Mean cell area (mm^2)	Porosity (%)
W0	15.6 ± 0.4^{ef}	2.6 ± 0.2^{g}	0.61 ± 0.12^{bc}	26.96 ± 4.03^{abcd}
W1	14.4 ± 0.4^{abc}	2.3 ± 0.2^{f}	0.61 ± 0.08^{bc}	31.96 ± 3.99^{de}
W2	13.8 ± 0.1^{abc}	2.8 ± 0.2^{h}	0.60 ± 0.05^{bc}	31.41 ± 0.72^{cde}
W3	13.4 ± 0.3^{a}	2.6 ± 0.2^{gh}	0.88 ± 0.04^{ef}	36.31 ± 2.07^{ef}
AW0	16.0 ± 0.5^{f}	1.9 ± 0.1^{cd}	0.43 ± 0.05^{a}	22.46 ± 1.08^{ab}
AW1	15.4 ± 0.2^{def}	1.7 ± 0.1^{abc}	0.48 ± 0.08^{ab}	24.82 ± 2.42^{ab}
AW2	14.5 ± 0.1^{bcd}	2.1 ± 0.2^{def}	0.50 ± 0.03^{ab}	22.04 ± 2.20^{a}
AW3	13.8 ± 1.0^{abc}	2.2 ± 0.2^{ef}	0.65 ± 0.05^{c}	35.19 ± 1.11^{ef}
SW0	13.6 ± 0.0^{ab}	1.6 ± 0.3^{ab}	0.55 ± 0.01^{abc}	25.40 ± 1.99^{abc}
SW1	14.4 ± 0.7^{abc}	1.4 ± 0.2^{a}	0.69 ± 0.02^{cd}	28.58 ± 4.17^{bcd}
SW2	14.7 ± 0.1^{cde}	1.8 ± 0.1^{bc}	0.80 ± 0.09^{de}	32.03 ± 1.57^{de}
SW3	13.9 ± 0.1^{abc}	1.9 ± 0.2^{cde}	0.97 ± 0.02^{f}	38.80 ± 2.52^{f}
P-value				
Type of dough	0.0839	**0.0000**	**0.0000**	**0.0014**
C. vulgaris level	**0.0202**	**0.0000**	**0.0000**	**0.0002**

*Means with different letters within a column were significantly different ($P<0.05$).

The chemical composition of the breads was significantly affected by the microalgae level, with exception of the fat content, and no significant differences were induced with the type of dough (Table 3). Despite the different water absorption of the diverse doughs, no pattern was identified among the samples. As expected, the protein as well as the ash contents were significantly increased with the microalga's addition due to the higher presence of these two components in *C. vulgaris*. This is also reported in the literature for other bakery products [12]. On the other hand, addition of *Chlorella vulgaris* did not significantly affected the fat content of the resulting breads.

Table 3. Chemical composition in g /100 g (as is) of breads supplemented with seaweed powder. Samples codes indicated the type of bread followed by a number that described the level of seaweed, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3)

Type of dough	Moisture	Protein	Ash	Total Fat	Carbohydrates
W0	35.39 ± 0.51^{bc}	12.17 ± 0.02^{a}	0.84 ± 0.05^{a}	0.24 ± 0.06	51.36
W1	35.16 ± 0.13^{b}	12.67 ± 0.03^{c}	0.99 ± 0.03^{b}	0.25 ± 0.05	50.94
W2	35.41 ± 0.29^{bc}	13.27 ± 0.00^{e}	1.10 ± 0.05^{cde}	0.22 ± 0.06	50.00
W3	35.49 ± 0.96^{bcd}	13.75 ± 0.06^{f}	1.12 ± 0.08^{def}	0.25 ± 0.07	49.38
AW0	35.30 ± 0.70^{b}	12.25 ± 0.03^{a}	0.86 ± 0.02^{a}	0.21 ± 0.04	51.28
AW1	33.89 ± 0.10^{a}	12.93 ± 0.06^{d}	0.99 ± 0.05^{bc}	0.25 ± 0.02	51.94
AW2	34.79 ± 0.32^{ab}	13.31 ± 0.11^{e}	1.04 ± 0.08^{bcd}	0.25 ± 0.02	50.60
AW3	35.01 ± 0.06^{b}	13.90 ± 0.03^{g}	1.21 ± 0.07^{f}	0.28 ± 0.03	49.61
SW0	35.68 ± 0.55^{bcd}	12.18 ± 0.10^{a}	0.83 ± 0.04^{a}	0.26 ± 0.10	51.05
SW1	36.46 ± 0.17^{cd}	12.50 ± 0.11^{b}	0.97 ± 0.05^{b}	0.26 ± 0.05	49.81
SW2	35.02 ± 0.70^{b}	13.22 ± 0.02^{e}	1.04 ± 0^{bcd}	0.27 ± 0.03	50.45
SW3	36.57 ± 0.06^{d}	13.66 ± 0.04^{f}	1.17 ± 0.02^{ef}	0.24 ± 0.09	48.36
P-value					
Type of dough	0.0043	0.3802	0.6067	0.8118	0.1112
Seaweed quantity	**0.3172**	**0.0000**	**0.0000**	0.9610	**0.0106**

*Means with different letters within a column were significantly different ($P<0.05$).

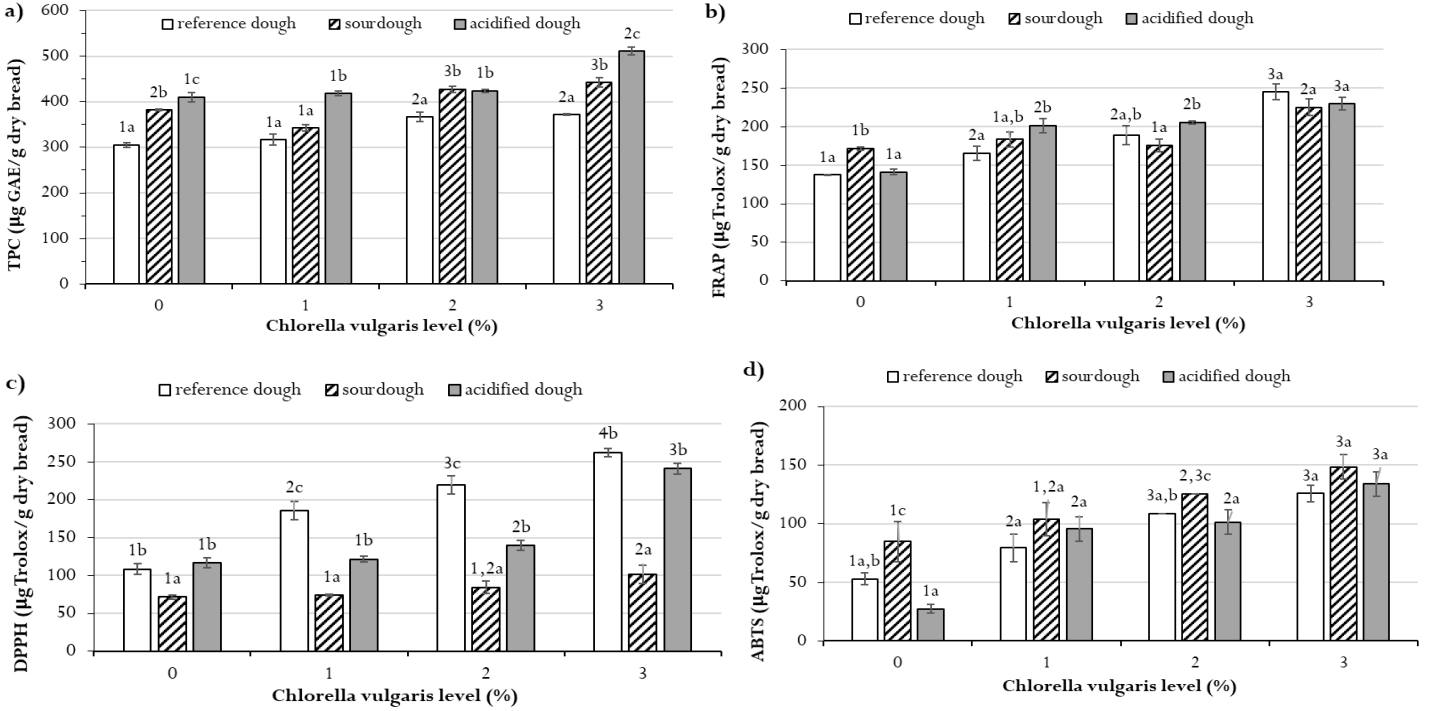
Variation of total phenolics and antioxidant activity among bread samples

The TPC and antioxidant activity of breads were significantly increased with increasing level of the microalgae (Figure 2). The application of General Linear Model revealed that only microalgae levels equal or greater than 2% were able to increase significantly the TPC. Furthermore, it was found that the type of dough plays an important role in the amount of TPC. The TPC increases in the following order: reference dough<sourdough<acidified dough. It is recognized that low pH values facilitate the extraction of phenolic compounds. Thus, sourdough and acidified breads theoretically should have more extracted phenolics in the free form compared to the control dough that has higher pH among the doughs. Since in the case of acidified dough the dough matrix is exposed for longer time to lower pH than in the sourdough, it is probable that this could explain the greater TPC content of acidifies dough. FRAP antioxidant activity was also significantly increased with increasing of the microalgae level compared to the reference (bread without *C. vulgaris*) regardless the type of dough used (Figure 2b). It seems that type of dough used for bread preparation played also a significant role in the amount of increase in the FRAP values (reference dough ≤sourdough ≤ acidified dough). DPPH and ABTS activity of breads augmented with increasing of the microalgae level for all the types of dough (Figure 2c, d), indicating higher levels of hydrophilic compounds like ascorbic acid and hydrophobic compounds like pigments, respectively. Again, the type of dough plays an important role in the increase observed. Sourdough seemed to increase ABTS values at a greater extent than acidified dough and reference dough, that showed a similar magnitude of increase. The opposite was observed when comparing the effect of dough type on the increase of DPPH values, with greater DPPH values in the reference dough followed by acidified dough and then sourdough. It was surprising the significant increase observed in DPPH values in breads made with 3% microalga and acidified dough. Likely, the combination of high amount of acetic acid and the low lactic acid content might be responsible of that response. Considering that the ABTS method has higher sensitivity towards hydrophobic compounds, results could be related with such compounds released by lactic acid

bacteria, whereas DPPH with higher sensitivity towards hydrophilic compounds were closely related to reference dough.

In the present study, the total phenolic content showed significantly moderate correlation with antioxidant activity of breads determined by FRAP (r= 0.541) and ABTS (r= 0.486) assays. Nevertheless, no correlation was observed between the antioxidant activity measured by DPPH assay and the TPC. FRAP antioxidant activity correlated very well with ABTS (r= 0.816) and moderately with DPPH (r= 0.520), while the data obtained from DPPH assay did not correlate with those gained from ABTS assay. Phenolic compounds must be first released from the bread matrix during extraction in order to be detected for their antioxidant activity. Their chemical structure as well as the food matrix interactions (such as with carbohydrates, proteins and lipids) are factors that impede bioaccessibility and bioavailability of phenolics [15,16]. This could explain the small increase in the amount of phenolic compounds with increasing of microalgae level as well as the different patterns among the three assays. The literature cited several possible processing conditions (such as mechanical processing, enzymatic and chemical treatments, thermal processing, pasteurization and sterilization, extrusion cooking, and bioprocessing, drying, ultrasounds, high pressure, pulsed electric fields, etc.) that affect the release of phenolics from the food matrix [16,34]. It is obvious that the type of fermentation plays an important role not only in the amount of phenolics but also in the antioxidant activity of the bread. Although the amount of free phenolic compounds detected in the acidified dough bread was higher than in sourdough and reference bread only FRAP assay data follow the same trend. However, with reference to DPPH and ABTS, reference dough and sourdough bread showed the highest antioxidant activity in each respective assay. According to Skendi, et al. [35], factors such as mechanisms of action of the applied assays, presence of different compounds with different antioxidant activity, synergistic/antagonistic effects between phenolic compounds have an important effect on the overall antioxidant activity of the extracts. Thus, fermentation type affects not only the amount of bioactive compounds [20] but also their profile since other compounds may be synthesized resulting in an alteration of the antioxidant activity[18].

Figure 2. Total phenolic content (TPC, expressed as µg GAE/g of bread dw, a), and antioxidant activity (ferrous reducing capacity (FRAP); b), DPPH free radical scavenging activity; c), and ABTS scavenging activity; d), expressed as µg Trolox equivalent (TE)/g of bread dw, for the different breads fortified with *C. vulgaris* at three addition levels. Different numbers above error bars within the same type of bread indicate significant differences ($P \leq 0.05$) among the means, as determined by the Duncan's multiple range test. Different letters above error bars within the same microalgae concentration indicate significant differences ($P \leq 0.05$) among the means, as determined by the Duncan's multiple range test.



Principal component analysis

A principal component analysis (PCA) was carried out with all the variables evaluated to identify possible discrimination among samples containing microalgae and the impact of the dough type (Figure 3). Two components were able to explain 63.3% of the samples' variability. Component 1 explained 37% whereas Component 2 described 26.3% of the variation.

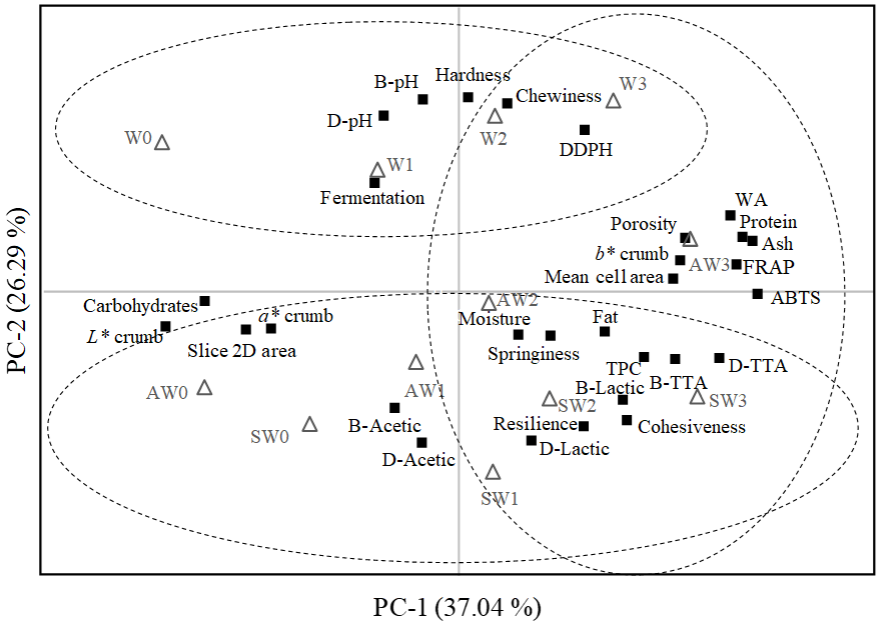


Figure 3. Plot (PC 1 × PC 2) of the set of bread samples and evaluated variables obtained by principal component analysis (PCA). Samples are labelled as in the text. pH, TTA and acids content preceded by D or B are referred to dough or bread, respectively. Dotted lines are grouping the samples obtained from different type of doughs.

The score plot revealed that samples were grouped based on the dough type, with clear discrimination between reference breads and those containing acidified doughs (sourdough or chemically acidified). Component 1 allowed discrimination of the breads obtained from different type of doughs, whereas Component 2 discriminated between reference breads and acidified breads (sourdough or chemically acidified). Breads containing microalgae were located in the positive axis of

Component 1, with exception of AW1, being discriminated by the lactic acid content and TTA of doughs and breads, the crumb structure (porosity and gas cells size), the textural properties, breads composition and the total phenolic content and antioxidant activities. However, Component 2 allowed better discrimination of the acidified breads that were grouped in the negative axis of PC2, with exception of AW3, owing their textural properties, acids content, total phenolic content and ABTS activity. Furthermore, reference breads were grouped in the positive axis of PC2, mainly due to their higher pHs, longer fermentation, higher crumb hardness and DDPH activity. Therefore, the PCA confirmed the role of acid doughs for promoting the antioxidant activity and TPC when incorporating *Chlorella vulgaris* into breadmaking process.

Conclusions

Incorporation of *C. vulgaris* into wheat breads allowed obtaining breads with innovative technological characteristics but also enriched in bioactive compounds. However, its supplementation to acidified doughs permitted to enhance the antioxidant activity. The incorporation of *C. vulgaris* into bread recipes required the water absorption adjustment but no further impact on dough stability was observed. Breads enriched with *Chlorella vulgaris* up to 3% were mainly characterized by intense green hue, slightly lower slice 2D area and higher crumb hardness that was associated to the thicker gas cell walls compared to the control bread. Particularly, in microalgae enriched breads, the use of chemically acidified doughs or sourdoughs allows enhancing the TPC and antioxidant activity in the resulting breads. Nevertheless, antioxidant assays suggested differences in the profile and amount of phenolics released since the increase in the antioxidant activity was different among the prepared breads. Thus, to optimize the health benefits of *Chlorella vulgaris* in breads, it would be advisable to use acidic doughs, prepared chemically or by applying sourdough fermentation.

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

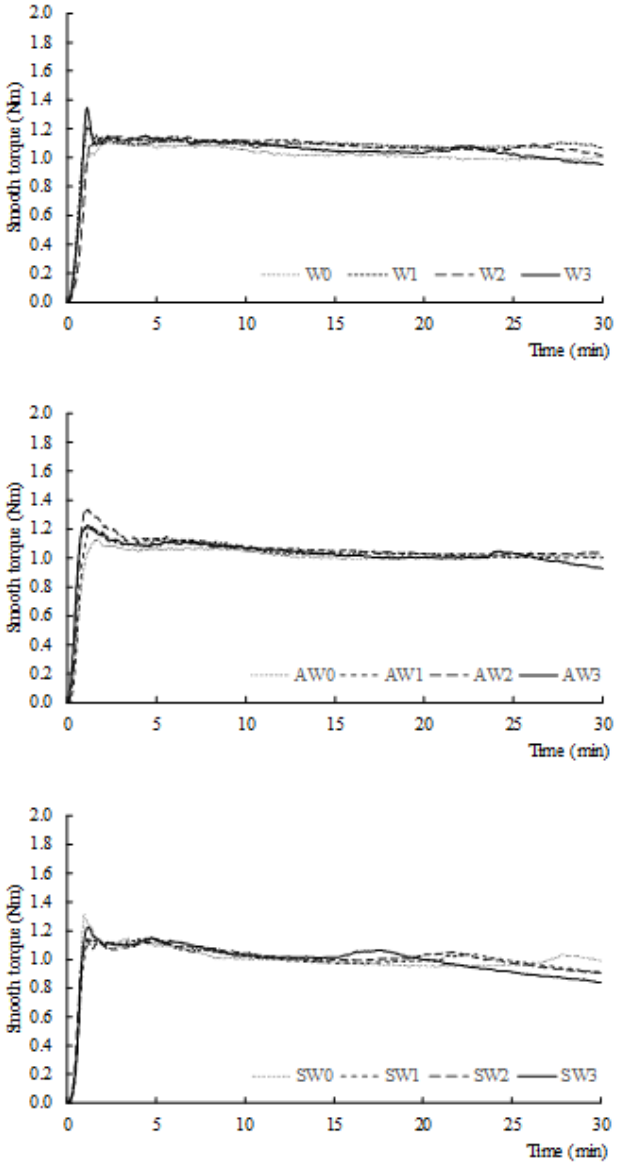

Figure S1. Plots recording dough consistency by using the Chopin Mixolab. Samples codes indicated the type of bread followed by a number that described the level of C. vulgaris, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

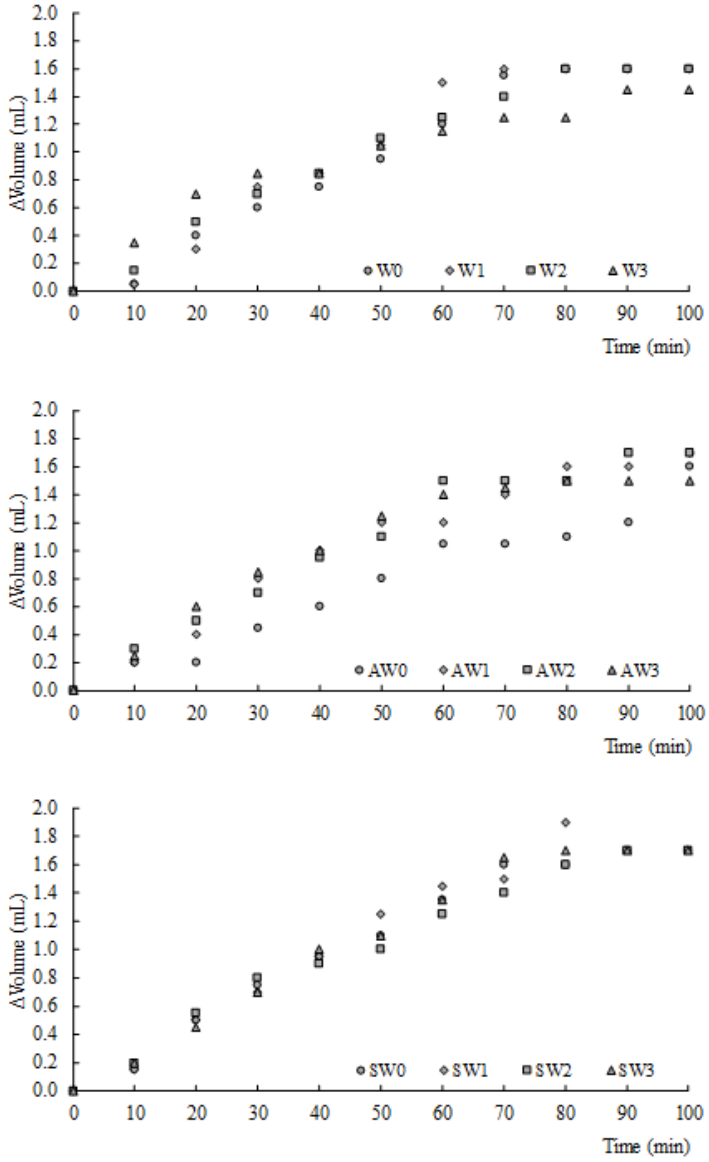

Figure S2. Effect of *C. vulgaris* powder and acidification on the fermentation kinetics of bread doughs. Samples codes indicated the type of bread followed by a number that described the level of *C. vulgaris*, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Table S1. Parameters defining the kinetic of dough fermentation and the bread textural properties. Samples codes indicated the type of bread followed by a number that described the level of *C. vulgaris*, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Type of dough	Fermentation kinetics			Textural properties			
	Time 2V (min)	Slope (mL/min)	Max. ΔV (mL)	Springiness	Cohesiveness	Chewiness (N)	Resilience
W0	60 ± 0^{d}	0.024 ± 0.001	1.60 ± 0^{abc}	0.992 ± 0.001	0.850 ± 0.006^{a}	2.2 ± 0.2^{e}	0.493 ± 0.007^{a}
W1	58 ± 1^{c}	0.018 ± 0.00	1.60 ± 0^{abc}	0.992 ± 0.001	0.912 ± 0.007^{b}	2.1 ± 0.2^{de}	0.512 ± 0.007^{b}
W2	60 ± 1^{cd}	0.019 ± 0.002	1.60 ± 0^{abc}	0.994 ± 0.002	0.942 ± 0.009^{cd}	2.7 ± 0.2^{f}	0.513 ± 0.005^{b}
W3	70 ± 0^{e}	0.011 ± 0	1.45 ± 0.07^{a}	0.994 ± 0.001	0.954 ± 0.006^{efg}	2.5 ± 0.2^{f}	0.538 ± 0.008^{d}
AW0	59 ± 1^{cd}	0.020 ± 0	1.60 ± 0^{abc}	0.993 ± 0.001	0.953 ± 0.007^{ef}	1.8 ± 0.1^{bc}	0.526 ± 0.008^{c}
AW1	40 ± 0^{a}	0.017 ± 0	1.80 ± 0^{c}	0.993 ± 0.001	0.950 ± 0.008^{de}	1.6 ± 0.2^{ab}	0.516 ± 0.011^{b}
AW2	41 ± 1^{a}	0.025 ± 0.001	1.70 ± 0^{bc}	0.993 ± 0.001	0.945 ± 0.002^{cde}	1.9 ± 0.2^{cde}	0.514 ± 0.012^{b}
AW3	40 ± 0^{a}	0.019 ± 0.00	1.50 ± 0.14^{ab}	0.992 ± 0.002	0.936 ± 0.008^{c}	2.0 ± 0.2^{de}	0.512 ± 0.011^{b}
SW0	50 ± 0^{b}	0.020 ± 0.00	1.70 ± 0^{bc}	0.993 ± 0.002	0.960 ± 0.007^{fgh}	1.4 ± 0.1^{a}	0.542 ± 0.004^{d}
SW1	49 ± 1^{b}	0.022 ± 0.00	1.80 ± 0.14^{c}	0.995 ± 0.002	0.963 ± 0.008^{gh}	1.4 ± 0.2^{a}	0.559 ± 0.005^{e}
SW2	50 ± 0^{b}	0.015 ± 0.002	1.70 ± 0.14^{bc}	0.992 ± 0.001	0.978 ± 0.011^{i}	1.7 ± 0.1^{bc}	0.546 ± 0.006^{d}
SW3	50 ± 0^{b}	0.018 ± 0.004	1.70 ± 0.14^{bc}	0.994 ± 0.002	0.966 ± 0.005^{h}	1.9 ± 0.2^{cd}	0.548 ± 0.005^{d}
P-value							
Type of dough	**0.0000**	0.3743	**0.0046**	0.1547	**0.0000**	**0.0000**	**0.0000**
C. vulgaris quantity	0.1202	0.1145	**0.0120**	0.4525	**0.0001**	**0.0000**	**0.0273**

Table S2. Crust and crumb color of breads supplemented with different levels of *C. vulgaris* powder. Samples codes indicated the type of bread followed by a number that described the level of *C. vulgaris*, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Type of dough	Crust color				Crumb color			
	*L**	*a**	*b**	Δ*E** crust	*L**	*a**	*b**	Δ*E** crumb
W0	68.37 ± 1.69^{h}	4.68 ± 0.93^{c}	32.81 ± 1,65	-	68.51 ± 2.22^{i}	-0.98 ± 0.14^{g}	14.96 ± 0.62^{a}	-
W1	51.01 ± 0.61^{ef}	-1.69 ± 0.85^{ab}	30.51 ± 0,57^{e}	18.66 ± 0.63^{bc}	48.87 ± 1.01^{f}	-6.91 ± 0.10^{a}	32.09 ± 0.61^{d}	26.73 ± 1.10^{d}
W2	45.17 ± 0.72^{d}	-1.91 ± 0.8^{ab}	27.93 ± 0,53^{d}	24.63 ± 0.69^{d}	39.83 ± 1.05^{c}	-5.76 ± 0.13^{c}	31.42 ± 0.49^{d}	33.05 ± 1.30^{f}
W3	39.32 ± 0.78^{b}	-2.57 ± 0.33^{ab}	23.52 ± 0,57^{b}	31.36 ± 0.81^{e}	34.16 ± 1.35^{a}	-4.97 ± 0.24^{d}	29.14 ± 0.65^{c}	37.39 ± 1.16^{h}
AW0	71.55 ± 1.95^{i}	5.34 ± 1.77^{c}	33.54 ± 2,24^{h}	4.42 ± 1.35^{a}	72.19 ± 1.30^{j}	-0.90 ± 0.11^{g}	13.88 ± 0.79^{a}	3.87 ± 1.44^{a}
AW1	53.09 ± 1.53^{f}	-0.32 ± 1.39	32.04 ± 0,42^{fg}	16.17 ± 1.19^{b}	53.69 ± 1.60^{g}	-6.21 ± 0.10^{b}	31.46 ± 0.49^{d}	22.81 ± 1.34^{c}
AW2	43.78 ± 1.89^{cd}	-0.62 ± 1.67^{b}	27.88 ± 0,93^{d}	27.22 ± 4.18^{de}	43.96 ± 0.93^{d}	-5.66 ± 0.13^{c}	32.03 ± 0.47^{d}	29.91 ± 0.91^{e}
AW3	41.31 ± 0.67^{bc}	-2.51 ± 0.33^{ab}	24.51 ± 0,47^{b}	29.21 ± 0.59^{ef}	38.29 ± 1.10^{c}	-5.00 ± 0.09^{d}	29.72 ± 0.37^{c}	33.88 ± 0.98^{fg}
SW0	65.03 ± 0.91^{g}	7.51 ± 0.91^{c}	36.19 ± 1,55^{i}	5.61 ± 1.75^{a}	63.75 ± 1.80^{h}	-0.95 ± 0.13^{g}	14.71 ± 1.23^{a}	6.40 ± 4.48^{b}
SW1	49.31 ± 0.86^{e}	-1.02 ± 0.67^{ab}	30.91 ± 0,49^{ef}	20.01 ± 0.83^{c}	46.83 ± 1.21^{e}	-5.76 ± 0.15^{c}	30.69 ± 1.08^{cd}	27.42 ± 0.83^{d}
SW2	43.25 ± 4.34^{cd}	-4.21 ± 7.79^{a}	25.73 ± 0,72^{c}	28.32 ± 5.49^{ef}	36.52 ± 2.45^{b}	-4.71 ± 0.24^{e}	29.26 ± 1.40^{c}	35.31 ± 1.70^{gh}
SW3	35.01 ± 6.41^{a}	-1.39 ± 0.25^{ab}	22.15 ± 0,34^{a}	35.59 ± 6.10^{f}	34.32 ± 1.34^{a}	-4.26 ± 0.31^{f}	27.50 ± 3.31^{b}	36.70 ± 1.14^{h}
P-value								
Type of dough	**0.0000**	0.5027	0.1077	**0.0015**	**0.0000**	**0.0000**	**0.0010**	**0.0000**
C. vulgaris quantity	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**

*Means with different letters within a column were significantly different ($P<0.05$).

II. Functional and nutritional replacement of gluten in gluten-free yeast-leavened breads by using β-conglycinin concentrate extracted from soybean flour[1]

*Johanan Espinosa-Ramírez[a,b], Raquel Garzón[a], Sergio O. Serna-Saldivar[b], Cristina M. Rosell[a]**

*[a]Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain. [b]Centro de Investigación y Desarrollo de Proteínas (CIDPRO) and Centro de Biotecnología FEMSA. Escuela de Ingeniería y Ciencias, Tecnológico de Monterrey, Av. Eugenio Garza Sada 2501, C.P. 64849 Monterrey, N. L., Mexico. *Corresponding author e-mail: crosell@iata.csic.es*

Abstract:

The search of proteins that could act as both structural agents and nutritional enhancers in gluten free bread is still a challenge for the food industry. The present study evaluated the inclusion of 10% β-conglycinin concentrate (βCC) extracted from defatted soybean flour to rice flour to improve the structure and protein quality of gluten-free yeast-leavened bread. βCC breadmaking performance was compared to vital gluten functionality. Batter pasting properties and bread characterization in terms of color, texture, image analysis, microstructure a,nd protein quality were assessed. βCC and gluten led to batters with higher peak viscosity and breakdown, although only βCC was able to increase the setback. The inclusion of 10% βCC in rice flour formulations led to breads with improved color parameters and protein quality. The texture properties of βCC breads did not present significant differences compared to vital gluten breads. The crumb analysis showed that βCC led to lower cell density with highest mean cell area. The micrographs showed that βCC was able to create a net-like structure similar to the one created by gluten, confirming that βCC is capable of acting as structuring agent and protein quality improver in gluten-free formulations.

Keywords: β-conglycinin, proteins, gluten-free, bread, rice flour, protein quality

Johanan Espinosa-Ramírez: Investigation, Formal analysis, Visualization, Writing- Original Draft

Raquel Garzon: Investigation, Formal analysis, Visualization, Writing- Original Draft

Sergio O. Serna-Saldivar: Writing- Review & Editing, Funding acquisition

Cristina M. Rosell: Conceptualization, Supervision, Writing- Review & Editing, Funding acquisition

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Introduction

The baking properties of wheat flour are determined by the unique characteristics of its proteins, namely gluten. After hydration and kneading of wheat flour, gluten proteins form a network responsible of the cohesiveness, viscosity and elasticity of the dough, making it capable of holding the gas produced during fermentation and leading to the characteristic foam structure of bread [1]. However, these proteins are not considered safe for patients with celiac disease, which is the most common food intolerance of the world population [2]. In these patients, gluten ingestion triggers an immune mediated response that causes villous atrophy in the small intestine and subsequent malabsorption. Therefore, celiac disease is frequently associated with deficiencies of macro and micronutrients [3]. The most efficient treatment for celiac is a strict withdrawal of gluten containing foods from the diet [2]. However, gluten-free baked goods are generally produced with refined starch, leading to products with low amounts of proteins, dietary fiber and micronutrients in comparison with their gluten product counterparts [4,5]. Hence, the nutritional deficiencies of the celiac patients cannot be mitigated with the current gluten-free products unless those are nutritionally closer to their gluten containing equivalents.

Rice flour is one of the most common ingredients in the production of gluten-free foods including bread [6] because it has bland taste, is colorless, inexpensive, easily digested, and present hypoallergenic properties [7]. However, due to the differences in its storage proteins, rice flour proteins are unable to develop a network with properties similar to gluten [8]. To improve the quality of the final products, several additives including hydrocolloids, enzymes, emulsifiers and proteins are supplemented in gluten-free formulations to increase the acceptability of consumers [9]. In this backdrop, proteins have been evaluated in rice-flour formulations to overcome the problems associated to the lack of structure, mimic the characteristics that gluten provides to bread and also to enhance their nutritional value [8,10,11,12,13]. Soybean has been one of the most evaluated sources of proteins for breadmaking due to their high lysine content, improving the essential amino acids content when combined with cereals [14], and more recently due to its potential to serve as structure-forming

agent in gluten-free formulations [8,15-19]. Nevertheless, in these studies the inclusion of additives such as hydrocolloids and enzymes was necessary to obtain the desired gluten-like network structure, since hitherto, no other protein has been able to fully replace gluten functionality.

In a recent model study, a β-conglycinin concentrate (βCC) proved to be an adequate structuring agent for yeast leavened breads produced with native corn starch [20]. The βCC was evaluated in a lean system where no other additives were used, which resulted in breads with higher 2D area, height, softness and cohesiveness compared to vital gluten breads. These results showed the potential of the βCC to improve the rise and quality properties of other gluten-free formulations acting as structure-forming agent. Moreover, since this βCC was obtained after the fractionation of soybean proteins [21], it could also contribute to improve the nutritional quality of rice flour breads.

The objective of this study was to evaluate the potential of βCC to mimic the gluten functionality in rice flour gluten-free leavened breads and to enhance the nutritive value of breads in terms of protein content and quality. Other structuring agents such as hydrocolloids or enzymes were not used during the process. Doughs and breads produced with rice flour and βCC or vital gluten were compared.

Material and methods

Materials

The rice flour was purchased from Harinera La Meta S.A (Lleida, Spain) (7.4% protein; 12.5% moisture). Vital gluten (77.4% protein; 8.3% moisture) was donated by Puratos (Groot-Bijgaarden, Belgium), whereas the β-conglycinin concentrate (βCC) was obtained according to the method proposed by Qi, et al. [21] with modifications reported by Espinosa-Ramírez, et al. [20]. The protein content of the lyophilized βCC containing 6.3% moisture was 87.5% (N·6.25).

Three formulations were tested for bread production: 1) 100% rice flour (negative control), 2) 90% rice flour + 10% vital gluten (positive

control), and 3) 90% rice flour + 10% βCC (experimental treatment). Vital gluten and βCC were blended with rice flour in a proportion 90:10 based on the results obtained in a previous study, where the substitution of corn starch with 10% βCC improved the height and texture of gluten-free breads [20]. Gluten was substituted in the same concentration for comparison purposes.

Flours characteristics

The water binding capacity (WBC) of the rice flour and two composite flours was determined according to Method 44-15.02 (AACC, 1999) [22]. This value was selected as the optimal hydration level for the breadmaking process. Results were expressed as percentage in flour basis (g water/100 g flour) in accordance to baker's formulations. Five replicates were made for each sample.

The pasting and apparent viscosity properties of the batters were determined by Rapid Visco Analyzer (RVA 4500, Perten Instruments SA, Stockholm, Sweden) following the method reported by Xing, et al. [23]. Batters were prepared according to baker's formulation with 100% flour, 1.5% salt, 1.0% sugar and the hydration selected from the WBC value for each flour. For the analysis, batters were diluted (8 g of bread batter and 12 g of water) and held for 60 s at 50 °C, then heated from 50 to 95 °C at 12 °C min^{-1} and held at 95 °C for 60 s. Thereafter, the batters were cooled to 50 °C at 12 °C min^{-1} and held at 50 °C for 2 min. The mixing speed was 600 rpm and the apparent viscosity was recorded during the heating-cooling cycle using Thermocline software for Windows (Perten Instruments SA). The onset temperature, peak viscosity at 95 °C, breakdown (difference between peak viscosity and trough viscosity), final viscosity and setback (difference between final viscosity and trough viscosity) were evaluated. The RVA determination was conducted twice for each batter.

Breadmaking process

For breadmaking, the lean baker's formulation consisted of 100% flour, 1.5% salt, 1.0% sugar and 1.0% dry yeast. The water hydration used was selected from the WBC value determined for each flour. Breadmaking was carried out following the mini-scale procedure described by Garzon, et al. [24]. Briefly, doughs were kneaded at 100 rpm for 90 s in a stirrer with a turbine accessory (IKA Eurostar 40, Staufen, Germany). After kneading, 2 g of dough were set in greased glass pans (diameter, 1.8 cm; height, 3 cm) and proofed for 40 min at 35 °C and 65% relative humidity. After fermentation, 100 μL of distilled water were added to the surface of the proofed dough to avoid dough surface dryness and then baked in an oven set at 130 °C for 10 min. Two batches were run for each formulation.

Bread characterization

Resulting breads were characterized in terms of color, texture and internal bread crumb structure. The color of the crumb was determined using a colorimeter (Chroma Meter CR-400/410, Konica Minolta, Tokyo, Japan) after white calibration (L^*= 96.9, a^*= -0.04, b^*= 1.84). Results were means of 4 replicates and expressed using the CIE-$L^*a^*b^*$ scale, where L^* indicates lightness, a^* redness to greenness, and b^* yellowness to blueness.

The texture parameters were determined according to Garzon et al. (2017) in a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) using a texture profile analysis (TPA) double compression test. Bread slices of 10 mm thickness were compressed to 50% of their original height at a test speed of 1 mm s^{-1} with a stainless-steel probe of 25 mm diameter. Texture was assayed in four slices of each batch. The parameters evaluated were hardness, springiness, cohesiveness, chewiness, and resilience.

The 2D area of breads and their crumb cell structure were evaluated by digital image analysis. For that, eight breads of each formulation were selected and cut with a scalpel to obtain longitudinal and cross section images captured at 1200 ppi using a flatbed scanner HP Scanjet G3110

(Hewlett-Packard, USA). A 10x10 mm field of view was evaluated for each image. Image were improved by Image J software (National Institutes of Health, Bethesda, MD, USA) using the Otsu's algorithm for assessing the threshold [25]. Data derived from the crumb structure analysis included: cell density (u/cm^2), mean cell area (mm^2) and cell circularity.

Protein quality of breads

The protein content of breads was determined according to the official method AOAC 920.87 [26]. The *in vitro* protein digestibility (IVPD) of lyophilized breads was assayed by the methods of Hsu, Vavak, Satterlee, & Miller (1977) and Bilgiçli, Ibanoglu, & Herken, (2007) with some modifications. Briefly, 1 mL of aqueous protein suspension containing 6.25 mg protein/mL was prepared. Samples were placed in a 37 °C water bath and the pH was adjusted to 8.00 using 0.1 N NaOH and/or 0.1 N HCl, while stirring. Trypsin at a concentration of 1.6 mg/ml was maintained in an ice bath and the pH was adjusted to 8.00 with 0.1 N NaOH and/or 0.1 N HCl. Then, 0.1 mL enzyme solution was added to the protein suspension, which was continuously stirred at 37 °C. The trypsin had an activity of 13,766 BAEE units/mg proteins. The pH drop was recorded at 15 s after the addition of the enzymatic solution and at 1-min intervals during 10 min. The IVPD percentage was calculated using Eq. (1) [27], where x is the change in pH after 10 min.

$$y = 210.464 - 18.1\,x \qquad \text{Eq. (1)}$$

Amino acids analysis of the flours used for breadmaking was performed using the official method AOAC 982.30 [26] coupled to an HPLC system. Protein digestibility corrected amino acid scores (PDCAAS) were calculated according to WHO/FAO/ONU [28] using the formulas:

$$\text{Limiting amino acid score} = \frac{\text{Limiting essential amino acid content of test protein}}{\text{Amino acid requirement pattern}} \qquad \text{Eq. (2)}$$

$$\text{PDCAAS} = (\%\ \text{protein digestibility} * \text{amino acid score})/100 \qquad \text{Eq. (3)}$$

Scanning electron microscopy

For scanning electron microscopy (SEM), fermented dough and bread samples were frozen in liquid nitrogen immediately after production and freeze-dried. Doughs and breads were fractured and placed on a metal stub. Then samples were sputter-coated with gold and palladium by ion sputter (Bio-Rad SC-500, Aname, Madrid, Spain). Sample images were recorded with a Hitachi S-4800 (Ibaraki, Japan) scanning electron microscope and observed at 10kV accelerating voltage with magnification level of 700x.

Statistical analysis

Analysis of variance (ANOVA) was used for the statistical analysis of the results. Means were compared using Tukey's test. Analyses were performed using Minitab 17 statistical software and 95% confidence.

Results and Discussion

Flours and batters' characteristics

Due to the important role of water in the quality of gluten-free bread products [10], the water binding capacity (WBC) of rice flour and blends was determined to select the optimum water absorption for the breadmaking process. The formulation containing 10% βCC presented the lowest WBC (Table 1). According to a previous work, the WBC of βCC was significantly lower compared to vital gluten (0.98 g/g vs 1.45 g/g) [20]. Therefore, the lower WBC of the blends containing βCC was attributed to the lower quantity of water bound by the βCC in comparison to rice flour and vital gluten. Crockett, et al. [29] added higher amounts of water in breadmaking formulations of rice flour supplemented with a soybean protein isolate. The WBC of a soybean protein isolate could be up to 5 times higher [30] when compared to the βCC used herein. Differences in the WBC according to the ingredients used in the formulation, highlight the importance of its assessment for each breadmaking system.

The RVA parameters and curves were obtained to evaluate the effect of the replacement of rice flour with proteins on the pasting properties of batters (Table 1; Figure 1). The onset pasting temperature did not show significant differences among flours ranging from 66.4 °C to 68.1 °C. The onset pasting temperature was governed by the starch type and coincided with the pasting temperature of rice flour [24,31]. Peak viscosity was higher for batters containing 10% proteins. When the final viscosity was evaluated, the batter composed of rice flour and gluten showed the lowest value. Other authors found that the rice flour replacement with protein isolates (5%) resulted in lower peak and final viscosity [13,15]. These authors related the lower final viscosity of protein-containing batters with the lower amount of gelatinized starch resulting from the partial replacement of the rice flour. Nevertheless, present results indicated that batters' performance is different than when only rice flour was tested. Conversely, batters containing 10% βCC presented higher values for final viscosity compared to those for gluten. Marco and Rosell [15] observed higher final viscosities when rice flour was supplemented with whey and egg proteins when compared to batters produced with other sources of protein, stating the different behavior of the proteins depending their nature. These authors associated this behavior to the heat-induced gelation capacity of whey and egg proteins. Soy proteins also form heat-induced gel structures, which get stiffer after cooling [32], that could explain the high viscosity of βCC batters despite the replacement of rice starch. When breakdown was compared, higher values were observed for batters containing added proteins, thus batters' stability during cooking was lowered with the proteins. Conversely, the setback values were only significantly modified when βCC was present in the batter, likely, this protein favored the amylose retrogradation.

Table 1. Effect of gluten and βCC (10% flour replacement) on the rice flour hydration and batter properties[a].

Parameter	Formulation		
	100% Rice flour	90% Rice flour + 10% Gluten	90% Rice flour + 10% βCC
WBC (g water/100 g flour)	134 ± 2[a]	130 ± 2[a]	121 ± 5[b]
Pasting properties			
Onset pasting temperature (°C)	67.3 ± 0.7[a]	68.1 ± 0.5[a]	66.4 ± 0.5[b]
Peak viscosity (mPa)	2221 ± 16[c]	2839 ± 175[a]	2554 ± 81[b]
Breakdown (mPa)	1323 ± 1[b]	2062 ± 185[a]	1936 ± 89[a]
Final viscosity (mPa)	1462 ± 7[b]	1333 ± 4[c]	1747 ± 11[a]
Setback (mPa)	564 ± 8[b]	556 ± 14[b]	849 ± 1[a]

[a]Values are means and standard deviation of at least two replicates; means with different letters within the same parameter differ significantly ($P < 0.05$). WBC= Water binding capacity; βCC= β-conglycinin concentrate; mPa= millipascal.

The RVA curves (Figure 1) for the batters showed plots similar to native rice flour pasting pattern. This pattern consists in an increment in viscosity to reach a peak viscosity during heating and breakdown on holding at 95 °C followed by setback during cooling [31]. However, even when the same trend was obtained for all batters, irregular lines and multiple peaks before and after the peak viscosity were observed especially in RVA curves from rice flour (Figure 1). These irregularities were attributed to destabilization of the batter during the heating stage. When proteins were blended with rice flour, the shape of the RVA curves became more regular, especially in gluten batters. The vanishing of irregular peaks in RVA curves of batters that contained gluten and βCC could be associated to a higher water absorption of the proteins in comparison to the rice flour components leading to the thickening of the suspension and thus to less destabilization. The higher stability in the RVA curves of βCC and gluten batters could also be associated to the formation of network structures during heating among proteins and rice flour components. Matos, et al. [16] associated the changes in the rheological behavior during heating of gluten-free batters supplemented with proteins, to the formation of a more rigid protein network promoted by the protein denaturation compared to the rice-based control. On the

other hand, the small irregular peaks in batters containing βCC could be related to the dissociation of proteins due to the heating treatment causing protein aggregation [16,32]. The aggregation of soybean proteins at temperatures lower than 95°C where the peak viscosity of the rice flour is obtained has been reported before [8,32].

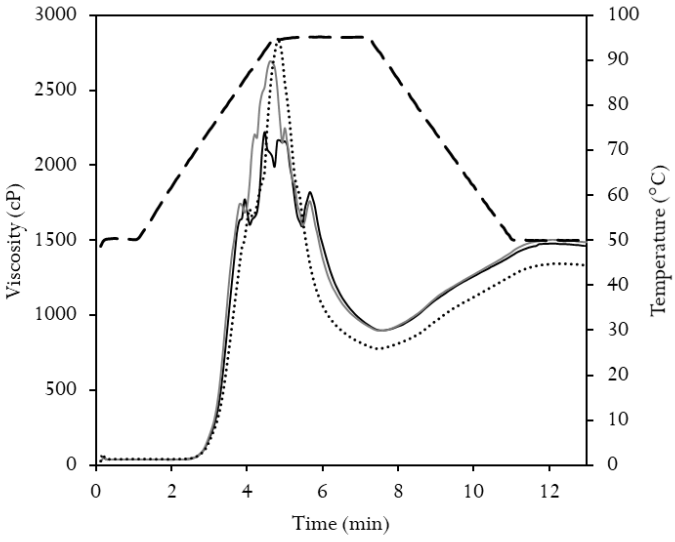

Figure 1. Viscosity profile determined by Rapid Visco Analyser of batters produced with 100% rice flour (black line); 90% rice flour + 10% gluten (dotted line); and 90% rice flour + 10% β-conglycinin concentrate (gray line). temperature profile. Values are means of two replicates.

Bread characterization

The effect of the replacement of rice flour with 10% proteins in the quality of breads was evaluated. The crumb color, textural parameters, 2D area and cell structure features are summarized in Table 2. The color of the crumb was affected by the addition of proteins. Breads that contained βCC or wheat gluten presented lower lightness and higher yellowness compared to rice flour counterparts. The differences in breads color were associated to the color of the proteins powders. Gluten (L^*=85.9 ± 0.3, a^*= -0.4 ± 0.0, b^*=13.8 ± 0.4) and βCC (L^*= 88.3 ± 0.1, a^*= -1.2 ± 0.0, b^*=14.1 ± 0.6) used herein had lower L^* and higher b^* values compared to the values for rice flour (L^*=92.8 ± 0.1,

a= -1.0 ± 0.0, *b*=5.4 ± 0.1). Previous reports have shown the same behavior in the color of breads produced with rice flour and supplemented with proteins and associated these changes to the color of raw materials [8,12]. Ziobro, et al. [33] also found that the addition of proteins modified the color of gluten-free breads by increasing the darkening and yellowness of crumbs. Moreover, according to these authors, the color changes were advantageous resulting in higher organoleptic acceptability by consumers. The *a** parameter presented values ranging from -1.53 to -1.11. These values were similar to those reported for rice flour breads [24]. The differences in the color of the raw materials compared to breads were associated to the baking process which enhanced non-enzymatic browning reactions, favored by the supplemented proteins [8].

Table 2. Effect of the substitution of gluten and βCC in the color, texture, 2D area and crumb structure features of yeast-leavened rice flour breads[a].

Parameter	Formulation		
	100% Rice flour	90% Rice flour + 10% Gluten	90% Rice flour + 10% βCC
Color			
*L**	79.2 ± 1.2[a]	72.5 ± 2.1[b]	72.9 ± 1.7[b]
*a**	-1.37 ± 0.03[ab]	-1.11 ± 0.29[a]	-1.53 ± 0.18[b]
*b**	7.8 ± 0.5[c]	13.7 ± 0.8[a]	10.1 ± 1.2[b]
Texture			
Hardness (g)	96.0 ± 5.0[b]	126.8 ± 22.0[a]	138.2 ± 15.7[a]
Springiness	0.95 ± 0.06[b]	1.00 ± 0.00[a]	1.00 ± 0.01[a]
Cohesiveness	0.68 ± 0.04[b]	0.75 ± 0.06[a]	0.71 ± 0.03[ab]
Chewiness (g)	61.6 ± 6.4[b]	94.0 ± 12.8[a]	98.3 ± 8.3[a]
Resilience	0.34 ± 0.04[b]	0.39 ± 0.03[a]	0.37 ± 0.02[ab]
2D Area (cm^2)	2.98 ± 0.23[b]	2.88 ± 0.21[b]	3.37 ± 0.26[a]
Crumb cell structure			
Cell density (u/cm^2)	13 ± 2[a]	12 ± 1[ab]	11 ± 2[b]
Mean cell area (mm^2)	1.99 ± 0.92[a]	2.31 ± 0.44[b]	3.12 ± 0.64[a]
Cell circularity	0.37 ± 0.04[a]	0.36 ± 0.03[a]	0.39 ± 0.04[a]

[a] Values are means and standard deviations of at least three replicates; means with different letters within the same parameter differ significantly ($P < 0.05$). βCC= β-conglycinin concentrate

Hardness and chewiness were increased due to the addition of gluten and βCC proteins (Table 2). Previous reports also found an increment in the

crumb hardness caused by the addition of proteins to rice flour formulations [8,13]. Although no significant differences were detected, breads containing βCC presented the highest hardness values. This could be associated to the low water binding capacity of the rice flour/βCC bread formulation (Table 1). Aprodu, et al. [10] found a negative relationship between the water absorption and the hardness of the crumb of gluten-free breads. Although, it must be stressed that the increment in the crumb hardness of breads that contained gluten or βCC could be also attributed to the formation of more rigid structures among the supplemented proteins and rice flour components during the thermal treatment of baking.

Interestingly, bread springiness, which is positively related to crumb elasticity, increased in breads that contained gluten or βCC (Table 2). Cohesiveness and resilience also increased due to the protein addition, but compared to rice flour breads, the increment was only significant when the supplemented protein was wheat gluten. High values of cohesiveness, springiness and resilience are desirable since they represent lower disaggregation during mastication and adequate crumb elasticity [34,35]. It must be remarked that no significant differences were found in these texture parameters when breads containing βCC were compared to those containing vital gluten. Moreover, βCC values were higher than those obtained for gluten-free formulations that used other non-gluten proteins [8,12,13,36] and most of the commercial gluten-free breads analyzed by Matos and Rosell [37].

The 2D area and crumb cell features were evaluated since they are related to the CO_2 binding ability during both proofing and baking. Breads produced with 10% βCC had the highest 2D area (Table 2). This is consistent with a previous report where βCC and corn starch were blended to produce model breads which showed higher 2D area in comparison to gluten-corn starch [20]. Authors attributed this significant improvement to higher retention of carbon dioxide by βCC proteins during proofing. The cell density, which represents the number of cells in one cm^2, presented lower values when proteins were blended with rice flour, being significant in βCC breads (Table 2). Likewise, βCC breads presented the highest mean cell areas among formulations. Ziobro, et al.

[33] also found that the addition of non-gluten proteins to gluten-free bread formulations resulted in crumbs with lower cell density and in most cases, higher cell or loci sizes. Marco and Rosell [8] found that the formation of networks during heating in gluten-free formulations was favored by the addition of proteins and hydrocolloids. This network gave strength to the expanding cells of the dough and led to an improved crumb structure with less number and larger size of gas cells [8]. In the present study no hydrocolloids were used. Therefore, the results support the assumption that βCC forms a strong network able to retain the gas released during proofing. The cells shape was similar for all breads, as the cell circularity did not show significant differences among formulations (Table 2).

Protein quality of breads

The protein content, amino acid profile, PDCAAS and IVPD of breads are depicted in Figure 2 and Table 3. Breads produced with composite flours containing βCC or gluten had almost twice the protein content of the rice flour breads. As expected, breads supplemented with βCC contained the highest protein content because the βCC contained higher protein purity compared to the vital wheat gluten. Matos and Rosell [4] analyzed eleven commercial gluten-free breads and found a protein content ranging from 0.9 to 15.1 g/100 g (dry basis, db) with a mean value of 4.8 g/100 g. The protein content reported for white wheat breads was around 13 g/100 g (db) [38]. Therefore, the bread produced with a composite flour containing 10% βCC and rice flour contained higher protein content compared to commercial gluten-free breads and wheat breads.

The amino acid profiles of breads are presented in Table 3. Breads containing gluten presented up to 99% and 50% more proline and glutamic acid + glutamine, respectively, compared to the other bread formulations. In contrast, gluten breads had the lower concentrations of the amino acids: aspartic acid + asparagine, arginine and lysine, among breads. This is consistent with the amino acid profile of gluten proteins, which is characterized by high levels of proline and glutamine and low contents of charged amino acids [39]. The unique amino acid composition and its sequence determine the type of inter- and intra-chain interactions

which promote the molecular weight distribution of gluten fractions, especially its polymeric nature, which is recognized as a determinant factor of dough properties and baking performance [1]. On the other hand, the amino acids that had a similar content in both gluten and βCC breads were alanine, glycine, threonine and valine. In contrast, 100% rice flour breads presented higher concentrations of these amino acids. The non-covalent interactions formed by the agglomeration of hydrophobic amino acids or electrostatic interactions and hydrogen bonds are necessary to stabilize the gluten network [40]. The amino acids that were similar among gluten and βCC formulations but were different to rice breads, could be related to important non-covalent interactions that lead to net-like structures. In this backdrop, the concentration of these amino acids would be essential to favor those net-forming interactions. Breads containing 10% βCC had the lowest content of sulfur amino acids among breads (Table 3) since soybean proteins have low concentrations of these sulfur-containing amino acids [40]. Disulfide bonds formed by the crosslinking of cysteine residues are the most important structural element of the gluten network [40]. However, the similar content of cysteine in breads produced with 100% rice flour or substituted with 10% gluten, proves that the presence of this amino acid is not enough to favor these interactions.

To evaluate the protein quality, the limiting amino acid was determined based on the FAO/WHO/ONU [28] requirement of a two-year infant (Table 3). In most instances, lysine is the limiting amino acid in wheat bread and other cereal-based foods [38]. However, when rice flour was blended with βCC, the lysine content increased 37% in breads. This led to an increment from 0.83 to 1.14 in the amino acid score of lysine. Lysine was the essential amino acid present in the lowest concentration compared to the requirement for infants. Therefore the limiting amino acid score was calculated for lysine, being rice flour and rice flour + 10% gluten the breads, which did not fulfill the WHO/FAO/ONU [28] requirements for 1-2 years infants (which covers the range appropriate for human adults) (Table 3). Even when the replacement of rice flour with 10% βCC led to lower levels of cysteine + methionine, the suggested requirement for sulfur amino acids [28] was still fulfilled by this composite bread. This proved that the selected level of substitution of

rice flour with 10% βCC led to the improvement of the amino acid profile of gluten-free breads based on rice flour.

The PDCAAS is a quality index for food protein sources that considers their digestibility and limiting amino acid score [28]. In the present study, the scores for lysine were used for the PDCAAS calculation (Table 3). Breads containing βCC presented the highest PDCAAS value, which was 20% higher compared to the 100% rice flour breads. The improvement in the PDCAAS was associated to the complementary effect of the rice proteins and the βCC rich in lysine as well as its adequate IVPD. Moreover, PDCAAS of βCC breads was 3.4 times higher than that reported by Villarino, et al. [38] for wheat bread. This proved that the substitution with 10% βCC led to the improvement of the protein quality of rice flour gluten-free formulations.

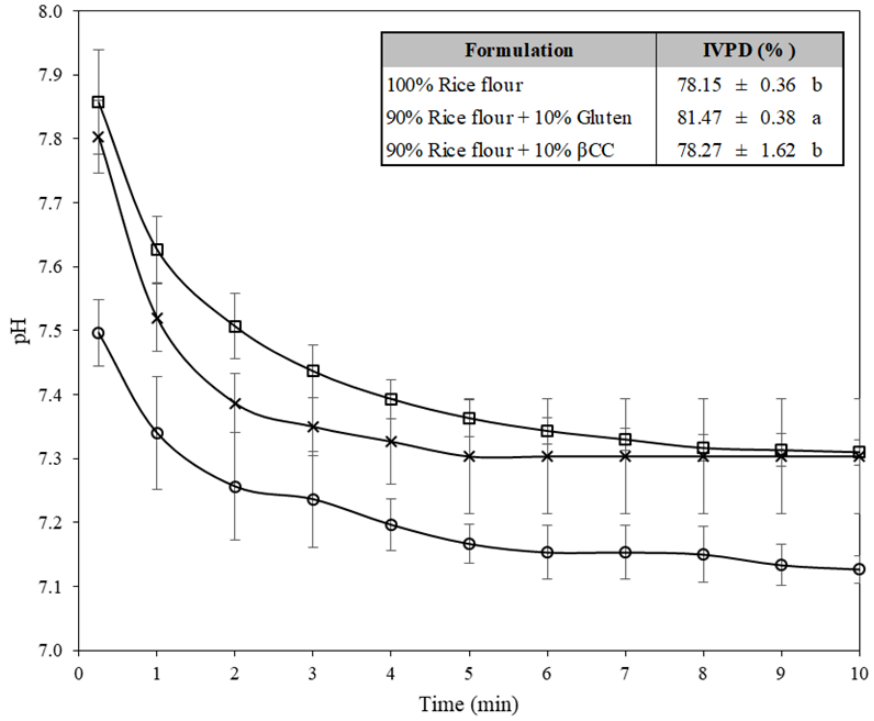

Figure 2. In vitro protein digestibility (IVPD) and pH drop in yeast leavened breads produced with 100% rice flour (□); 90% rice flour + 10% gluten (○); and 90% rice flour + 10% β-conglycinin concentrate (×).

Table 3. Effect of the substitution of gluten and βCC in the protein quality characteristics of rice flour yeast-leavened breads[a].

Parameter	Formulation			WHO/FAO/ONU suggested requirement for essential amino acids[c]
	100% Rice flour	90% Rice flour + 10% Gluten	90% Rice flour + 10% βCC	
Protein (%, db)[b]	7.81±0.15[c]	15.31±0.11 [b]	16.10±0.15[a]	
Amino acid content (g/100 g protein)				
Alanine	5.78	4.45	4.66	
Arginine	8.06	5.70	8.40	
Aspartic Acid + Asparagine	9.14	6.01	10.78	
Glutamic Acid + Glutamine	17.88	26.83	20.36	
Glycine	4.70	3.97	3.79	
Histidine	2.42	2.26	2.42	1.8
Isoleucine	4.44	4.15	4.77	3.1
Leucine	8.60	7.73	8.24	6.3
Lysine	4.30	2.99	5.91	5.2
Methionine + Cysteine	2.82+2.42	2.15+2.27	1.72+1.52	2.6
Phenylalanine + Tyrosine	5.65+3.23	5.51+3.41	5.85+3.31	4.6
Proline	4.30	8.57	4.74	
Serine	4.84	4.25	4.36	
Threonine	3.63	3.05	2.96	2.7
Tryptophan	1.08	1.06	0.85	0.74
Valine	6.32	5.19	5.10	4.2
Limiting amino acid score	0.83 (lys)	0.57 (lys)	1.14[d]	
PDCAAS	0.65	0.47	0.78	

[a] βCC= β-conglycinin concentrate; PDCAAS= Protein digestibility corrected amino acid score.

[b] Values are means and standard deviations of three replicates; means with different letters within the same parameter differ significantly ($P < 0.05$).

[c] Suggested requirements for 1-2 years infants. Values are expressed in g/100 g protein.

[d] Value was truncated to 1.0 for the calculation of PDCAAS as recommended by WHO/FAO/ONU, 2007.

Microstructure of doughs and breads

Scanning electron microscopy was used to evaluate the effect of the addition of βCC on the microstructure of fermented doughs and breads (Fig. 3). Micrograph of the 100% rice flour fermented dough showed an amorphous structure where starch granules were held together by the proteins, but no gas cells were observed (Fig. 3A). On the other hand, the structure of fermented doughs containing gluten or βCC was significantly different. These doughs presented a continuous protein network, where gas cells were trapped covered by a veil of rice starch granules (Fig 3B-C). Compared to gluten, a higher size of gas cells in βCC doughs and also more uniform distribution of the starch granules over the entire protein network was observed.

In bread micrographs, a continuous structure resulted from gelatinized starch and denatured proteins in which air cells were visible (Fig. 3D-F). Therefore, the use of the water binding capacity as an indicator of the water required for forming the gluten free structure allowed an adequate interaction of the components after heating However, several differences were noticed in the microstructure depending on the proteins added. For 100% rice flour breads continuous zones with no air cells were observed, and wherever pores, those were smaller (Fig. 3D), in comparison to the formulations with proteins. Conversely, βCC and gluten breads presented the same net-like structure, but βCC displayed higher number of larger pores and thinner lamellae. Previous reports highlighted the importance of the addition of proteins to gluten-free formulations to form web-like structures and stabilize gas cells [8,42]. However, these authors used several additives including hydrocolloids and crosslinking enzymes to obtain improved aerated structures similar to those produced in wheat bread. The breads produced herein had lean formulations and no hydrocolloids/enzymes were used. Notwithstanding, βCC breads produced a web-like structure similar to those produced with gluten-rice flour and to wheat bread [42].

The development of the net-like structures observed in SEM images after breadmaking could be the reason of the increased hardness and chewiness observed in breads containing wheat vital gluten or βCC, but also of their

improved values of springiness, cohesiveness and resilience (Table 2). Moreover, these results confirmed the high capacity of βCC proteins to give the strength needed to hold the expanding CO_2 during fermentation and baking, which led to the observed higher 2D bread crumb area and mean cell areas (Table 2). Therefore, βCC serves as structuring agent and has the potential to form net-like structures similar to gluten during breadmaking.

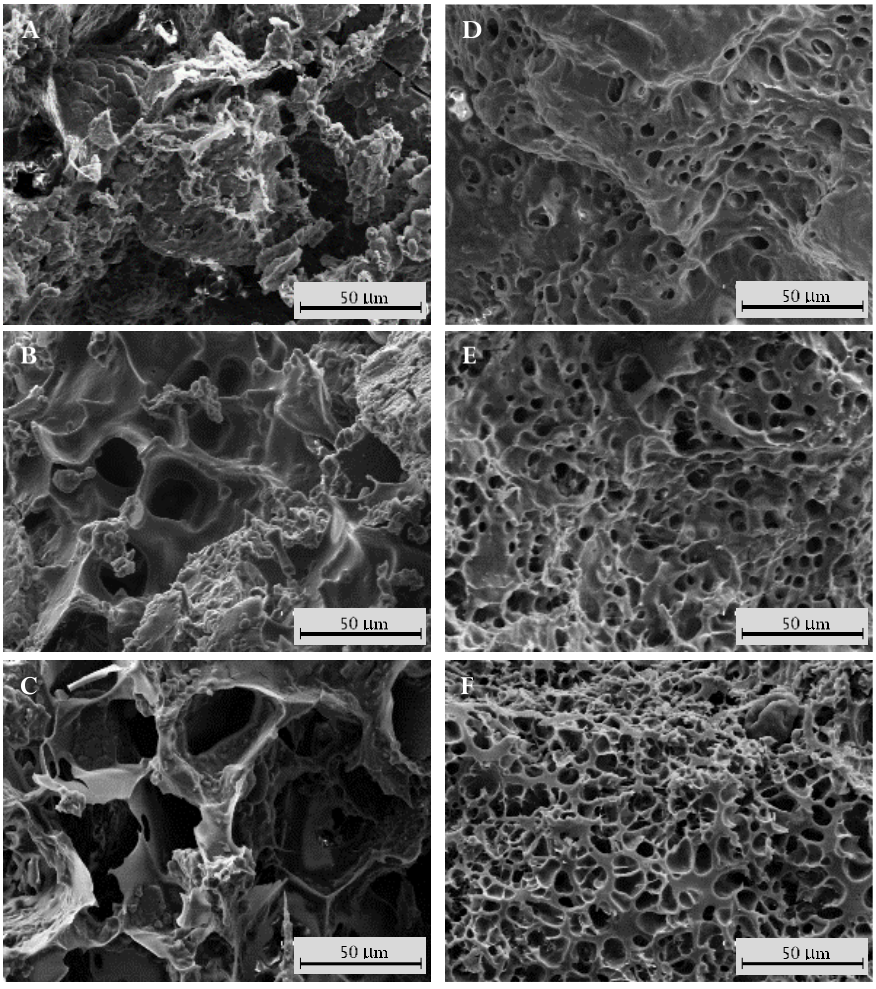

Figure 3. Scanning electron microscope images of the fermented doughs (A-C) and yeast-leavened breads (D-F) of formulations with 100% rice flour (A and D), 90% rice flour + 10% gluten (B and E), and 90% rice flour + 10% β-conglycinin concentrate (C and F) at 700x magnification.

Conclusion

Rice-based yeast-leavened breads with adequate features (volume and texture) were produced from batters using the water binding capacity of the flours as the parameter to set the optimum amount of water. βCC (10%) when incorporated to rice flour for the production of gluten-free bread provided the required network for holding the carbon dioxide during proofing and also for allowing expansion during baking. In fact, SEM micrographs reveal that βCC brought about a net matrix similar to the one obtained in the presence of wheat gluten, and without the addition of any hydrocolloid. Breads containing 10% βCC showed improved quality in terms of color, texture, 2D area, crumb structure and protein quality in comparison to the bread counterpart elaborated with 100% rice flour. It is noteworthy to remark that a concentration of 10% βCC allowed to reach a complete balance of essential amino acids in rice flour formulations. Overall, the use of this ingredient is useful to fulfill both technological and nutritional challenges in gluten-free breads.

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III. Understanding emulsifiers effect on bread aeration during breadmaking [1]

*Raquel Garzón[a], Isabel Hernando[b], Empar Llorca[b], Cristina M. Rosell[a]**

*[a]Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain. [b]Food Microstructure and Chemistry Research Group, Department of Food Technology, Universitat Politècnica de València, Camino de Vera, s/n, 46022, Valencia, Spain. * Corresponding author e-mail: crosell@iata.csic.es*

Abstract:
BACKGROUND: Much research has been done to explain emulsifiers action during breadmaking, but there is still plenty unknown to elucidate their functionality despite their diverse chemical structure. The aim of the present study was to provide some light about the role of emulsifiers on air incorporation into the dough and gas bubbles progress during baking and their relationship with bread features. Emulsifiers like diacetyl tartaric acid ester of monoglycerides (DATEM), sodium stearoyl lactylate (SSL), distilled monoglyceride (DMG-45 and DMG-75), lecithin and polyglycerol esters of fatty acids (PGEF) were tested in very hydrated doughs. RESULTS: Emulsifiers increased the maximum dough volume during proofing. Emulsifiers increased the number of bubbles incorporated during mixing, observing higher number of bubbles, particularly with PGEF. Major changes in dough occurred at 70 K when bubble size augmented, becoming more heterogeneous. DMG-75 produced the biggest bubbles. As a consequence, emulsifiers tend to increase the number of gas cells with lower size in the bread crumb, but led to greater crumb firmness, which suggested different interactions between emulsifiers and gluten, affecting protein polymerization during baking. CONCLUSION: Bubbles progress during baking allowed discriminate among emulsifiers, which could explain their performance in breadmaking.

Keywords: β-conglycinin, proteins, gluten-free, bread, rice flour, protein quality

Raquel Garzon: Investigation, Formal analysis, Data curation, Visualization, Writing- Original Draft
Isabel Hernando: Writing- Review & Editing
Empar Llorca: Investigation, Writing- Review & Editing
Cristina M. Rosell: Conceptualization, Supervision, Writing- Review & Editing, Funding acquisition

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Introduction

Bakery products are extensively consumed worldwide due to their nutritional and physical characteristics [1]. Among the diversity of bakery products obtained from either different raw ingredients or making processes, the most appreciated products are the sponge baked wheat bread, with low density and soft crumb. In the course of flour and water mixing, gluten formation and aeration brought about during kneading will be responsible of the subsequent cellular structure of the baked bread [2]. Air incorporated into the dough during mixing must be kept through the breadmaking process to attain low density breads. Bread contains about 70% of gas that comes from the initial aeration and the fermentation, both are important stages to take into account during the making process [3]. Because of that air bubbles incorporation during mixing have been the focus of many studies that stated the influence of mixer type and mixing time [4,5], besides the important role of ingredients[6,7]. Certainly, the progress of those initial nuclei bubbles throughout fermentation when carbon dioxide is generated [8] and final expansion of the gases occluded into the bubbles during baking determines the diversity of cellular structures encountered on bread crumbs [3]. Bubbles are very fragile and whatever changes in their number and size will have a direct impact on the internal crumb structure [9].

Nowadays, large-scale production and consumers demand for higher quality, homogeneity and longer shelf life that have been achieved with the use of processing aids such as enzymes, hydrocolloids, emulsifiers, etc. to adjust doughs properties. These additives are essentials for improving dough properties and final quality of fresh product [10]. Specifically, emulsifiers are active surfactant composites used in breadmaking for their ability to stabilize dough, a thermodynamically unstable system, through their interactions with gluten proteins [11]. During mixing, the use of emulsifiers increases the strength and the extensibility of the dough; in the fermentation stage they improve gas retention and avoid dough collapse [11,12], leading to softer bread crumbs [13], although their effect is greatly dependent on the wheat flour protein content [14] and proofing duration [15]. Those studies confirmed the effect of different emulsifiers in breadmaking processes, specifically in improving the internal structure of bread [16]. In spite of the knowledge acquired on

emulsifiers action during breadmaking, they are still attracting research due to there is still much unknown to explain their functionality despite their chemistry diversity. For instance, despite the impact of dough aeration into bread crumb features, there is no information about the role of emulsifiers on dough aeration and the bubbles number and size along the process. To understand the role of emulsifiers on determining the cellular structure of bread crumb, the main objective of this study was to assess the amount of gas occluded into the dough and bread along bread making and how several emulsifiers with diverse chemical structure affected the bubble size distribution.

Materials and methods

Breadmaking wheat flour was supplied by Harinera La Meta (Lleida, Spain) and compressed yeast by (DHW Europe, Germany). The selected emulsifiers included: diacetyl tartaric acid ester of monoglycerides (DATEM), sodium stearoyl lactylate (SSL) and distilled monoglyceride (with potassium citrate added) with two different particle sizes 45 microns (DMG-45) and 75 microns (DMG-75), which were provided by Danisco (Grindsted, Denmark), defatted hydrolyzed sunflower lecithin (Tricalcium phosphate) from Lasenor (Barcelona, Spain), and Polyglycerol esters of fatty acids containing polysorbate 80 (PGEF) from Palsgaard (Juelsminde, Denmark).

Gas bubbles during fermentation and baking

A very hydrated dough recipe containing wheat flour, water (900 ml kg^{-1} based on wheat flour weight) and 10 g kg^{-1} compressed yeast was used. Emulsifiers were added at 5 g kg^{-1} (f.b.) whenever tested. Ingredients were mixed during 3 minutes at 328 rpm in a mixer (RZR-1 Heidolph, Schwabach, Germany).

The gas released and dough development characteristics during fermentation were recorded using the Rheofermentometer F3 (Chopin, France), slightly modifying the instructions given by supplier. Briefly, hydrated dough (315 g) were confined in a glass recipient. The tests were performed on dough at 30 K for 3 hours, with a slight cylindrical weight. Registered parameters included: Hm (mm), maximum dough

fermentation height; T1, the time (min) at which Hm is attained; H′m (mm) maximum height of gaseous release; T′1, the time (min) at which H′m is reached; Tx, the time (min) at which gas starts to escape from the dough, thus when porosity of dough develops. All determinations were made at least in duplicate, and the average values were adopted.

A microscope was used to follow bubble changes of dough during baking as previously described Rodríguez-García, et al.[17] For that purpose, doughs were prepared as described before but without the addition of yeast to follow behavior of bubbles from air incorporation. Microbaking was performed using a system controller unit for heating and freezing stages (Analysa-LTS350, Linkam, Surrey, UK) mounted under the lens of a light microscope (Nikon ECLIPSE 80i, Nikon Co., Ltd., Tokyo, Japan). The temperature profile settings were from 30 K to 105 K increasing at 1.5 K min^{-1}. Samples were captured at ×4 magnification (objective lens ×4/0.13∞/– WD 17.1, Nikon). During microbaking, a video film was recorded with an attached camera (ExWaveHAD, model no. DXC-190) and images were acquired every 10 K. The analysis software (Linksys 32, Linkam) was directly interfaced with the microscope, enabling temperature control and image recording control. Duplicates were recorded. The number, size and distribution of the bubbles in the dough were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Bread making and characterization

A scale-down breadmaking method was carried out [18] to identify the emulsifiers effect. Recipes were prepared as described before, and then four grams of dough were placed in previously oiled cylindrical glass molds (17 mm x 300 mm, diameter x height). They were fermented for 100 min at 30 K and finally baked at 130 K for 11 min. Two batches were run for each sample.

Texture profile analysis of crumbs was carried out in a TA-XTPlus (Stable Micro Systems, Surrey, UK). A 10 mm thick slices were compressed twice with a 0.6 mm diameter probe up to 50% at 1 mm s^{-1} speed. The registered parameters were crumb hardness (g), springiness, cohesiveness, chewiness (g) and resilience. In order to study cell crumb

distribution and morphogeometric characteristics of the loaves, both cross and longitudinal sections of breads were captured using a scanner (HP Scanjet G3110, Hewlett-Packard, USA) with 600 dpi resolution. The 2D area and perimeter of longitudinal section was assessed using ImageJ software. The same software was used to analyze the cell crumb distribution in 10x10 mm crumb cross-sections. Image section was improved by splitting RGB channels and selecting the channel with greater contrast between background and object. Finally, Otsu algorithm (predefined by the software) was applied to convert image into a binary image and particle analysis of the image was carried out. The parameters assessed were cell/cm^2, mean area (mm^2) and circularity (from 0, rectangle, up to 1, perfect circle). Six slices were used for each determination.

Statistical analysis

Experimental data were statistically analyzed by analysis of variance (ANOVA) using Statgraphics Centurion XVI.I 16.1 software (Statistical Graphics Corporation, UK), to identify significant differences among them. Cluster analysis and principal components (PCA) were also performed to discriminate among emulsifiers with the tested variables.

Results and Discussion

Dough development and gaseous release characteristics

To evaluate the action of diverse emulsifiers on dough performance during breadmaking, very hydrated doughs were used. The effect of emulsifiers on gas retention during dough fermentation was recorded in the rheofermentometer plots (Figure 1). After an initial elapsed time, a steady increase of dough volume was displayed, but certain variation was observed in the presence of emulsifiers (Figure 1a). Lecithin and PGEF delayed the onset of volume increase compared to the control and the other emulsifiers. All emulsifiers increased the proofing rate, calculated as the initial slope of dough volume increase (Table 1). The maximum dough development (Hm) reached in the presence of the emulsifiers was higher than the one observed in the control dough, being greater in the case of PGEF (34.6 mm), followed by SSL and DMG-75 (33.0 mm and

32.4 mm, respectively). The presence of polysorbate blended with the PGEF might contribute to the high volume obtained due to its action as dispersing agent. This result agrees with those obtained by Gómez et al. [19] where polysorbate addition to low hydrated doughs led to higher dough volumes than other emulsifiers as DATEM and SSL. Nevertheless, the time (T1) required to reach the maximum dough development was higher in the presence of emulsifiers than in the control, confirming that emulsifiers are much more effective when longer dough fermentations are applied[19]. This improvement has been ascribed to the emulsifiers ability for strengthening the gluten network, increasing dough extensibility [19] and dough volume [16], which in turn was attributed to the formation of aggregates with gluten proteins [20]. However, that effect cannot be explained only by the emulsifier chemical structure, given that distilled monoglycerides with different particle size (DMG-45 and DMG-75) produced different responses. Dough stability during fermentation was greatly dependent on the emulsifier tested, and only lecithin and DMG-45 extended the stability of the dough longer than the control.

Regarding the gas production during fermentation (Figure 1b), the most evident effect was the decrease in the initial CO_2 production when emulsifiers were present. It seems that emulsifiers, independently of their chemical structure, affected the initial release of carbon dioxide. Taking into account that no sugar was added in the recipe, possible explanations could be related to either some interactions between emulsifiers and the free sugars, available in the flour for proofing, that decrease their readiness for the yeast or due to physical constraints derived of the more ordered and stronger protein structure in the presence of emulsifiers [21]. As the proofing progresses, main difference was observed during the last hour of fermentation when a decrease on the CO_2 production was observed, due to dough permeability to gas in some of the doughs. Doughs with DMG-45, DMG-75 and lecithin showed greater permeability than the control, which resulted in a decrease of the ability

to retain CO_2 at the end of the fermentation. The highest CO_2 production was with DATEM addition.

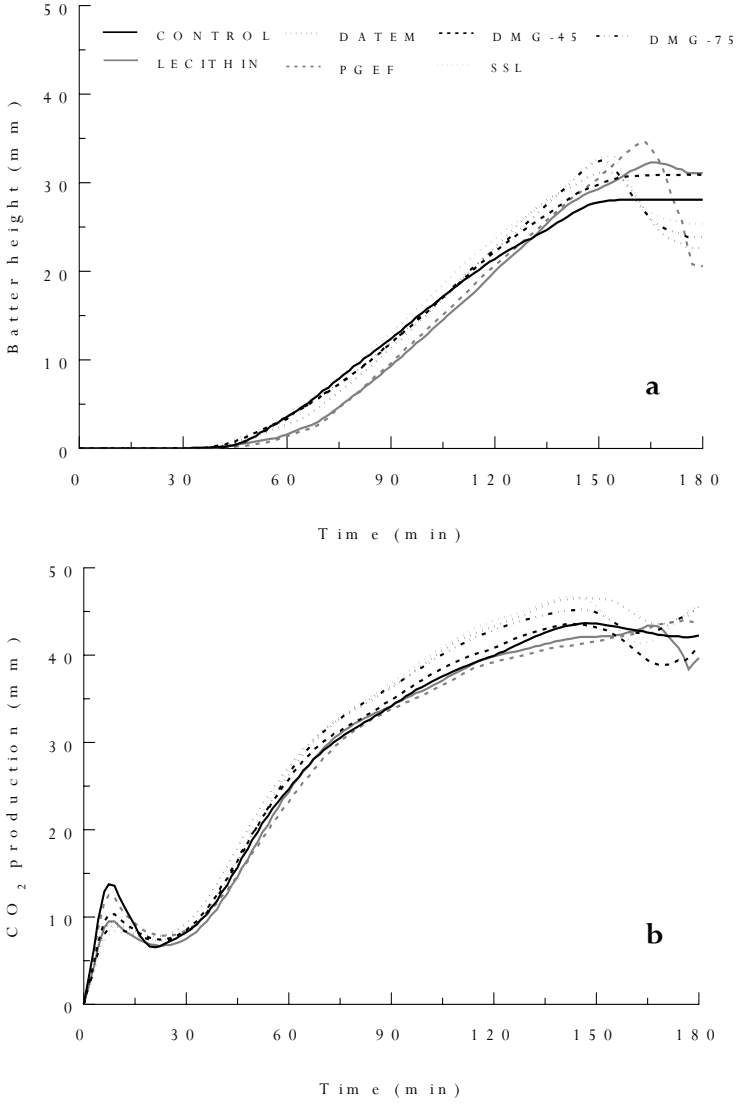


Figure 1. Rheofermentometer curves consisted of dough development time curves (a) and gas release curves (b).

Table 1. Analysis of fermentation stage of batters containing emulsifiers by reofermentometer.

	Dough development			Gas behaviour		
	Hm (mm)	T1 (min)	Proofing rate (%)	H'm (mm)	T'1 (min)	Tx (min)
Control	28.5±0.5^{a}	145±0^{a}	30.79	43.7±1.0^{a}	146±3^{a}	140±1^{b}
DATEM	31.4±1.0^{b}	167±9^{c}	33.44	47.0±0.8^{c}	161±7^{c}	145±2^{c}
DMG-45	31.0±1.0^{b}	169±4^{c}	31.13	43.6±0.1^{a}	140±6^{a}	136±5^{a}
DMG-75	32.4±0. 8^{b}	150±2^{b}	31.62	45.8±1.3^{b}	161±8^{c}	134±0^{a}
Lecithin	32.3±0.6^{b}	165±3^{c}	33.77	43.4±0.8^{a}	165±4^{c}	136±3^{a}
PGEF	34.6±0.9^{d}	164±6^{c}	34.77	44.0±0.5^{ab}	176±7^{d}	175±5^{d}
SSL	33.0±0.8^{c}	153±4^{b}	31.46	44.5±0.9^{b}	150±3^{b}	148±4^{c}

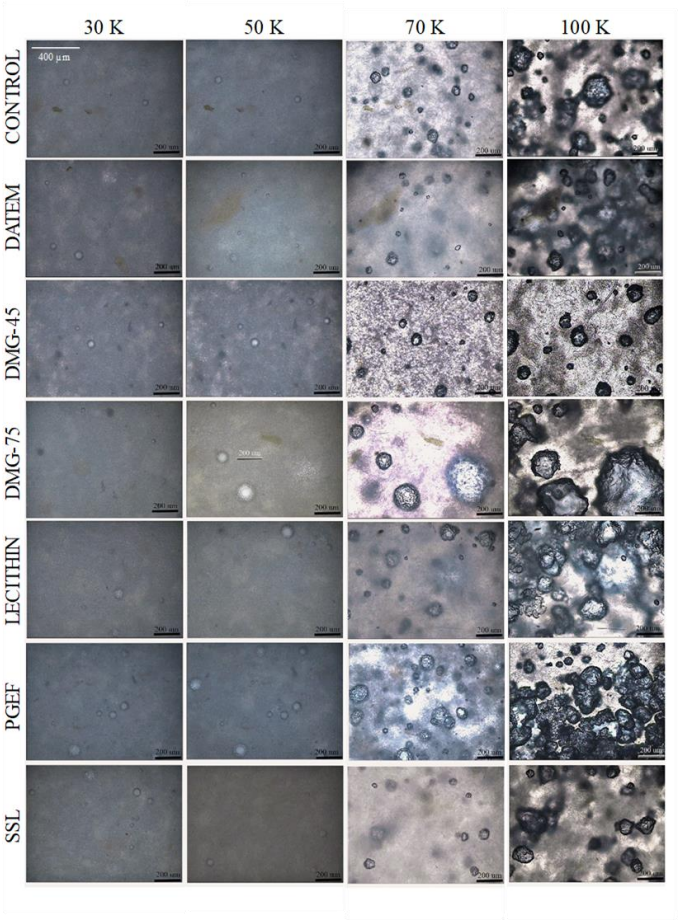
Mean ± standard deviation. Different letters within the same parameter differ significantly ($P<0.05$)

Microscopy and analysis image of simulated microbaking

The ability of the emulsifiers to stabilize the gas bubbles, incorporated into the doughs during mixing, was continuously monitored under a microscope. Very hydrated doughs were subjected to a steady temperature increase to simulate the baking process and consequently the capacity of the dough to hold the gas. Turbin-Orger, et al. [22] suggested that the liquid fraction present in the dough influence the cellular structure by affecting the connectivity of bubbles and their possible coalescence. In this study, very hydrated doughs were used to discard the possible interference of liquid effect. The captured images of doughs along temperature increase are shown in Figure 2. Initially, differences in the structure of the doughs were barely visible. Junge, et al. [23] reported that emulsifiers increase the incorporation of gas bubbles during mixing, but they did not find modifications during the baking stage. However, dough images (Figure 2) showed progressive changes with the temperature increase and major changes were observed when reaching 70

K. Babin, et al. [24] reported that the cell structure stabilization occurs with the temperature range 50–70 K when main changes associated to starch granule swelling and gluten cross-linking are produced. In all cases, the bubble size augmented as the temperature increased and their number and size were dependent on the type of emulsifier. The most important differences were observed when using DMG-75: bigger bubbles were observed at low temperature (40 K) if compared to the doughs prepared with the other emulsifiers, and these bubbles were really big at 70 K, giving place to the biggest bubbles at 100 K.

Figure 2. Captured images of gas bubbles during simulated microbaking at microscope



Quantitative analysis of the bubbles distribution and size is shown in Figure 3, where distributions were ordered from smaller to larger bubble width when temperature increased. In all the samples, the addition of emulsifiers increased the number of bubbles incorporated during mixing if compared to control, which may be due to the lower surface tension induced by the addition of emulsifiers. Kokelaar, et al. [25] showed that addition of some emulsifiers as SSL and DATEM originated more and smaller bubbles during mixing, because of the lower surface tension of dough inducing the subdivision of the entrapped air bubbles. When comparing the doughs prepared with the different emulsifiers (Figure 3), the dough formulated with PGEF presented greater incorporation of air bubbles during mixing, as the diagram corresponding to this emulsifier shows greater frequency of bubbles at the beginning of the micro baking process. Through temperature rise, all the samples, including control, showed an increase in the amount of detected bubbles, and bubbles size distribution became more heterogeneous due to expansion and interaction of the bubbles. The doughs prepared with DATEM, Lecithin and DMG- 45 presented a frequency distribution similar to that obtained for the control dough; in fact, the size of the bubbles increased in a uniform, controlled way (Figure 2). All these doughs showed small bubbles at low temperatures and a tendency to regular distribution of bubbles during heating; moreover, bubbles exceeding 120.000 μm^2 were not generally detected regardless of the heating temperature. Nevertheless, the samples prepared with SSL, PGEF and DMG-75 exhibited bigger bubbles, over 120.000 μm^2. Specifically, DMG-75 dough diagram presented very big bubbles, which continued interacting and coalescing even at 100 K. When temperature reached 100 K the samples containing SSL, PGEF and DMG-75 presented coarser distribution of bubbles, while DATEM, DMG-45 and lecithin had more bubbles but smaller ones. When baking temperature rises, the bubbles expand increasing the coalescence due to Ostwald maturation [26], where big bubbles grow up at the expense of small ones, consequently there is an increase in its size. With the addition of the emulsifiers, this phenomenon often decreased, due to the stabilization of the interface. [27] However, in the present work, it can be observed that depending on the emulsifier used in the dough formulation, the expansion of bubbles is controlled in a different way, being DATEM, DMG-45 and lecithin more effective for controlling this mechanism. It must be stressed that besides

the different chemical structure of the emulsifiers, their physical structure must be considered, since DMG-45 and DMG-75 induced different bubble stabilization.

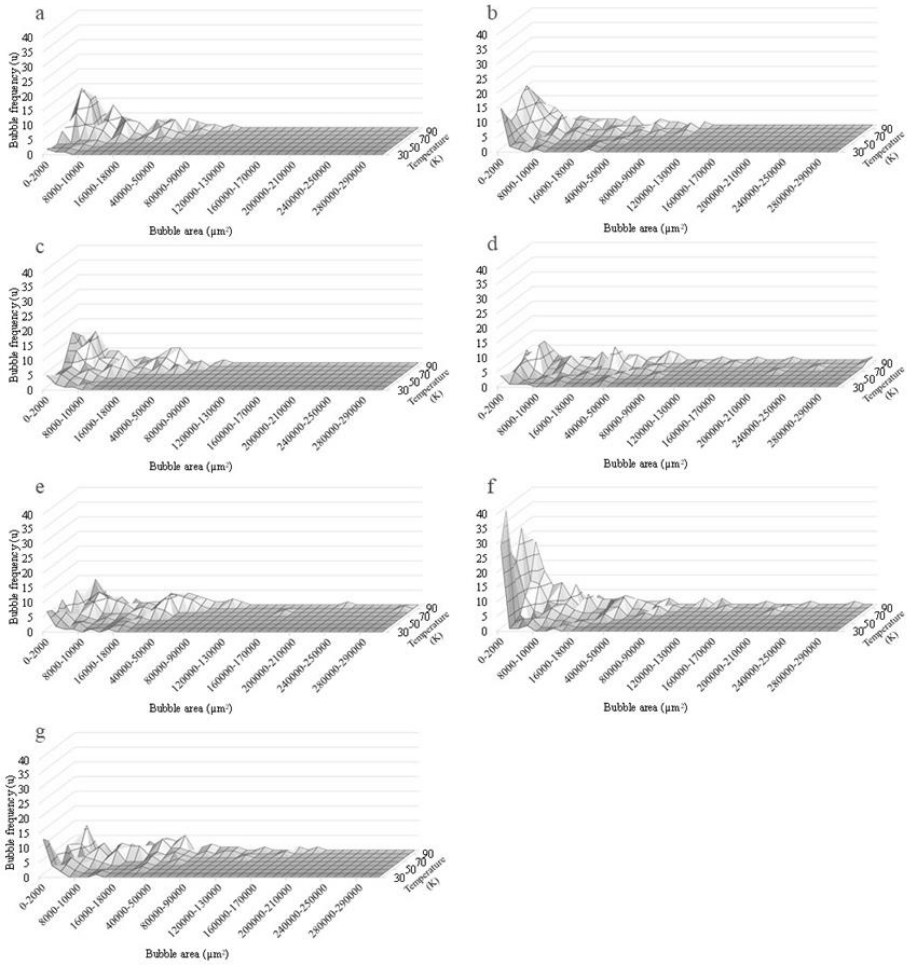


Figure 3. Bubble size distribution during baking for each emulsifier: Control (a), DATEM (b), DMG-45 (c), DMG-75 (d), Lecithin (e), PGEF (f) and SSL (g).

Image digital analysis and texture profile of breads

The effect of emulsifier addition on technological characteristics is summarized in Table 2. Compared to the control, significantly smaller longitudinal area was produced by PGEF addition, which meant a reduction in the size of the loaves. The rest of the emulsifiers did not significantly modify this parameter. Previous studies reported that adding SSL and DATEM resulted in higher area and volume of breads, due to the increase in dough aeration and volume [28,29]. Probably the use of high hydrated doughs is responsible for the differences with previous studies. In addition, a negative correlation (r=-0.8754) was observed between the longitudinal area of the small-scale breads and the maximum height of the proofed dough (Hm). This correlation indicated that dough volume increased during fermentation with emulsifiers addition but likely they did not confer enough resistance to improve final volume. Likewise, no significant differences were found in the longitudinal perimeter, except with DATEM and PGEF that gave smaller values.

The analysis of the bread cross section revealed significant differences in the number of gas cells (cell cm^{-2}) and mean cells area (mm^2) on account of emulsifiers addition (Table 2, Figure 4). The greater number of cells, the less mean cell area and vice versa. DMG-45, DMG-75, DATEM and Lecithin showed greater cell number with smaller area than the control. In the case of distilled monoglycerides emulsifier (DM) with different particle size (DMG-45 and DMG-75), no significant difference was observed in these parameters, showing that particle size did not affect the cell number and area. Emulsifiers did not induce a significant effect on the circularity compared to the control. However, significant differences between DMG-75 (0.60) and PGEF (0.74) were found. Perfect circularity is difficult to obtain in bread, due to the pressure differences in the gas bubbles and changes occurred during process [25].

All samples showed significant differences in all texture parameters compared to control (Table 2). With the hydrated recipe used very soft crumbs were obtained, and emulsifiers increased the crumb hardness although variation ranged from 79 to 100 g. The highest hardness was obtained with the PGEF, followed by SSL and DATEM. DMG-45, DMG-75 and lecithin were the emulsifiers that less rise the crumb hardness.

Hardness showed a strong positive correlation (r=0.9373) with the maximum height of dough (Hm) during fermentation, but that contrasts with results obtained when optimum hydration of wheat flour (500-600 ml kg^{-1}) was used [12]. Dough hydration affects the size of the bubbles diameter [30], leading to higher bubbles, but since all recipes were prepared with the same hydration it should be expected no additional effect due to the liquid phase. Considering the high number of smaller cells mostly found in the breads containing emulsifiers, the hardness increase must respond to the thickness of the cell walls. Therefore, in this study the interaction of emulsifiers with proteins and starch leading to the cell walls had greater impact on texture than the bubbles feature. It has been previously reported that a higher degree of gluten polymerization during baking results in higher firmness of the baked products [11]. At the same time, emulsifiers, like SSL or DATEM interact with gluten, changing the solubilization of polymeric aggregates and that interaction was dependent on the type of emulsifier [20], particularly SSL reduces the incorporation of gliadins into the gluten network having a direct effect on the subsequent polymerization during baking [11], and in turn affecting crumb firmness. Therefore, at the level of hydration used in the present study, emulsifiers contributed to increase dough aeration and in consequence the number of gas cells in the crumb, but simultaneously their interaction with gluten changed the proteins polymerization during baking affecting cell walls thickness and in turn crumb firmness.

Considering the other texture parameters, chewiness was significantly higher in the samples with emulsifiers, except with DMG-75, than in the control, and resilience decreased especially in samples with distilled monoglycerides (DMG-45 and DMG-75), which again differed than the previously reported with optimum hydrated doughs[12], confirming the important role of water on the dough aeration and crumb texture.

Table 2. Emulsifiers effect on loaves morphogeometrics characteristics, cell crumb distribution and texture profile of small scale breads.

Sample	Control	DATEM	DMG-45	DMG-75	Lecithin	PGEF	SSL
Longitudinal section							
Area (cm^2)	5.37 ± 0.48^{bc}	5.31 ± 0.24^{bc}	5.09 ± 0.19^{ab}	5.12 ± 0.32^{ab}	5.00 ± 0.24^{ab}	4.95 ± 0.20^{a}	5.50 ± 0.30^{c}
Perimeter (cm)	1.25 ± 0.04^{c}	1.17 ± 0.07^{ab}	1.23 ± 0.05^{bc}	1.25 ± 0.07^{bc}	1.23 ± 0.1^{bc}	1.11 ± 0.04^{a}	1.20 ± 0.08^{bc}
Cross section							
Number of cells cm^{-2}	10 ± 2^{a}	13 ± 2^{bc}	15 ± 1^{cd}	16 ± 2^{d}	15 ± 2^{cd}	9 ± 2^{a}	12 ± 3^{ab}
Mean cell area (mm^2)	3.31 ± 0.81^{b}	1.92 ± 0.89^{a}	1.57 ± 0.47^{a}	1.61 ± 0.40^{a}	1.88 ± 0.34^{a}	3.23 ± 1.70^{b}	2.48 ± 0.48^{ab}
Minimum cell area (mm^2)	0.15 ± 0.02^{c}	0.14 ± 0.04^{c}	0.10 ± 0.03^{ab}	0.06 ± 0.02^{a}	0.12 ± 0.03^{bc}	0.13 ± 0.02^{bc}	0.12 ± 0.03^{bc}
Maximum cell area (mm^2)	11.80 ± 3.61^{bc}	7.88 ± 1.79^{ab}	7.22 ± 1.94^{a}	9.12 ± 2.60^{abc}	10.13 ± 2.37^{cd}	17.73 ± 4.77^{d}	13.67 ± 3.65^{abc}
Circularity	0.68 ± 0.16^{ab}	0.68 ± 0.12^{ab}	0.62 ± 0.11^{ab}	0.60 ± 0.11^{a}	0.70 ± 0.06^{ab}	0.74 ± 0.07^{b}	0.72 ± 0.05^{ab}
Crumb texture							
Hardness (g)	55 ± 4^{a}	85 ± 6^{c}	79 ± 3^{b}	79 ± 3^{b}	80 ± 4^{bc}	100 ± 6^{e}	93 ± 5^{c}
Springiness	0.95 ± 0.01^{c}	0.93 ± 0.01^{c}	0.94 ± 0.02^{a}	0.86 ± 0.06^{ab}	0.94 ± 0.04^{c}	0.91 ± 0.02^{bc}	0.85 ± 0.08^{c}
Cohesiveness	0.83 ± 0.03^{c}	0.73 ± 0.02^{b}	0.72 ± 0.03^{a}	0.66 ± 0.05^{a}	0.73 ± 0.04^{b}	0.74 ± 0.03^{b}	0.64 ± 0.04^{b}
Chewiness (g)	35 ± 5^{a}	56 ± 6^{bcd}	53 ± 5^{bc}	47 ± 5^{ab}	56 ± 3^{bc}	58 ± 6^{d}	51 ± 7^{b}
Resilience	0.49 ± 0.03^{c}	0.38 ± 0.01^{b}	0.37 ± 0.04^{a}	0.29 ± 0.03^{a}	0.39 ± 0.04^{b}	0.40 ± 0.03^{b}	0.26 ± 0.01^{b}

Mean ± standard deviation. Different letters within the same parameter differ significantly ($P<0.05$)

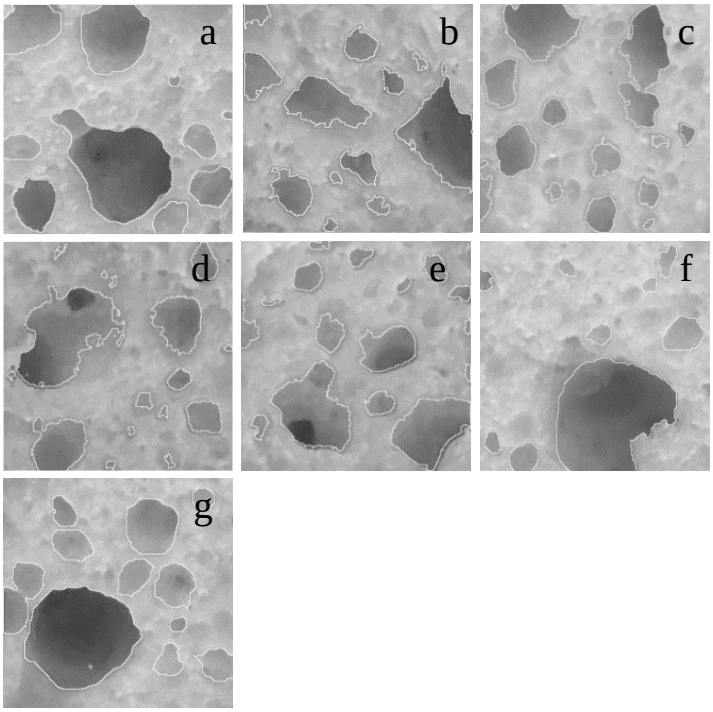


Figure 4. Captured images and bubbles count of small scale breads. a: Control, b: DATEM, c:DMG-45, d:DMG-75, e: Lecithin, f: PGEF, g: SSL.

Statistical analysis

In order to understand the effect produced by the emulsifiers and the differences or correlations between them, a cluster analysis (Figure 5) and an analysis of principal components (Figure 6) were carry out. Cluster analysis showed the discrimination between breads containing emulsifiers and the control by combining the two observations that were closest to form the groups. Three well differentiated groups were drawn, with the control and DMG-75 being more separated from the rest of the samples. The other emulsifiers were closer in relation to the analyzed variables, evidencing more similar effects in the doughs and final product. According to their performance with dough and bread, the closest emulsifiers were DATEM and lecithin, followed by DMG-45, SSL and finally PGEF. In this study, the closeness observed between lecithin and DATEM was attributed to the production treatment of lecithin that

included a hydrolysis stage, thus it behaves as a monoglyceride despite of being from a diglyceride.

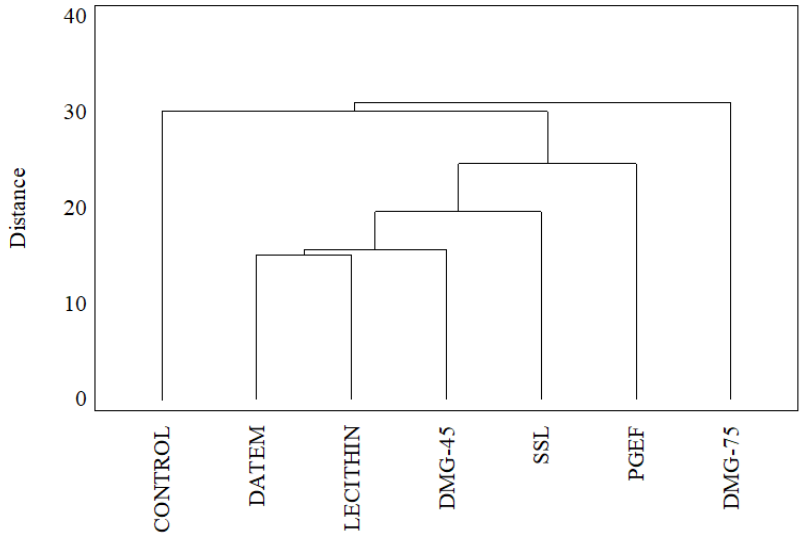


Figure 5. Cluster statistical analysis by using closest neighbor method.

Principal component analysis (PCA) of the experimental data obtained containing emulsifiers resulted in two principal components explaining 48.3 and 22.0 % of the data variation (Figure 6). Thus, the model explained 70.3% of the total variation in data. The first principal component weight (PC1) was defined by the longitudinal 2D area, the longitudinal 2D perimeter and cell cm^{-2} in the positive axis, and on the negative axis were located resilience, cohesiveness, springiness, Tx and mean cell area. Component 2 (PC2) was defined by T1, bubbles cm^{-2}, longitudinal 2D area, H′m and the mean bubble area. DATEM, Lecithin, SSL, and PGEF were found in the negative PC1 component where the majority of dough and bread responses were located. As shown in cluster analysis (Figure 5), DMG-75 was the furthest emulsifier attending to its experimental responses, particularly longitudinal 2D area and perimeter, and H′m. Results obtained from DATEM and Lecithin were explained due to responses to chewiness, cohesiveness, resilience, cell circularity and Tx. However, PGEF and SSL, adjacent in cluster analysis, were related with the mean cell area, hardness, T′1 and Hm. Eventually,

DMG-45 position was related to T1 and the number of gas cells cm^{-2}. Overall, emulsifiers could be grouped into four categories attending to the responses obtained with dough and bread performance. In the first group, DATEM and Lecithin due to their effect on crumb texture and dough permeability; second group would include SSL and PGEF that showed bigger bubbles, with less and bigger gas cells and higher crumb hardness; third group with an intermediate behavior respect to the previous ones DMG-45; and finally DMG-75, with a more distant behavior than the control, which led to big bubbles.

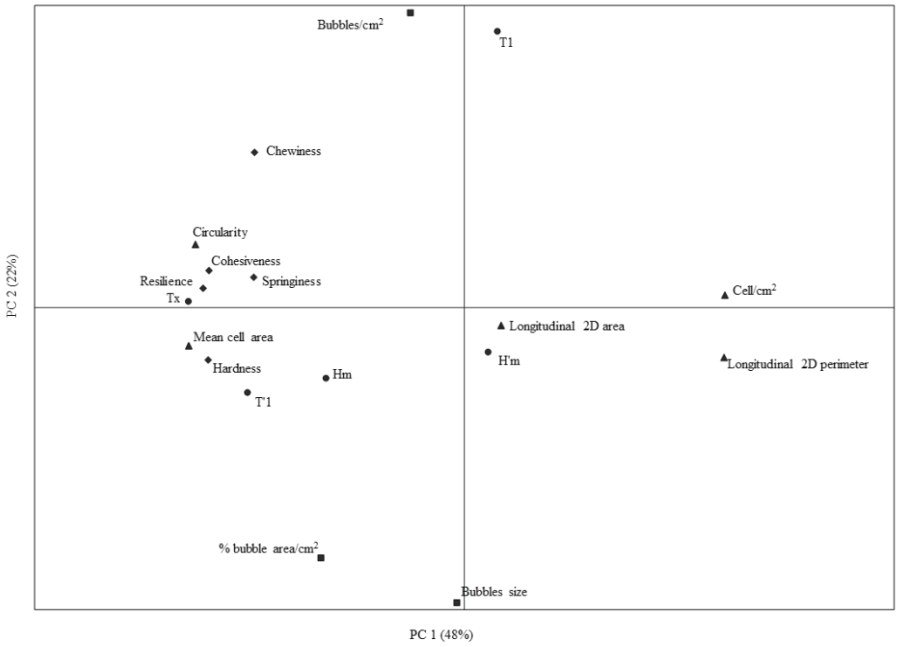


Figure 6. Score plot from a principal component analysis of the combination of components weight (■ simulated microbaking, ◆ texture properties, ● rheofermentometer variables and ▲ digital image analysis of breads) and principal components (× emulsifiers).

Conclusions

Emulsifiers role on the progress of bubbles during proofing and baking was evaluated. Emulsifiers showed different functionality that was attributed to their diverse chemical structure and also physical

characteristics (particle size). Furthermore, results shown that emulsifiers functionality was dependent on the dough hydration. All emulsifiers studied, increased the maximum dough volume during proofing, but showing different effect on dough permeability or ability to retain CO_2. Digital image analysis of the recorded baking under microscope, allowed quantifying both bubbles number and size and understand emulsifiers role on aeration. Emulsifiers allowed greater air incorporation into the dough observing higher number of bubbles, particularly with PGEF. Major changes in dough occurred at 70 ºC when bubble size augmented and became more heterogeneous, and emulsifiers affected the size and number of bubbles, with DMG-75 producing the biggest bubbles. In bread, emulsifiers tend to increase the number of gas cells with lower size, but that gave greater crumb firmness, which suggested different interactions between emulsifiers and gluten, affecting protein polymerization during baking. Despite the diverse chemical structure of the emulsifiers, experimental data following dough proofing and bread features allowed to discriminate among them.

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Capítulo 4

Establecer posibles relaciones entre las características tecnológicas y las propiedades del bolo, la masticación y la deglución de la miga.

I. Modifying gluten-free bread's structure using different baking conditions[1]

Raquel Garzón, Cristina M. Rosell

Institute of Agrochemistry and Food Technology (IATA-CSIC). Agustín Escardino, 7, Paterna, Valencia, Spain

Abstract:
This study evaluated the impact of different gluten-free bread's inner structure on oral processing and texture perception. Four rice-based breads were prepared varying water hydration (H: 85 or 100 g of water/100 g of flour) and fermentation time (F: 30 or 75 minutes). Texture and structure of the bread crumbs, bolus properties (after three chews and at swallowing point) and perceived sensations using Temporal Dominance of Sensations were measured. Long fermentation breads (H100F75 and H85F75) had bigger air cells and less density than short fermentation breads (H100F30 and H85F30). High hydration breads were harder, chewier, less cohesive, and resilient. Bolus from H100F75 and H85F75 breads was moister, softer, less dense, than the H100F30 and H85F30 bolus, which was more consistent and adhesive. Breads differed in the amount of saliva required for chewing and the chewing number before swallowing. Thus, by varying breadmaking conditions like hydration and fermentation time it was possible to change the texture of gluten-free breads and in consequence the texture sensations perceived during consumption.

Key words: crumb, structure, bread, *in vitro* chewing, texture

Raquel Garzon: Investigation, Formal analysis, Data curation, Visualization, Writing- Original Draft
Cristina M. Rosell: Conceptualization, Supervision, Writing- Review & Editing, Funding acquisition

[1] These results have been submitted as a part of the following publication:
P. Puerta [1,a], R. Garzón [1,a], C.M. Rosell[1], S. Fiszman[1], L. Laguna[1*], and A. Tárrega[1]. Modifying gluten-free bread's structure using different baking conditions: impact on oral processing and texture perception. *LWT. In press.*
[a] These authors have equally contributed to the work

Introduction

The increasing demand for gluten free products, and particularly bread, has promoted numerous researches for improving their quality. Despite the extensive research carried out in gluten free breads, it is still an important challenge to replace gluten with other structure-forming ingredients able to keep the carbon dioxide during proofing and baking [1]. In breads, gluten forms a network that provides cohesiveness and helps CO_2 retention, produced during fermentation, creating an open crumb structure [2]. The absence of gluten dramatically affects the dough characteristics, leading to batters instead of doughs that have very limited capacity for holding the carbon dioxide released by bakers' yeasts [3]. Gluten free breads are characterized by low volume, a crumbly texture, pale colour, and poor flavour compared their gluten containing counterparts [1,4]. Because of that intensive efforts are still addressed to improve the quality of these products.

Commercial gluten-free-breads are made using a long list of ingredients, including hydrocolloids as structuring agents, but also acidifiers, emulsifiers, leavening agents and preservatives [4,5]. This extensive list of ingredients is against the current food trend that looks for lean and clean labels [6]. Because of that it has become rather important to investigate the impact of breadmaking conditions on the gluten-free bread characteristics [7]. Recently, Cao, et al. [8] investigated the effect of fermentation time on gluten containing breads, reporting an increase of bread volume and softer texture when optimizing the fermentation time. Nevertheless, this strategy has not been tested in gluten-free breads. In the case of gluten free breads, it has been reported the importance of water hydration or the amount of water added in the recipe when making bread [9]. By changing the water hydration, it is possible to modify the bread volume and the texture of gluten-free breads [10-13]. Therefore, breadmaking conditions could be investigated as an alternative to modify texture properties of gluten free products.

Considering the impact of mastication on absorption and likely incidence on health, this fact becomes of crucial importance in foods addressed to people suffering any ailments. In fact, gluten intolerant consumers are suffering of vitamin and mineral deficiencies when diagnosed [14] and

commercial gluten free products show a very diverse nutritional pattern [4] leading to unbalanced diets. An efficient adsorption would be strongly desirable in this type of products.

This study's hypothesis was that the modification in the structure of gluten-free breads achieved by varying breadmaking conditions can affect the texture of the bread and its breakdown pattern in mouth. Therefore, this study aimed to study how structural changes in gluten-free breads, caused by different baking conditions (level of dough hydration and fermentation time), could affect the *in vitro* disintegration, and those results have been correlated with oral processing (bread breakdown and bolus formation) and sensory perception.

Materials and methods

Rice flour was purchased from Harinera La Meta, S.A. (Lleida, Spain). Corn starch was acquired from EPSA Aditivos Alimentarios (Valencia, Spain). Compressed yeast was kindly donated by Lessaffre (Lille, France). Hydroxypropylmethylcellulose (HPMC) (Methocel K4M) was supplied by Dow Chemical (Midland, MI, USA). The salt, sugar and seeds oil were purchased from the local market.

GF Breadmaking

Preliminary tests were carried out testing the effect of hydration and proofing time in order to obtain different crumb texture. From those, conditions were selected to obtain four different types of bread. The basic gluten free formulation was the following, on flour/starch blend basis: 70% rice flour, 30% corn starch, 3% of compressed yeast, 1.5% of salt, 2% of seeds oil, 2% of HPMC, 1% sugar. Water hydration was 85% or 100%, further referred as H85 or H100, respectively. Solids were blended in a kneader with a dough hook, then water was added and mixed for 8 minutes. Then 600 g of dough were moulded (20 x 10 x 10 cm, length, width, height) and fermented in a controlled cabinet (Salva, Guipúzcoa, Spain). The fermentation times were 30 or 75 minutes at 35 °C to obtain different crumb grain structures, which were referred as F30 or F75, respectively. Finally, the dough was baked at 185 °C during 45 minutes in a convection oven (Eurofours, Gommegnies, France). The bread quality attributes were evaluated after cooling for 1 h at room

temperature. Combining both levels of hydration and fermentation times, four breads were obtained: H85F30, H85F75, H100F30, and H100F75.

Characterisation of instrumental quality parameter of gluten free breads

Breads were characterized regarding moisture content, crumb texture, crumb grain characteristics and density. The moisture content of gluten free bread samples was determined following the International Cereal Chemistry (ICC 110/1, 1994). Texture profile analysis (TPA) was performed using a TA-XT.plus texturometer (Stable Microsystems, Surrey, UK) equipped with a 5-kg load cell and 75-mm aluminium cylindrical probe. Crumb slices of 2 cm thickness and after removing the crust were used for texture evaluation. The test speed was 2.0 mm/s with a trigger force of 5 g and crumb slices were compress to 50% of its original height. Eight replicates from two different batches of baking were analysed and averaged. Parameters recorded were hardness (maximum force at the first compression), cohesiveness (calculated as the ratio between the areas under the second and the first peak), springiness (calculated as the ratio between the distances at which force is maximum during the second and the first cycle), chewiness (calculated from the multiplication of cohesiveness per hardness), and resilience (calculated by dividing the upstroke area of the first compression by the downstroke area of the first compression). The grain characteristics of gluten free breads were studied by digital image analysis as it has previously reported [1]. Briefly, bread slices were captured using a flatbed scanner (HP Scanjet 4370, Hewlett–Packard, USA) in the RGB standard format with 1200 dpi of resolution. Image analysis of bread slices was carried out by Fiji Image J software (Schindelin et al., 2012). For image segmentation and crumb grain characteristics determination images contras were improve and was use a pre-defined algorithm "Otsu". The crumb grain measurements included: cell density (cell/cm^2), mean cell area (mm^2), surface porosity (total cell area/ total slice area in percentage) and mean cell shape using the circularity factor (0- perfect circle to 1 perfect rectangle). Results are the average of two replicates from two different sets.

Crumbling was use as a descriptor to simulated mastication without any liquid and to determine how the process affected the crumbling.

Crumbling was carried out by ULTRA-TURRAX® Tube drive (IKA, Germany) equipped with BMT-20 tube for grinding using 15 glass balls. For the analysis 1 g of each sample was introduced and grinded during 15 or 30 seconds. Then the resulting crumb particles dispersion were scanned with the same conditions describe above and further analysed using Fiji Image J software. Crumbling, expressed as percentage, was calculated as total aggregates area divided by number of aggregates. Four samples from different batches were evaluated. The crumb density was obtained from the weight and volume of a cylinder of the central part of the crumb (diameter = 3.3 cm: height = 2 cm). Three replicates were analysed for each sample.

Bread bolus characterisation

Bolus characterisation and sensory evaluation were performed with the central part of the crumb (diameter = 3.3 cm; height = 2 cm). Eleven healthy subjects with complete dentition participated in the study. Subjects placed the entire sample in the mouth, chewed it and spitted the bolus out for bolus characterisation. Particle size distribution and number of particles were obtained for each bread and subject. The median particle area (a50) or particle area corresponding to 50% of total area occupied, was calculated. The interquartile ratio (a75/a25) between 75 and 25% of the cumulative area occupied by particles was determined. Consistency and adhesiveness of bolus obtained at swallowing point were measured using a TA.XT plus Texture Analyser (Stable Micro System, Godalming, UK), fitted with a TTC Spreadability Rig containing a male cone (90° and 40 mm diameter) that matches a glass containing the female cone fixed in an HDP/90 platform. The force when penetrating the sample with the male cone at a constant rate of 2 mm/s until a depth of 28 mm was recorded.

Statistical analysis

For each quality parameter, a one-way analysis of variance (ANOVA) was applied using Statgraphics Centurion XVII (Statistical Graphics Corporation, UK). Fisher's least significant difference test was used to assess significant differences ($P < 0.05$) among samples that might allow discrimination among them. Additionally, Pearson correlation analysis

was applied to establish possible relationships between the texture profile and both crumb grain structure and crumbling of the gluten-free breads.

Results

Gluten-free bread characteristics

Slices of gluten free bread images were captured to observe differences between the different samples (Figure 1). By controlling process conditions (hydration and proofing time) it was possible to obtain breads with different appearance and size. The loaves with less hydration (H85F30, H85F75) originated larger slices. Moreover, the increase of fermentation time (H85F75 and H100F75) increased the height of slices, compared with those obtained with shorter fermentation but same hydration (H85F30 and H100F30).

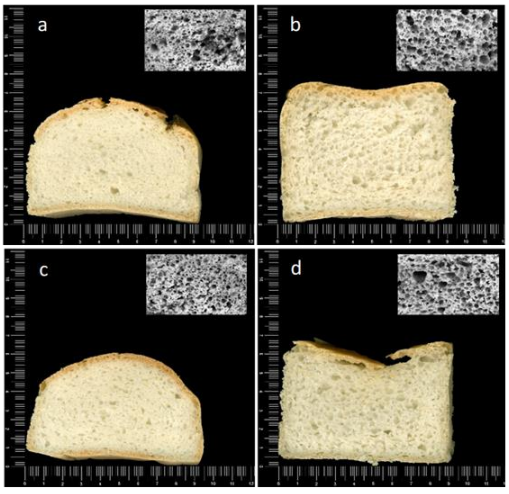

Figure 1. Scanning of the sliced gluten-free breads: a) H85F30, b) H85F75, c) H100F30 and d) H100F75. Units in the scale are expressed in cm. H85 and H100: addition of 85 or 100g of water/ 100 of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 minutes, respectively.

Texture parameters of gluten free breads were assessed using only the crumbs (Table 1). Crust was previously removed to eliminate the mechanical contrast that in oral processing could elicit increasing chewing effort [15]. Human mastication implies that spongy products like bread crumb are submitted to a severe compression, and consequently to a large deformation [16]. As it was expected, all texture values were affected by

hydration level and fermentation time ($P<0.05$), except springiness. Morreale, et al. [11] reported strong and moderate correlations between hydration level and some instrumental parameters. Particularly, authors found a strong positive link between water quantity and crumb hardness of gluten free breads. Long fermentation time gave softer crumbs than short fermentation time when the same amount of water was added in the process, which considering the highest height observed, it was indicative of higher dough expansion leading thinner gas cells. Cohesiveness and resilience were similar in all the samples, apart from H100F30 that was statistically significant lower. High cohesiveness is desirable because higher crumb cohesion represents a lower disaggregation during mastication [17]. Therefore, different textural parameters were obtained using the same lean formulation but changing process parameters, which might affect oral processing. Longer fermentation resulted in gluten-free breads with a more open structure (bigger air cells and less density) with crumbs presenting lower instrumental hardness values.

Moisture content of the breads was significantly dependent on the fermentation time, resulting in higher moisture content when extending the proofing time. Nevertheless, when crust was removed, the moisture content of the crumbs was significantly affected by hydration level and fermentation time. Specifically, as expected, higher hydration (100%) gave crumbs with higher moisture content and also fermentation time increased the moisture retention. This different moisture content of the crumb could affect the swallowing points [15]. Considering the possible impact of internal crumb structure on the mastication process, crumb structure was evaluated, assessing the number and size of the gas cells due to their impact on the internal crumb structure [18]. Moreover, crumb grain structure was modified only when fermentation time change during the process, but no significant differences were observed due to the different hydration. The number of cells increased with short fermentation times, but with lower mean cell areas. Nevertheless, circularity was similar even with the used fermentation time or water amount.

To evaluate physical disruption of the crumb that could occur during mastication, a test to force crumb rupture was set up. In addition, two different times (15 s and 30 s) were defined to simulate the impact of shorter or longer mastication. Crumbling parameter was intended to

identify possible differences in the disintegration of the samples, with higher percentage corresponding to higher tendency of the crumb to be disrupted that should indicate that shorter mastication would be needed (Table 1 and Figure 2). After breaking, crumbs were reduced into particles with smaller size. Unexpectedly, longer stress time did not conduct significantly modify the crumbling parameter, which could indicate that aggregates were linked by high cohesive forces, likely the plasticizer role of water contributed to that behaviour. In fact, bread de-structuration has been related to hydration and crumb fragmentation [19]. Hydration level significantly affected the crumbling tendency of the crumbs, and fermentation time only affected significantly when crumbs were subjected to longer physical stress (30 s) (Table 1). Breads with longer fermentation time showed higher crumbling, which agrees with the fragile and thinner cell wall structure that is brought about with higher expansions. The crumb disruption was better observed when the distribution of the area of the aggregates was analysed (Figure 2). The mean area of the aggregates increased with the hydration level and the proofing time, and that trend was observed after samples stressing for 15 or 30 s. The particle area distribution decreased when increasing the hydration level or the proofing time, shifting to smaller particle areas when using high hydration and longer proofing. In addition, that dispersion was reduced when crumbs were subjected to longer stressing (30 s). Samples with less water amount (H85F30 and H85F75) originated great dispersion of aggregates with larger area. On the other hand, long fermentation time produced greater number of aggregates with respect to their counterparts obtained with the same hydration. Considering that large size particles after chewing have been related to low postprandial insulin responses [20], lower hydration and shorter fermentation might result in better postprandial physiology.

To study the relation between different instrumental quality parameters commonly used to characterize breads as textural parameters or crumb grain structure and crumbling, a correlation a Pearson correlation analysis was made (data not shown). Hardness were moderated significant and positively correlated with cell density and crumbling 30". Nevertheless, a weak negative correlation between chewiness and mean cell area and with surface porosity was found. Presumably, low cell areas mean thinness cell walls and reducing the chewiness.

Table 1. Instrumental quality parameters of gluten free breads.

	H85F30	H85F75	H100F30	H100F75	*P-value*	
Texture profile					*Hydration level*	*Fermentation time*
Hardness (g)	4085 ± 337^{b}	1551 ± 236^{a}	4972 ± 697^{c}	1667 ± 362^{a}	**0.0329**	**0.0000**
Springiness	0.851 ± 0.045^{a}	0.860 ± 0.087^{a}	0.875 ± 0.070^{a}	0.906 ± 0.046^{a}	0.1346	0.4153
Cohesiveness	0.726 ± 0.028^{b}	0.758 ± 0.041^{b}	0.692 ± 0.027^{a}	0.733 ± 0.024^{b}	**0.0135**	**0.0037**
Chewiness (g)	2424 ± 379^{b}	1011 ± 191^{a}	3041 ± 474^{c}	1112 ± 272^{a}	**0.0262**	**0.0000**
Resilience	0.400 ± 0.024^{b}	0.417 ± 0.030^{b}	0.370 ± 0.023^{a}	0.398 ± 0.015^{b}	**0.0080**	**0.0168**
Moisture						
Whole bread (%)	41.96 ± 0.57^{a}	50.85 ± 0.15^{c}	46.10 ± 0.87^{b}	47.50 ± 0.21^{b}	0.8273	**0.0297**
Crumb (%)	50.23 ± 0.49^{a}	50.92 ± 0.13^{ab}	51.00 ± 0.18^{b}	54.30 ± 0.14^{c}	**0.0189**	**0.0217**
Crumb density	0.54 ± 0.02^{d}	0.29 ± 0.01^{a}	0.49 ± 0.01^{c}	0.41 ± 0.01^{b}	**0.0486**	**0.0679**
Crumb grain structure						
Cell density (cell/cm^{2})	30 ± 0	15 ± 1^{a}	26 ± 1^{b}	15 ± 1^{a}	0.1503	**0.0001**
Surface porosity (%)	33.62 ± 0.27^{ab}	37.85 ± 1.52^{b}	32.79 ± 2.48^{a}	36.93 ± 2.29^{ab}	0.6154	**0.0382**
Mean cell area (mm^{2})	1.17 ± 0.17^{a}	2.47 ± 0.11^{b}	1.25 ± 0.07^{a}	2.50 ± 0.47^{b}	0.9128	**0.0005**
Circularity	0.552 ± 0.008^{a}	0.531 ± 0.009^{a}	0.535 ± 0.018^{a}	0.522 ± 0.001^{a}	0.1299	0.0632
Crumbling						
Crumbling 15" (%)	95.35 ± 2.84^{a}	96.98 ± 0.83^{ab}	97.37 ± 1.18^{ab}	98.76 ± 0.48^{b}	**0.0286**	0.1257
Crumbling 30" (%)	94.50 ± 1.24^{b}	97.60 ± 0.72^{a}	97.75 ± 0.38^{a}	98.22 ± 0.44^{a}	**0.0026**	**0.0044**

Means with different letters within the same parameter differ significantly (*P<0.05*)

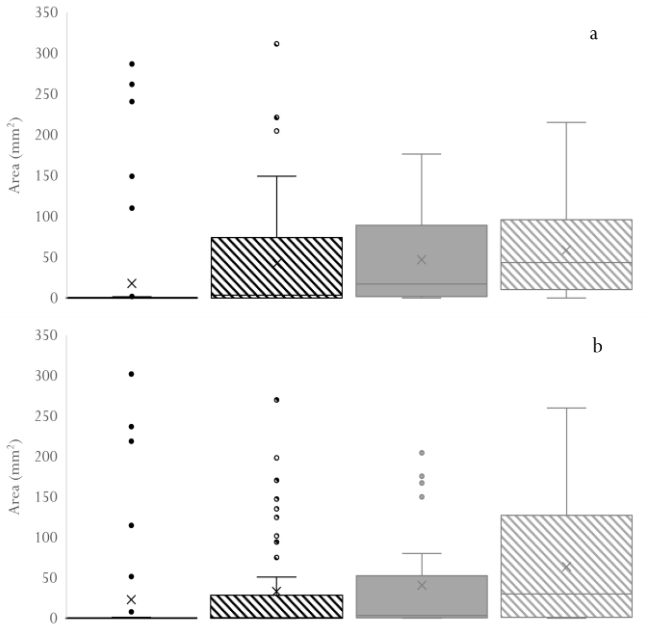

Figure 2. Areas of the aggregates obtained after grinding for 15 (a) or 30 (b) seconds a portion of gluten free bread crumbs. Black and grey boxes are for 85% and 100% hydration respectively; solid fill and downdrawn diagonal pattern fill are for 30 or 75 minutes of fermentation, respectively.

Chewing, and swallowing activity and bolus characterization

To test the impact of the structural features of the gluten-free breads, those were subjected to chewing and swallowing. Results (not shown) indicated that the number of chewing cycles, swallows, time to the first swallow, and total chewing time significantly varied among breads ($P < 0.005$), particularly due to the fermentation time. Increasing fermentation time resulted in breads (H100F75 and H85F75) that required fewer chews, swallows, time to the first swallow, and chewing time than breads with short fermentation.

The median particle area (a50) and interquartile ratio (a75/a25) after three chews also showed significant differences ($P < 0.001$) among samples for both parameters (data not shown). Consistency and adhesiveness values of bolus obtained at swallowing point were also determined, observing that long-fermented breads (H85F75 and H100F75) had less consistent bolus and less adhesive at swallowing than shorter fermented breads (H100F30 and H85F30).

Relationship between structural features and sensory perception

A correlation matrix was carried out to establish possible relationships between physical features of the gluten free breads and parameters related to sensory perception during chewing and swallowing (Table 2). Whole bread moisture content was strongly and negatively correlated with bolus consistency and adhesiveness, which was due to the high moisture content of the bread with low hydration and shorter fermentation time (H85F75) that led to more consistent and adhesive bolus. It should be stressed that the crumb structure had a significant impact on the parameters recorded during chewing and swallowing [21,22]. Specifically, cell density was highly positively correlated with a50, a75/a25, bolus adhesiveness, chewing cycles and swallows, and total chewing time. Conversely, mean cell area was strongly negatively correlated with a50, a75/a25, chewing cycles and swallows, and total chewing time. Therefore, bigger air cells area favoured chewing and swallowing, and low number of chews was required to disaggregate the structure. Gao, et al. [23] found that the crumb of steamed bread with lower porosity and smaller cells area required longer chewing time than baguette bread crumb (higher porosity and bigger cells area). The authors reported greater stickiness of the crumb to the teeth with the steamed bread, finding it more difficult to move the bolus. Although gluten free crumbs have been described as crumbling [1], results indicated that extending the fermentation time could improve the viscoelasticity of the crumb. Crumb density was positively correlated with bolus consistency, chewing cycles, time first swallow and total chewing time. Closed crumbs like the ones obtained with shorter fermentation were more consistent and harder to chew and swallow. Significant positive correlations were also observed between the crumbling 15 s and 30 s with the bolus moisture. Therefore, those gluten free breads that gave bigger particles with the simulated mastication showed higher moisture content in the bolus. It seems that initial hydration applied to obtain the breads and the crumb moisture was not significantly affecting the bolus moisture, but high amount of saliva is directly related with the size of the aggregates formed during chewing.

Table 2. Pearson correlation coefficients (r) between instrumental quality parameters and bolus characteristics, chewing and swallowing activity.

	a50	a75/a25	Bolus consistency	Bolus adhesiveness	Bolus moisture	Saliva uptake	Chewing cycles	Swallows	Time first swallow	Total chewing time
Hardness	0.9065	0.9335	0.7809	0.7774	-0.6426	-0.4228	0.9424	0.892	0.9305	0.94
Springiness	-0.4804	-0.5382	-0.3296	-0.5009	0.8167	-0.6902	-0.3302	-0.4555	-0.1765	-0.358
Cohesiveness	-0.6764	-0.6855	-0.598	-0.5106	0.2205	0.8062	-0.7965	-0.67	-0.8634	-0.7791
Chewiness	0.8883	0.9165	0.7599	0.7511	-0.6069	-0.456	0.9319	0.8734	0.926	0.9282
Resilience	-0.5489	-0.5616	-0.4669	-0.3662	0.064	0.8663	-0.6886	-0.5415	-0.773	-0.6678
Whole bread moisture	-0.9186	-0.8663	**-0.9928****	**-0.9682***	0.7847	0.2225	-0.8993	-0.936	-0.8784	-0.9031
Crumb moisture	-0.5799	-0.6531	-0.3808	-0.5356	0.8005	-0.4884	-0.4663	-0.5484	-0.331	-0.489
Cell density	**0.9956****	**0.9982****	0.9324	**0.9606***	-0.8845	-0.145	**0.9676***	**0.9907****	0.9146	**0.9750***
Surface porosity	-0.9354	-0.9456	-0.8484	-0.8274	0.6495	0.4714	-0.9758	-0.9276	**-0.9736**	**-0.9721***
Mean cell area	**-0.9786****	**-0.9939****	-0.8824	-0.9032	0.8128	0.2413	**-0.9728***	**-0.9686***	-0.9318	**-0.977***
Circularity	0.8513	0.8573	0.7992	0.898	**-0.9999*****	0.3338	0.7391	0.8457	0.6248	0.7599
Crumbling 15s	-0.683	-0.7061	-0.6037	-0.7425	**0.9572***	-0.5677	-0.5383	-0.6713	-0.3971	-0.5645
Crumbling 30s	-0.7452	-0.7196	-0.7786	-0.8638	**0.9503***	-0.4158	-0.6169	-0.7526	-0.5068	-0.6389
Crumb density	0.9315	0.8902	**0.9720***	0.9172	-0.6645	-0.4645	**0.9607***	0.9444	**0.9717***	**0.9570***

*****$P < 0.05$, ******$P < 0.01$ and *******$P<0.001$ (Fisher test).

Conclusions

Changes in the structure of gluten-free breads were achieved by varying two conditions: water hydration and fermentation time. Both conditions influenced mechanical properties of food bolus, but only fermentation time influenced oral behaviour and sensations perceived during mastication. The evaluation of the bread structure confirmed its influence on the sensory perceptions and the chewing and swallowing performance. Therefore, innovations in gluten free breads could be obtained by modulating the breadmaking conditions, which allowed modifying the crumb texture using very lean recipes.

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DISCUSIÓN GENERAL

La búsqueda por la innovación, mejora y control de la calidad de los productos derivados de cereales ha sido uno de los objetivos principales tanto a nivel industrial como en investigación. En general, los principales rasgos característicos que describen el pan son el volumen y la textura, y ambos intervienen en la definición final de la morfogeometría y la distribución alveolar [1,2]. Es más, estos atributos influyen en las decisiones que toma el consumidor a la hora de adquirir el producto. Por otra parte, la constante evolución del mercado y preferencias de los consumidores hacen que sea necesaria la búsqueda de nuevos ingredientes y/o aditivos que amplíen o renueven la oferta. Tal es el caso de la utilización de ingredientes que modifiquen el perfil nutricional de los panes con o sin gluten y/o aditivos que mejoren el proceso o el producto final [3,4]. La modificación de los ingredientes o aditivos utilizados, así como el proceso seguido modifican dichos atributos. De hecho, la modificación de la formulación implica la adaptación del proceso y viceversa. En consecuencia, es necesario conocer y caracterizar los cambios que afectan a la formación de la estructura, y para ello es necesario registrar las modificaciones ocasionadas en todas las etapas de elaboración al modificar la formulación o el proceso. Existen numerosos métodos, ampliamente estudiados, que permiten caracterizar los cambios originados durante la etapa del amasado, la fermentación o la cocción, así como para caracterizar la textura o el volumen. Sin embargo, la aparición del análisis digital de imagen amplía la posibilidad de registrar los cambios ocurridos en la estructura de estos productos de manera objetiva y no destructiva. Por ello, los resultados obtenidos en las diferentes partes de la tesis han estado centrados en comprender la funcionalidad de esta nueva herramienta para estudiar el impacto de los cambios en la formulación o en el proceso en la formación de la estructura (Tabla 1). Para ello, los resultados han sido comentados en detalle en los cuatro capítulos y permitieron la consecución del objetivo de la tesis.

Tabla 1. Resumen de la aplicación del ADI durante los capítulos de la tesis.

	Captura y procesado	Aplicación ADI
Capítulo 1	Escáner (distintas resoluciones) Distintos algoritmos	Optimización de la captura y el procesado. Capacidad de identificar estructuras de la miga de pan con y sin gluten Identificación de cambios en formulación y proceso
Capítulo 2	SEM. Adaptación del umbral para cada imagen	Cambios en la estructura durante la gelatinización de distintos almidones
Capítulo 3		
Capítulo 3-I	Escáner. Escáner. Mejora de la imagen, mismo umbral (valor de t), excepto sin microalga.	Cambios producidos en la estructura de las migas originados por el cambio de tipo de masa (referencia, masa madre o acidificada) y la adición de microalga.
Capítulo 3-II	SEM. Cualitativo. Escáner. Mejora de la imagen, mismo umbral.	Masa fermentada. Estudio de la red proteica al adicionar agentes estructurantes en panes sin gluten. Pan. Estructura final de los productos.
Capítulo 3-III	Microscopio óptico. Mismo umbral para todas las imágenes. Escáner. Mejora de la imagen, aplicación del algoritmo "otsu".	Simulación de la cocción (microscopía). Cuantificación de los núcleos de aire formados durante el amasado y las modificaciones durante la cocción. Pan. Cambios en la estructura y relación con lo obtenido durante la microsimulación de la cocción.
Capítulo 4	Escáner. Mejora de la imagen, mismo umbral.	Caracterización de los panes y correlación del ADI con las propiedades del bolo, masticación y deglución.

En primer lugar (**Capítulo 1-I**) se evaluaron y definieron las condiciones de captura y el procesado posterior de las imágenes, además de validar el análisis digital de imagen como herramienta para discernir entre las distintas estructuras originadas en pan con y sin gluten. El estudio mostró que la resolución afecta ($P<0,05$) a la fidelidad del análisis de imagen a la hora de identificar la estructura del pan. Por tanto, la resolución influye en la cantidad de información y en la capacidad posterior para cuantificar los objetos. Por ejemplo, con menor resolución se redujo el número de alveolos obtenidos en el recuento ya que no se fue capaz de identificar aquellos de menor tamaño, lo cual coincidió con resultados previos obtenidos en panes dulces [5]. Por otra parte, el estudio del efecto del algoritmo utilizado en el análisis es dependiente del producto que se pretende analizar tal como propusieron Gonzales-Barron & Butler [6], recomendando la utilización del algoritmo "otsu" para analizar la distribución alveolar del pan. A pesar de los resultados previamente reportados para la estructura de la miga de panes de trigo, en el presente estudio se analizaron 8 algoritmos distintos en pan con y sin gluten, sin detectarse diferencias estadísticamente significativas entre los distintos algoritmos utilizados. Este resultado se atribuyó a que los algoritmos se adaptan a las características iniciales de la muestra, estableciendo umbrales distintos dependiendo de las características de la imagen analizada. En este sentido, es importante que, a la hora de aplicar el análisis digital de imagen, se preste especial atención en el umbral seleccionado (valor de t) e intentar utilizar el mismo algoritmo para que los resultados sean comparables, eliminando de este modo el posible sesgo producido al aplicar el algoritmo en distintas imágenes con, por ejemplo, tonalidades iniciales distintas.

Una vez comprendida la importancia de la resolución y el umbral aplicado en ADI, los conocimientos adquiridos permitieron comprobar si el análisis de imagen fue capaz de identificar cambios en la estructura originados por modificaciones en la formulación **(Capítulo 1-II)**. En este sentido se probaron distintos hidrocoloides (HPMC y CMC), emulgentes (DATEM, SSL, lecitina y esteres de poliglicerol de los ácidos grasos), dos lipasas comerciales distintas y una lipasa con α-amilasa, y cuatro masas madres de distintos cereales. En el caso de la validación en estructuras sin gluten se utilizaron distintos hidrocoloides y combinaciones de estos (HPMC, goma xantana + goma guar y Ultracel™)

con proteínas de distintos orígenes (guisante y huevo). En ambos casos, el análisis de imagen fue capaz de discriminar entre todas las formulaciones propuestas. Es decir, la aplicación de un mismo umbral (valor de t) permitió distinguir y comparar las distintas estructuras independientemente de la complejidad de la formulación, del cambio realizado en el proceso o del tamaño de la muestra. En el caso de los panes con gluten, la adición de hidrocoloides o enzimas originaron migas con mayor número de alveolos, pero de menor tamaño que el control. Esto se debió principalmente a la interacción de estos con la matriz almidón-gluten, que permitieron reforzarla [7]. Además, las enzimas originaron alveolos más redondeados que en el resto de los casos estudiados. Sin embargo, la adición de emulgentes, excepto la lecitina, mostraron migas más abiertas. Las masas madres consiguieron modificar la estructura, creando migas más cerradas que el control, sin embargo, debido probablemente a la aplicación del mismo valor de t (umbral) durante el ADI, no se pudieron cuantificar dichos cambios. Lo que demuestra la importancia del control del valor del umbral aplicado, siendo necesario adaptarlo en el caso de que el color inicial de las migas sea muy variable. La estructura obtenida en los panes sin gluten también se modificó atendiendo a la formulación utilizada. Sin embargo, el efecto más acusado en los cambios de estructura fue el causado por la adición de proteína de origen vegetal o animal. En el caso de la adición de huevo (propiedades emulsionantes [8]), las migas fueron más uniformes y cerradas que con el uso de proteína vegetal; siendo el hidrocoloide Ultracell™ el que originó migas más abiertas, es decir, independientemente del origen de la proteína utilizada. Por lo que, el ADI resultó una herramienta suficientemente específica y no destructiva, que permitió caracterizar el impacto del cambio de la formulación en la distribución alveolar de panes con y sin gluten. Por otra parte, en el **Capítulo 1-III** tras poner a punto y validar la metodología para elaborar panes a pequeña escala (4 gramos de masa por pieza), se estudió el efecto de la velocidad de amasado. El perfil de consistencia de la masa se modificó a medido que se incorporó mayor cantidad de energía durante el amasado (mayor velocidad). Esto resultó en panes con diferentes texturas y estructuras. Siendo, los panes de velocidades intermedias los que presentaron alveolos de menor tamaño y migas más homogéneas, características de pan de molde. En el caso de los panes amasados a velocidades muy altas (200 y 240 rpm), la energía incorporada durante esta etapa pudo ser excesiva dañando la red de gluten

e induciendo a la coalescencia de los alveolos. Es decir, mediante la velocidad de amasado es posible modular la incorporación de núcleos de aire durante el amasado, modificando la estructura final de los productos.

Como se ha comentado anteriormente, es importante conocer y comprender el impacto de los distintos ingredientes en la formación de la miga. Gran parte de la responsabilidad a la hora de fijar la estructura final de los productos viene dada por el componente mayoritario de los cereales, el almidón. En panes con harina de trigo, durante el amasado y gracias a la energía aportada por la amasadora se forma la red de gluten que contiene los gránulos de almidón en su interior [9]. Estos gránulos serán los responsables durante la cocción de ayudar a fijar la estructura conseguida en las anteriores etapas [10]. Esto es debido a que al aumentar suficientemente la temperatura el almidón gelatiniza, consiguiendo así mantener la estructura porosa. Estudiar mediante una metodología rápida los cambios ocurridos en el almidón durante el calentamiento y poder relacionarlo con la estructura y características del gel formado, fue el objetivo del **Capítulo 2**. Actualmente, las harinas y almidones empleados en la producción de pan con y sin gluten son de distintos orígenes. Por ello, se estudiaron los cambios originados en cuatro almidones (trigo, arroz, maíz y patata) mediante un ensayo rápido de 90 segundos ("Rapid Force Analayzer", equipo Amylab de Chopin). Las micrografías obtenidas a partir de los geles obtenidos a los 90 segundos mostraron que la estructura fue dependiente del tipo de almidón estudiado. Además, cada uno de los almidones durante el calentamiento con agitación registró una fuerza diferente, estando correlacionada positivamente ($P< 0,05$) con la firmeza de los geles obtenidos. Lo más destacable fue que el tiempo para alcanzar la gelatinización y formación de la estructura del gel, varió en función del tipo de almidón. El almidón de patata mostró una estructura claramente formada a los pocos segundos de comenzar el ensayo, sin embargo, el almidón de trigo no mostró una estructura bien formada hasta el final del ensayo. El estudio resultó altamente innovador, dado que se ha extendido la aplicación de un equipo de recientemente lanzamiento a la discriminación entre almidones distintos.

La elaboración de pan a nivel industrial es una adaptación del proceso tradicional realizado durante muchos años. Sin embargo, no existe un

manual que poder aplicar en todos los casos, es más, en la mayoría de las ocasiones se utilizan las mismas temperaturas de horno para elaborar distintos productos, modificando exclusivamente los tiempos que permanecen estos en el horno. Algunos estudios intentan comprender el efecto de la variación de la temperatura en la cocción de los panes. Zhang & Datta [11], observaron como el aumento excesivo de temperatura durante la cocción disminuye el volumen de las piezas. Therdthai, et al. [12], recomiendan que la mejor forma de realizar la cocción es introduciendo gradientes de temperatura, comenzando por temperaturas más bajas que van subiendo a lo largo de la etapa de cocción (de 115ºC a 176ºC). Probablemente, estos hallazgos están relacionados con la velocidad a la que se fija la estructura, o lo que es lo mismo a la temperatura a la que gelatiniza el almidón durante la cocción, dejando de ser una masa elástica y no permitiendo la expansión en el horno. Por lo tanto, determinar las diferencias en la velocidad de gelatinización y formación de la estructura de los distintos almidones, podría ser el punto de partida para aproximarse a la temperatura óptima de cocción.

Los cambios en la estructura y su formación durante las distintas etapas del proceso, en panes con y sin gluten, debidos a la modificación de la formulación por la adición o sustitución de ingredientes y/o aditivos se estudió en el **Capítulo 3** (C3-I, C3-II y C3-III). El ingrediente seleccionado para estudiar los cambios originados en pan con gluten fue la microalga *C. vulgaris* (**C3-I**). El pan es un producto básico ampliamente consumido y versátil, permitiendo la incorporación de distintos ingredientes que permitan mejorar su perfil nutricional o modificar sus características. En los últimos años ha aumentado el interés en la microalga *C. vulgaris* debido principalmente a su alto contenido en proteína, compuestos fenólicos, vitaminas, minerales y perfil lipídico [13]. Para comprobar su efecto en la formación de la estructura se realizó la caracterización de las masas, el seguimiento de la fermentación y se evaluaron las características finales de los panes (a nivel tecnológico y nutricional) tras adicionar distintos niveles de *C. vulgaris* (0, 1, 2 o 3 %), en presencia o ausencia de masa madre o en masa ácida (acidificada químicamente). La incorporación de *C. vulgaris* incrementó los compuestos bioactivos del pan, pero fueron dependientes del tipo de masa utilizada. Sin embargo, fue necesario adaptar el proceso, puesto que se modificaron las características de las masas (absorción y pH) lo que

originó cambios en la cantidad de agua final añadida y el tiempo de fermentación. Las algas y microalgas son una fuente de hidrocoloides, cuando estos son adicionados es necesario adaptar la cantidad de agua para obtener la misma consistencia debido a su elevada absorción de agua [14]. La adición de la microalga modificó las características tecnológicas de los panes. A medida que se incrementó la cantidad de *C. vulgaris* añadida, disminuyó el área 2D de las rebanas, aunque la porosidad de la estructura y tamaño de los alveolos fue mayor. Es decir, pese a que todos los panes se elaboraron a partir de masas con igual consistencia e igual incremento de volumen durante la fermentación, la estructura final de los productos fue diferente, posiblemente motivado por el debilitamiento de la red de gluten que provocó *C. vulgaris*, limitando su expansión final en el horno [15]. Por tanto, pese a la adaptación de las características de las masas y de las distintas etapas del proceso, la adición de esta microalga modificó la estructura de los panes, originando migas más abiertas y heterogéneas, independientemente de la formulación base utilizada.

Uno de los principales problemas a los que se enfrenta la industria a la hora de elaborar panes sin gluten, es la ausencia de las proteínas que conforman el gluten y que dotan a las masas, de la red responsable de dar las características viscoelásticas necesarias durante el proceso [16]. La propuesta estudiada en el **C3-II** fue la sustitución del 10% de la harina de arroz por β-conglicinina (βCC), para evaluar su capacidad como agente estructurante. Para entender la función de la sustitución del 10% de la harina de arroz por βCC como agente estructurante, los panes se compararon con un control 100% harina de arroz y con pan con un 10% de sustitución con gluten vital. La cantidad óptima de agua en la formulación se determinó mediante la capacidad de retención de agua de las mezclas, ya que estudio previos indicaron que de la cantidad de agua añadida en las formulaciones de panes sin gluten depende, en gran medida, el éxito en la elaboración de los productos [17]. De hecho, Espinosa-Ramírez, et al. [17] describieron que la capacidad de retención de agua de βCC fue menor que de gluten vital, debido a la menor formación de enlaces con el agua. Los resultados obtenidos en la presente investigación también reflejaron esa incapacidad de la βCC de formar enlaces con el agua en comparación con la harina de arroz o el gluten vital. La adición de βCC originó una red de proteína continua, semejante a la

observada con el gluten vital, pero con una estructura más homogénea. De hecho, el análisis digital de imagen mostró como la βCC no solo fue capaz de crear una red proteica durante el amasado y mantenerla durante la fermentación (mayor área 2D de las rebanadas), si no que esta se tradujo en una distribución alveolar más abierta (alveolos de mayor tamaño) y homogénea en la miga de los panes sin gluten obtenidos. Sin embargo, también aumentó la dureza de las migas, asociado a su menor capacidad de retención de agua[16] o a que la formación de alveolos de mayor tamaño pudo originar paredes alveolares más gruesas que opusieron mayor resistencia durante el análisis TPA de textura. Por lo que la βCC es una alternativa viable para la producción de panes sin gluten, dado su carácter estructurante y además mejora el perfil nutricional de estos.

El **C3-III** se centró en el estudio de la adición de aditivos utilizados habitualmente por la industria de panificación. Concretamente, el papel de diferentes emulgentes (DATEM, SSL, monoglicéridos destilados con diferente tamaño de partícula (DMG-45 y DMG-75), lecitina de girasol y esteres de poliglicerol de los ácidos grasos conteniendo polisorbato (PGEF) en la estabilización de las masas con hidratación elevada. En procesos industriales estos aditivos son esenciales para mejorar tanto las propiedades de la masa como la calidad final del producto [18], debido a que estas sustancias surfactantes son capaces de crear interacciones con las proteínas del gluten [19]. El papel de los emulgentes estudiados mostraron diferentes funcionalidades dependiendo de la etapa del proceso. En general todos mostraron un buen comportamiento durante la fermentación, mejorando la red de gluten, aunque el efecto fue más visible en tiempos de fermentación largos. El PGEF originó el mayor volumen durante el seguimiento de la fermentación, debido probablemente a la presencia de polisorbato. Gómez, et al. [20] atribuyeron la mejora del volumen durante la fermentación, a la presencia de polisorbatos, debido probablemente a su capacidad como agente dispersante. Sin embargo, la adición de emulgentes retrasó el tiempo para alcanzar el máximo volumen durante la fermentación, debido a posibles interacciones con los azúcares libres que ralentizaron la fermentación. Por microscopía óptica se realizó la simulación del proceso de horneado y cuantificó el número e incremento de tamaño de los núcleos de aire creados durante el amasado, al incrementar la temperatura hasta los

100°C. El recuento de burbujas formadas desde el inicio mostró la capacidad de los emulgentes para estabilizar las oclusiones de aire formadas durante el amasado. Kokelaar, et al. [21] explicaron que la capacidad de estabilizar las burbujas es debida a que los emulgentes disminuyen la tensión superficial de las masas y por lo tanto permiten incorporar mayor cantidad de aire al aplicar agitación. Sin embargo, esta capacidad se vio modificada dependiendo del emulgente estudiado, puesto que al alcanzar los 70°C, cuando aproximadamente se fija la estructura, el comportamiento fue distinto. Algunos mostraron mayor capacidad a la hora de reforzar la red de gluten, tal fue el caso de DATEM o lecitina de girasol, que mantuvieron más constante el número y tamaño de las burbujas. Sin embargo, otros emulgentes tales como SSL o PGEF, originaron burbujas de mayor tamaño, formadas a expensas de las más pequeñas al producirse la expansión de los gases en el calentamiento. Este fenómeno ha sido descrito como la maduración de Ostwald, donde las partículas de mayor tamaño, enérgicamente favorecidas, atrapan a las más pequeñas aumentando de tamaño [22]. Finalmente se caracterizaron los panes, y se observó una relación directa entre lo observado durante la simulación al microscopio y el producto resultante final de la panificación (con levadura). Los emulgentes que mostraron mayor tamaño de burbuja durante la simulación (SSL, PGEF), también originaron alveolos de gran tamaño, al igual que en el caso del control. Sin embargo, los que mantuvieron el número y tamaño durante la simulación (DATEM y lecitina de girasol), originaron mayor número de alveolos, pero de menor tamaño. De esta forma se reforzó la teoría de que algunos de los emulgentes estudiados tuvieron capacidad para reforzar la red de gluten mediante la creación de interacciones con las proteínas, en masas batidas con alta hidratación, permitiendo conservar las burbujas originadas durante el amasado sin producirse la maduración de Ostwald.

Por tanto, el Capítulo 3 confirmó que cualquier cambio realizado en la formulación originó cambios en la estructura, no siendo atribuibles a un solo fenómeno. En el caso de la adición de aditivos o ingredientes que disminuyen el pH, es necesario no solo estudiar los cambios de estructura originados durante la fermentación, que podría verse afectada por inhibición de la actividad de la levadura, si no el efecto de estos sobre la masa (absorción de agua y estabilidad) y su posible impulso posterior en el horno. Con el análisis de imagen se pudieron cuantificar todos los

cambios ocurridos durante el proceso y en el producto final, permitiendo dar explicación a algunos de los cambios originados. Es más, conocer la variación de la estructura al realizar un cambio, permitiría controlar todo el proceso de forma eficiente, así como actuar y modificar aquello que causa los cambios no deseados en la estructura.

Cuantificar los cambios originados en la estructura durante el proceso ha sido uno de los objetivos principales durante la presente tesis. Sin embargo, cuando se desea explotar al máximo el potencial de una nueva herramienta de análisis y mostrar si el ensayo se aproxima a lo percibido sensorialmente, se abordó su correlación con la percepción de los consumidores. Esto se llevó a cabo con distintos parámetros instrumentales, incluyendo el ensayo TPA de distintos alimentos sólidos [23,24], incluidos entre ellos el pan [25,26] o las características de las masas panarias con lo percibido sensorialmente [27]. Lampignano, et al. [28] intentaron establecer posibles correlaciones entre la estructura de panes con gluten obtenidos con distintas cantidades de levadura y la percepción sensorial descrita por los consumidores. En dicho estudio no encontraron correlaciones entre la microestructura de los panes y la calidad general del pan percibida durante en análisis sensorial, siendo los atributos de olor y sabor los que se vieron más afectados y no los relacionados con la textura o estructura de la miga. El hecho de modificar la cantidad de levadura pudo producir que los cambios que se percibieron en mayor medida por los panelistas no estuvieran relacionados con la estructura, si no con sus características organolépticas modificadas por las distintas cantidades de levadura. Por ello en el **Capítulo 4** se produjeron cuatro panes sin gluten con características estructurales distintas, modificando únicamente las etapas del proceso (hidratación y fermentación). Las modificaciones propuestas se realizaron con el fin de no modificar el sabor y olor de los productos, tratando así, que el cambio de estructura fuera el componente principal durante el estudio de la masticación, deglución y características del bolo. La modificación del proceso originó cambios cuantificables en el perfil de textura TPA, la humedad de la miga, la densidad y la distribución alveolar. Las correlaciones existentes entre las características tecnológicas y las características del bolo, la masticación y la deglución confirmaron el impacto de la estructura sobre el proceso de masticación y deglución. Los tres parámetros obtenidos a partir del ADI de la miga (porosidad, área media de alveolo y circularidad) obtuvieron

correlaciones estadísticamente significativas con algunas de las características estudiadas, destacando la porosidad y el área media de los alveolos, que estuvieron implicadas tanto en las características del bolo como en la masticación y la deglución. Es decir, a mayor densidad alveolar, los alveolos fueron de menor tamaño, lo que se tradujo en un mayor tamaño de las partículas del bolo tras las tres primeras masticaciones, por lo que se requirió más tiempo para masticar y deglutir. Esto pudo ser debido a que la mayor densidad alveolar origina bolos más adhesivos (r=0,9606*), que dificultan la masticación y deglución de este [29]. Otra posible explicación estaría relacionada con el aumento del grosor de las paredes alveolares (no incluido en este estudio), relacionada con panes con menor número de alveolos, es decir estructuras más cerradas [30].

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CONCLUSIONES

Las conclusiones de la presente tesis doctoral se detallan a continuación:

El análisis de imagen se confirma como método no destructivo para discriminar la estructura de los panes, siendo necesario la definición previa de los ajustes en la captura de la imagen, el umbral que se aplica para generar la imagen binaria y el algoritmo utilizado para su cálculo. La funcionalidad del análisis de imagen se ha validado con pan de molde con y sin gluten.

El seguimiento de la gelatinización y gelificación de diversos almidones mediante el análisis de las micrografías de microscopía electrónica de barrido permitieron entender los cambios microestructurales que se producen durante estos fenómenos y la discriminación entre almidones mediante un ensayo de 90 segundos.

La morfogeometría y microestructura de la miga resultante de incorporar la microalga *C. vulgaris* (3%) pudo caracterizarse mediante el análisis digital, a pesar del impacto de la misma sobre el color de la miga. La inclusión de la microalga generó una disminución del área 2D de la rebanada, relacionada con el volumen del pan, así como mayor dureza de miga asociada al grosor de las paredes de la miga.

La optimización de los ajustes previos al análisis de imagen permitió la caracterización de las estructuras de masa y miga resultantes en ausencia de gluten, a pesar del elevado contenido de agua y la densidad de las mismas. Esto se confirmó el papel estructurante como mimético del gluten de la β-conglicinina en masas de harina de arroz. Los panes obtenidos con 10% β-conglicinina mostraron un incremento del área 2D y mejora de la estructura de miga respecto a sus homólogos obtenidos con 100% harina de arroz.

El análisis de imagen también resultó útil para detectar las modificaciones promovidas por la adición de aditivos en los procesos de panificación. Dicha aplicación se validó comparando el impacto de distintos emulgentes sobre la incorporación de aire en las masas y la expansión de las burbujas de aire provocada por los procesos de calentamiento. Aunque todos los emulgentes ejercieron un papel estabilizante de las masas, se pudo discriminar la funcionalidad de cada uno de ellos atendiendo al número y

tamaño de las burbujas tras la gelatinización del almidón (70°C), así como al número y tamaño de los alveolos en las migas de pan resultantes.

Los resultados obtenidos mediante el análisis de la imagen de las migas de pan sin gluten y su disgregación física *in vitro* permiten explicar el comportamiento de los panes durante el proceso de masticación y deglución. Por tanto, el análisis de imagen de las migas de pan podría utilizarse como técnica predictiva del comportamiento de los productos de panadería en las evaluaciones sensoriales focalizadas en textura y comportamiento oral de estos productos.

PUBLICACIONES

La autora, Raquel Garzón Lloría, ha obtenido los derechos por parte de las editoriales para incluir los artículos en los que se ha publicado la información de esta tesis doctoral, utilizando el formato original de la revista.

Microstructure and its relationship with quality of confectionary and bakery products

11

Cristina M. Rosell and Raquel Garzon
Institute of Agrochemistry and Food Technology (IATA-CSIC), Paterna, Spain

11.1 Introduction

Bakery and confectionary products play a main role in human nutrition worldwide. Bakery products constitute staple foods in many parts of the world, in some others they are used as nutrient carriers to balance nutritional deficiencies, and they are even pleasure commodities. Conversely, confectionary products are mainly consumed for pleasure or convenience due to the fact that they are mostly high-caloric foods (Rosell and Garzon, 2015). Whichever motivation explains their consumption, it is well-known that bakery and confectionary foods cover an extensive number of specialties that vary around the world; some of them have been extensively known but some others are autochthonous or a country's specialties. Those products can differ in the raw materials and preparation process, and both of factors determine their sensory characteristics. One of the main attributes within the sensory characteristics of this kind of products is their texture. In fact, one of the primary actions when buying those products is to check their softness by finger-pressing the surface. Texture is an important component for the consumers' perception of baked goods quality. Texture is considered a multidimensional attribute that comprises a number of different textural properties (Bourne, 2002). In a more specific definition, texture is primarily the response of tactile senses to physical stimuli that result from contact between some part of the body and the food. In case of bakery and confectionary products, the textural attributes that better define its quality and even freshness are crispness and crumb firmness. Texture is in turn largely connected to the mechanical properties, which in principle are directly derived from the internal food microstructure. Despite the importance of microstructure of bakery and confectionary products in defining the quality, there is scarce information about the relationship between them. The quality of bakery and confectionary products is mainly assessed through volume and texture, and microstructure is only determined to complement those parameters or to support texture information.

The present chapter compiles the information reported on microstructure of bakery and confectionary products, the main techniques used for assessing their microstructure, as well as a brief mention of the ingredients and process, their impact on microstructure, and some relationships identified between quality parameters and microstructure.

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11.2 Microstructure evaluation techniques for bakery and confectionary products

The quality of bakery and confectionary products is determined by the quality and quantity of the raw materials and the processing characteristics. There are a number of parameters, which are commonly related to food quality, particularly bakery and confectionary products. Volume, texture, color, and moisture content are among the most used quality parameters. Food microstructure, which is defined as the spatial arrangement of its structural components and their interactions (Herremans et al., 2013), also have significant impact on the physical, sensory, and textural properties of bakery and confectionary products. Microstructure of bakery products determines the appearance, texture, shelf-life, taste perception, and rheology of the products (Jekle and Becker, 2011; Aguilera, 2005).

Different techniques have been applied to determine the microstructure of these types of products. The most used techniques are listed in Table 11.1. Confocal laser scanning microscopy (CLSM) has been very useful for assessing the distribution of macrocompounds after staining proteins, starch, lipids, and carbohydrates (Alvarez-Jubete et al., 2010, Rodriguez-Garcia et al., 2012, Hager et al., 2011). This technique allows obtaining optical sections through a three-dimensional specimen (Arendt et al., 2009). Scanning electron microscopy (SEM) has been used for examining the surface and cross-section structure of cereal grains and also particle size distribution in flours (Hera et al., 2013, Protonotariou et al., 2015, Ahmed et al., 2015), flour-water systems, the structure of doughs and gluten network (Bache and Donald, 1998, Correa et al., 2010), the influence of additives on crumb microstructure (Bahal et al., 2013, Bárcenas and Rosell, 2005), and the effect of process in bakery products (Baier-Schenk et al., 2005, Baixauli et al., 2007). Similarly, environmental SEM has been used to directly analyze bakery products, without the necessity of drying the samples (Létang et al., 1999, Duta and Culetu, 2015).

Overall, these techniques provide very sensitive information of tiny sections of the food matrixes. In consequence, microscopy techniques have been applied mainly to identify interactions among ingredients or their distribution in the matrix. Generally, those techniques that do not require sample preparation are preferred over those that require some pretreatment. Consequently, SEM of freeze-dried samples are often reported, or cryo-SEM whenever possible. Only when the target is the functionality of specific biopolymers, CLSM is the preferred choice.

11.3 Ingredients and processing of bakery and confectionary products

To understand the basis of the quality of bakery and confectionary products it becomes primordial to know the main ingredients and the changes that they undergo during processing. A very brief mention to ingredients and additives that could be used in the production of bakery and confectionary products is included in the next section.

Table 11.1 The most used microscopy techniques in bakery and confectionary products

Technique	Sample	Sample pretreatment	Stain	Coating	Magnification	Condition	Author reference
CLSM	Gluten-free bread	–	Nile blue (0.1% w/w)	–	–	Excitation: 488 nm argon laser (fat); 633 nm helium neon laser (protein and starch). Objective 63×	Alvarez-Jubete et al. (2010)
CLSM	Cake	–	Nile red and Rhodamine B (proteins and carbohydrates)	–	–	Excitation: Ar laser line: 488 nm	Rodriguez-Garcia et al. (2012)
CLSM	Gluten-free bread	–	Fluorescence isothiocyanate (proteins and starch); aniline blue (β-glucan)	–	–	Excitation line: 405 and 488 nm. Objectives 10× and 20×	Hager et al. (2011)
CLSM	Crumb bread	Immersed in a 2% agar solution	Fuchsin acid (proteins)	–	–	Excitation 568 nm; emission 620 nm. Objective 10× and 40× water immersion	Renzetti et al. (2008)
Cryo-SEM	Dough, prefermented frozen dough, batter	Immersed in liquid nitrogen and fixed with OTC compound	–	Gold	–	10 kV	Baier-Schenk et al. (2005); Baixauli et al. (2007); Bonet et al. (2007)
Cryo-SEM	Wheat flour, dough, bread, cookies, cakes	Immersed in liquid nitrogen and cryofixed in slush nitrogen	–	Gold	–	15 kV	Sarabhai and Prabhasankar (2015); Rodriguez-Garcia et al. (2012); Bárcenas and Rosell (2005); Rojas et al. (2000)
ESEM	Dough	No pretreatment	–		200× and 2000×	–	Létang et al. (1999)
ESEM	Oat cookies	No pretreatment	–			25 kV	Duta and Culetu (2015)
SEM	Rice flour	–	–	Gold	50×, 700×, and 1000×	–	Ahmed et al. (2015)

(*Continued*)

Table 11.1 (Continued)

Technique	Sample	Sample pretreatment	Stain	Coating	Magnification	Condition	Author reference
SEM	Wheat flour, crust layers	Freeze-dried	–	Gold	–	10 kV	Altamirano-Fortoul et al. (2015); Angelidis et al. (2015)
SEM	Pseudocereals and gluten-free bread	Fixed with aluminum specimen stub	–	–	–	1 kV	Alvarez-Jubete et al. (2010)
SEM	Mixed dough, prefermented dough, bread	Freeze-dried	–	Gold	–	5–10 kV	Bahal et al. (2013); Calderon-Dominguez et al. (2003); Brennan et al. (1996)
SEM	Cookies	Freeze-dried	–	Gold	1000×	15 kV	Dachana et al. (2010); Filipcev et al. (2011)
SEM	Muffin crumb	–	–	–	–	15 kV	Jyotsna et al. (2011)
SEM	Gluten-free bread	Freeze-dried	–	Platinum	50×, 150×, 500×, 2000×, and 4500×	15 kV	Kawamura-Konishi et al. (2013)
SEM	Gluten-free cookies	–	–	Gold	–	30 kV	Park et al. (2015)
SEM	Dough with celluloses	Fixed in 10% glutaraldehyde, submerged in acetone	–	Gold	–	–	Correa et al. (2010)
Fluorescence microscope	–	Embedded in Tissue-Tek O.C.T. compound and frozen, fixed in cryostat	Acid magenta (10 min)	–	–	Using WU (blue) range 330–385 and 420 nm	Maeda et al. (2015)
Light microscopy	Bread	Soaked 15 min under vacuum in issue-Tek O.C.T	Light green (30 min)	–	–	–	Hug-Iten et al. (1999)

11.3.1 Bakery and confectionary ingredients

Flour is the major ingredient for making confectionary and bakery products and the main ingredient responsible for texture and crumb structure. Water is essential for making bakery products, because it is responsible of many interactions between ingredients after hydration. Some baked products require yeast as an essential ingredient. In leavened products yeast is in charge of the production of carbon dioxide derived from the alcoholic fermentation of sugars, which provides bread loaf volume and crumb structure, and contributes to the typical bread flavor (Cho and Peterson, 2010). Other leavening agents that can be used to produce carbon dioxide are chemical leavenings. They are more useful for making cookies or cakes and can be divided into three groups depending on the type of gas produced: sodium bicarbonate, potassium bicarbonate, and ammonium bicarbonate (De Leyn, 2014b). Among other ingredients one may cite sweeteners such as sugar, which affects the taste, color, texture, and appearance, and also contributes to fermentation (Pareyt and Delcour, 2008); and lipids (butter, vegetable shortenings, etc.), which impart sensory attributes such as mouthfeel, textural properties, and structure (Marangoni et al., 2014).

Apart from the aforementioned ingredients, there are numerous functional additives that are used to facilitate processing, to compensate for variation in raw materials functionality, to guarantee constant quality, and to preserve freshness and product properties. In this category one may cite enzymes or processing aids such as amylase, protease, oxidase, and transglutaminase, among others (Rosell and Dura, 2015). Transglutaminase, alpha-amylase, xylanase, and protease affect significantly the viscoelastic properties of dough and can change bread quality parameters such as volume or crumb structure (Caballero et al., 2007). The industrialization of the breadmaking process and the consumer demand for high quality and longer shelf life have increased the use of those processing aids and some other additives like emulsifiers that affect starch and proteins, producing softness, longer shelf-life, and increase in the volume, stability, and structure (De Leyn, 2014a). Finally, other modifiers that are useful in the production of these products are hydrocolloids like xanthan gum, hydroxypropyl methylcellulose (HPMC), guar gum, and others. They modify the rheology and the texture of the products, and their functionality is mainly related to their ability to bind water, and subsequently changes in dough rheology, freshness, and shelf-life have been described (Poonnakasem et al., 2015, Bárcenas and Rosell, 2005, Correa et al., 2010). Similarly, in special types of products like gluten-free, the functionality of the hydrocolloids is essential because they are acting as gluten replacers, and thus give the structure of the baked products (Lazaridou et al., 2007, Demirkesen et al., 2010, Rosell et al., 2001).

11.3.2 Flour microstructure

Since flour is the main ingredient in bakery and confectionary products, a special emphasis is given to it and all the changes that mechanical and temperature constrains induce in its structure. It must be stressed that the term flour is very

extensive, given the variety of sources and thus the differences in chemical composition. Nevertheless, when referring in general to flour attention drives to wheat flour, because it is the main cereal used for making baked goods. Because of that, this section will refer to wheat flour, although changes induced in proteins and starch when subjected to mechanical or temperature stresses can be extrapolated to any other type of cereal flour.

The knowledge of the composition of wheat grain is important in order to understand wheat flour microstructure. Wheat grain is divided into three main parts: endosperm, peripheral layers, and germ. Endosperm is the largest morphological component (82% of the grain). Peripheral layers or bran (15%) that surround the endosperm and germ (3% of the grain) are removed during milling to obtain refined wheat flour (Evers and Millar, 2002). Those parts are very well integrated in the kernel structure. In fact, SEM images of the wheat flour (Fig. 11.1A) have revealed that endosperm tissue is composed of large aggregates ($\geq$200 μm length) and protein matrix embedding endosperm starch granules (Rojas et al., 2000, Angelidis et al., 2015) but it changes with intense milling. Nevertheless, wheat kernels are not consumed directly; they undergo milling in which the initially ordered structure becomes disorganized and broken into smaller particles. The particle size affects the physicochemical properties of the flour.

Flour fractionation according to particle size distribution has been employed to obtain special flours for different end-use applications or for nutritional improvement. For instance, fine particle size flours have better protein quality, as determined by sodium dodecyl sulfate sedimentation value, higher damaged starch and falling number, and also lower ash content and improved baking performance for some type of products (Sakhare et al., 2014). Sakhare et al. (2015) used microstructure analysis to study the distribution of major constituents in the flour mill streams during wheat milling. Their results revealed that as milling intensity increased, more deformed and damaged starch granules were obtained, probably due to repetitive grinding. Besides, special techniques such as jet milling have been studied using different air pressures and it was observed that large aggregates were gradually reduced in size, depending on the intensity of the process, and starch granules were separated from the protein matrix (Angelidis et al., 2015). The effect was also tested on whole-meal flour, in which aleurone layer was broken down to small particles (about 20−180 μm) (Protonotariou et al., 2015). Jet milling on the whole

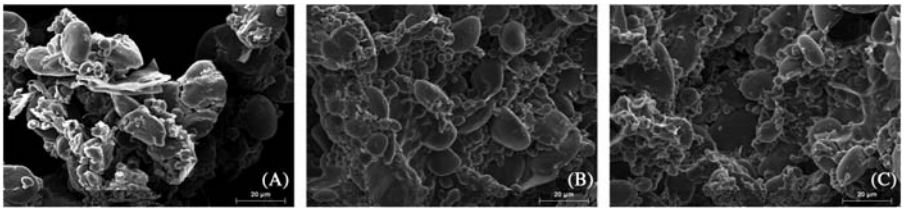

Figure 11.1 Scanning electron micrographs of flour (A); well-developed dough (B); and overmixed dough from wheat (C).

wheat flour also had an impact on the flour composition, because an increase in the total fiber content and digestible starch was observed as the severity of the jet milling treatment increased and also more starch granules were separated from the protein matrix (Protonotariou et al., 2015).

11.3.3 Microstructure changes through processing

The process to obtain bakery products is a dynamic system, where physical and chemical changes occur on the flour components; such changes determine the final microstructure of the baked products (Rosell, 2011). To understand the microstructure of baked products, a very brief description of the processing is included. There are three important stages that involve the major part of the changes during processing, which include mixing, proofing, and baking.

The first important stage is mixing. Even as the first stage its incidence on the final structure of the product is crucial, and undermixing or overmixing can make significant differences. During this stage the hydration of the ingredients, their uniform distribution in the dough, dough development (gluten formation), and air incorporation into the dough (Fig. 11.1B) occur. The creation of gas bubbles and their retention depend entirely on the mixing characteristics; this is necessary to define the cellular structure in baked products. Air incorporated into the dough, and more specifically oxygen, is responsible for the dough oxidation, which is needed to form disulfide bonds, linking protein chains and in consequence, increasing the strength of the gluten dough, which is crucial for the development of a gluten network.

To obtain a correct dough development it is necessary to adjust and optimize mixing time and hydration (Rosell and Collar, 2009). The structure of white bread dough with short mixing (undermixed) consists of large starch granules (over 10 μm) and smaller ones (<10 μm), a coarse veil (proteins) covering the structure, and many intact flour particles (Hoseney, 1994). When bread dough is well developed a compact system (Fig. 11.1B) with small and large starch particles embedded and distributed on the surface (protein cover disappears) (Maeda et al., 2015) and an even network of starch and proteins is obtained (Rojas et al., 2000). If mixing continues (overmixed), an open system with holes is obtained, in which the proteins and the starch particles are not embedded (Calderon-Dominguez et al., 2003, Létang et al., 1999) (Fig. 11.1C). The distribution and morphology of water−flour dough depends on the water content; the veil protein covering starch granules are much less visible in dough with less hydration (Létang et al., 1999). The same trend has been observed even in more complex formulations. Calderon-Dominguez et al. (2003) observed that dough of sweet yeast bread containing flour, sugar, shortening, milk, yeast, and water exhibited the same structure observed with white bread dough during mixing.

There are products with high quantity of shortening like puff or short pastry, in which the distribution of gluten, starch, and butter is very important. Kokawa et al. (2015) visualized by fluorescence fingerprint the microstructure difference in puff and short pastry due to their different processing. Puff pastry exhibited a structure

like a gluten network spread in the direction of dough extension, whereas in short pastry small and large clumps without a continuous gluten network were observed, owing to fat to avoid its formation.

The use of different additives to improve bakery product quality is a common practice. For instance, some enzymes are added during mixing to improve dough development. Bahal et al. (2013) observed that addition of lypoxygenase resulted in a more uniform dough surface, which persisted after fermentation, and also improved gas retention. The use of glucose oxidase has been studied by Bonet et al. (2007) to improve dough obtained from damaged wheat flour as an alternative to dough conditioners such ascorbic acid, azodicarbonamide, or potassium bromate. These authors observed that untreated damaged flour led to a discontinuous network with large and small fragments of gluten, but the use of glucose oxidase induced the formation of a protein network with a continuous structure similar to that of the dough from sound wheat. Disrupted doughs resulted in softer doughs with low stability, and to improve their performance the use of modified celluloses has been proposed (Correa et al., 2010). Depending on the structure of the modified celluloses the dough characteristics can be modulated. The addition of microcrystaline cellulose resulted in a more disaggregated structure of bread dough, whereas carboxymethylcellulose made difficult the gluten film formation and some types of HPMC led to filamentous microstructure, resembling a gluten network (Correa et al., 2010). The addition of hydrocolloids (carrageenan, xanthan gum, and HPMC) modifies the interaction between starch granules and protein matrix; structures are more closely linked in the presence of hydrocolloids (Fig. 11.2).

In the case of the batters, after mixing the dosing type could change the structure of muffins (Baixauli et al., 2007). Batter resulting from manually dosing displays

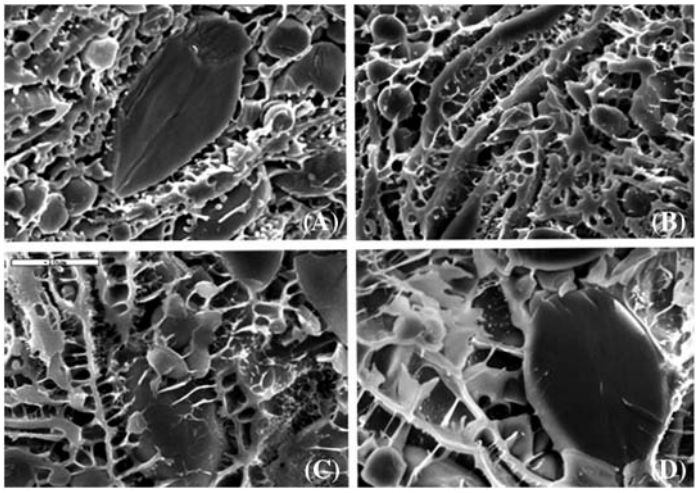

Figure 11.2 Scanning electron microscopy micrographs of different doughs (3500 ×). Control dough (A); with addition of κ-carrageenan (B); Xanthan gum (C); and HPMC (D).

two types of starch granules (rounds and lentil shape) immersed in the reticular structure of proteins and soluble solutes that form the matrix. Conversely, when the dosing is automatic greater compactness results along with a decrease in the fat globules size (Baixauli et al., 2007).

The second important stage is the fermentation or proofing. Fermentation aims to increase dough volume but also to generate small metabolites that will contribute to taste and flavor of final products. During fermentation sugars are transformed into carbon dioxide and alcohol due to the action of yeast, and also short fatty acids are released due to the contribution of lactic acid bacteria. In the absence of sugars, enzyme action on the starch polymer releases sugars and dextrins (fermentable - carbohydrates) that would be also substrates for yeast. The carbon dioxide released during the fermentation moves toward the gas nuclei, formed during mixing, leading to dough expansion and consequently, volume increase of the dough (Fig. 11.3). However, there are also unyeasted bakery and confectionary products, in which the gas required for volume increase is chemically produced from leavening agents like sodium bicarbonate, potassium bicarbonate, and ammonium bicarbonate (De Leyn, 2014b), among others.

Freezing has become a common practice to extend the shelf-life of bakery and confectionary products. This practice allows obtaining fresh products at any time of the day. In some cases, the bakery pieces are stored frozen after proofing. Micrographs of dough after proofing showed a porous structure and without the presence of ice crystals, but frozen storage might influence the dough properties and final quality (Baier-Schenk et al., 2005). In fact, after freezing dough cell walls exhibit small ice crystals ($\leq$100 μm); the number and size of crystals increase upon storage and they become more spherical (Baier-Schenk et al., 2005, Huen et al., 2014, Zounis et al., 2002). To avoid the formation of ice crystals during frozen storage, Huang et al. (2008) proposed the addition of transglutaminase and observed that the gluten network was less fractured and disrupted than samples without transglutaminase. Other alternatives like the addition of diacetyl tartaric acid ester of monoglycerides (DATEM) and guar gum were also studied, but larger amount

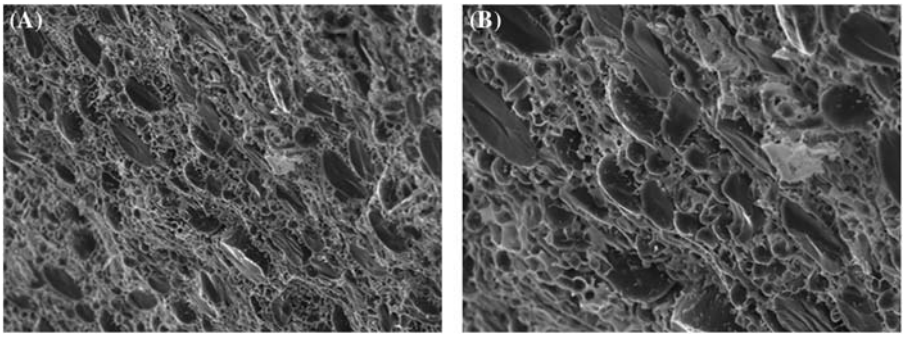

Figure 11.3 Cryo-SEM micrographs of fermented dough. Continuous and well-distributed matrix with firmly embedded starch granules (A: 750× and B: 1500×).

of void among starch granules and gluten network were observed with DATEM, and more dense structure was obtained with guar gum (Ribotta et al., 2004).

Finally, the last stage is baking, where dough is subject to temperature increase, resulting in gas expansion and simultaneous stretch of the dough. Dough extensibility depends on protein coagulation and starch gelatinization, and the former is influenced by water availability. When temperature is high enough, water evaporation takes places with the subsequent drying of the dough, leading to a porous structure (crumb) (Lucas, 2014). Meanwhile, temperature on the dough surface reaches much higher values, inducing caramelization and Maillard reactions that would lead to crust formation and release of flavor compounds (Lucas, 2014).

11.4 Microstructure and quality of bakery and confectionary products

Product composition determines the microstructure, and one of the main differences in bakery and confectionary products is the presence or the absence of gluten. Gluten creates a viscoelastic matrix that allows dough expansion, leading to aerated and open structures. Conversely, the absence of gluten tends to produce uneven and collapsed structures (Matos and Rosell, 2012). Because of that this section will present separately the microstructures of gluten-containing products and of those that lack gluten.

11.4.1 Gluten-containing products

A soft internal structure, namely crumb, surrounded by a dried outer part, which is called crust, is the very characteristic of breads. When going into detail through micrographs, a complex structure of an open sponge with numerous cavities inside large gas cells is observed (Fig. 11.4A). In that matrix starch granules are predominant, showing a veil on the surface comprised by gelatinized starch and degraded

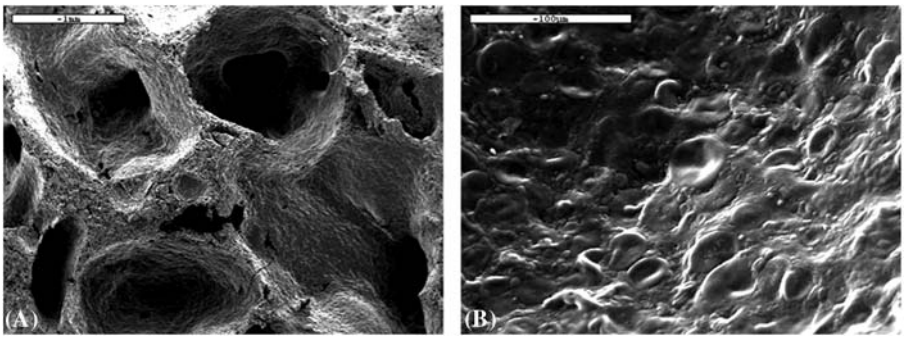

Figure 11.4 Scanning electron micrographs of wheat bread crumb at 35 × (A) and cell gas surface at 500 × (B).

proteins (Bahal et al., 2013, Brennan et al., 1996, Bárcenas and Rosell, 2005) (Fig. 11.4B). Therefore, starch granules and proteins are the main players in defining the structure of bakery and confectionary products. Their structure commonly consists of a number of gelatinized starch granules that together with denatured proteins form a smooth gel covering intact starch granules.

Cookies and cakes have similar microstructure to gelatinized starch granules, protein aggregates, sugar, and lipids embedding in the protein matrix (Chevallier et al., 2000, Rajiv et al., 2012, Rodriguez-Garcia et al., 2012). Starch granules are more extensively gelatinized in the center of the cookie than in the bottom or the top of it (Dachana et al., 2010, Nandeesh et al., 2011). Conversely, flat bread microstructure displays significant differences from those of other bakery products. No such gas cells could be observed in the microstructure of baked parotta (a type of flat bread), maybe because there is no fermentation and no gas formation in the process (Prabhasankar et al., 2003).

The addition of modifiers changes bakery product microstructure. When guar galactomannan is added to dough, for example, the hydrocolloid is dispersed into the matrix and mixed with the starch granules and protein matrix (Brennan et al., 1996). Addition of lipoxygenase improves microstructure, resulting in a better network of gluten with embedded starch granules; moreover, dough shows a more continuous and smooth surface that without enzymes (Bahal et al., 2013). Use of HPMC in bread formulation also leads to a more continuous surface; it seems that HPMC enfolds all the other bread constituents (Bárcenas and Rosell, 2005).

Apart from additives, there is a trend to improve the nutritional quality of bakery products by adding other types of cereals or ingredients, which significantly affect the microstructure. The use of wholegrain buckwheat flour and rye in biscuits was studied by Filipcev et al. (2011) who observed smooth and round spherical starch granules with different sizes. Other flours like the finger millet flour decreased the number of air cells, which is indicative of poor air incorporation during mixing (Jyotsna et al., 2011). In addition, the incorporation of wheat bran to improve fiber content of products induces the disruption of the protein matrix (Nandeesh et al., 2011). Flours from pulses have also been incorporated with the objective of nutritional enrichment of cookies. Rajiv et al. (2012) studied the use of green gram flour for this purpose and observed that the protein matrix structure was disrupted when this ingredient was added to the formulations. In order to decrease the fat content in cakes, Rodriguez-Garcia et al. (2012) replaced part of the oil with inulin. These authors observed a continuous matrix with embedded starch granules coated with oil; when fat replacements increased, starch granules appeared as separated structure. It seems that hydrophobic or hydrophilic ingredients can be incorporated in the dough or batter matrix up to a specific level but beyond that biphasic systems are obtained, which completely change their role and functionality in the dough or batter. This has been observed with inulin (Rodriguez-Garcia et al., 2012) and other hydrocolloids like xanthan gum, guar gum, and HPMC (Rosell et al., 2011).

11.4.2 Nongluten-containing products

Products without gluten have very different microstructure, since the absence of this protein prevents the development of a gluten-like network, and dough consistency is more similar to that of a batter. There are several alternative flours and starches for making gluten-free bakery products, but to form a network-like structure and retain gases it is necessary to use gluten mimetics like hydrocolloids. One of the most widely used gluten-free flours is rice flour, owing to its hypoallergenic proteins and bland taste. Rice flour particles are irregular, polyhedral in shape, and indeed, the average particle size of rice flours is 40−125 μm (Ahmed et al., 2015, Dixit and Bhattacharya, 2015). The micrographs of rice flour−water dough shows the formation of a cohesive network but not enough to obtain acceptable bakery products. Dixit and Bhattacharya (2015) added whey protein concentrate and Xanthan gum to obtain a more cohesive structure. The same approach was followed by Shanthilal and Bhattacharya (2015), using Arabic gum with rice flour. It was found that the flour particles were amply coated with gluey material, which improved the particle binding. Rice-based gluten-free breads have a very irregular cellular structure, and different approaches have been reported for smoothing the structure. Kawamura-Konishi et al. (2013) used proteases to improve the quality of rice-based flour. These enzymes induced protein hydrolysis, decreasing the hydrophobic nature of the rice proteins, which seems to favor the connection between protein matrix and starch granules. As a result, bread with a more regular structure and higher volume was obtained, with a subsequent decrease of the crumb hardness. Marco and Rosell (2008) designed protein-enriched gluten-free bread, containing composite rice flour with soybean and in the presence of transglutaminase to promote protein cross-linking. Similarly, transglutaminase has been added to other gluten-free flours like buckwheat, brown rice, and corn flour (Renzetti et al., 2008).

In the case of gluten-free cakes, gluten mimetic is usually added as structuring agent. The addition of Xanthan gum increases the size of the cell area in the cake crumb compared either with a sample without hydrocolloid or gums like guar, and κ-carrageenan (Turabi et al., 2010). Not only the recipes affect the microstructure of cakes—the process conditions can modulate the characteristics of the final product. For instance, cakes baked in a conventional oven exhibit more deformed starch granules compared with cakes baked in an infrared-microwave combination oven (Turabi et al., 2010).

Nutritional improvement also has been a concern in the case of gluten-free bakery products. To improve fiber content in gluten-free bread and cookies, Hager et al. (2011) and Duta and Culetu (2015), respectively, added oat β-glucan. Micrographs of the final products showed that oat starch granules were rounded and with irregular shapes and sizes up to 10 μm. In cookies, the structure consisted of starch granules grouped in clusters with the protein and fiber matrix (Duta and Culetu, 2015). In the case of bread, CLSM revealed that proteins appeared like clouds and a big blue signal that confirmed the presence of β-glucan in the breads. Incorporation of finger millet flour, which is a good source of minerals, has been used to increase the nutritive value of gluten-free muffins (Jyotsna et al., 2011).

The presence of finger millet flour in muffins led to a few large air bubbles, indicating that the air incorporation was low and some of the gelatinized finger millet starch granules appeared in the form of a thin sheet (broken matrix) (Jyotsna et al., 2011).

Pseudocereals are potential ingredients to obtain gluten-free bakery products and in the last decades have attracted much attention. Particle-size distribution of the pseudocereal flours differs considerably. Buckwheat flour exhibits the smallest particle size followed by amaranth and quinoa; and all of them have smaller particle size than rice flour (Alvarez-Jubete et al., 2010). In amaranth and quinoa, starch granule size is significantly smaller ($< 2\ \mu m$) than other studied flours, and their shape is polygonal (Alvarez-Jubete et al., 2010). Microstructure analysis of breads made with pseudocereal flours shows only partial gelatinization, with a great number of starch granules that retain their integrity, but the overall structure is homogeneous with a good distribution of fat, proteins, and starch, resembling the structure of gluten-containing products (Alvarez-Jubete et al., 2010). Another alternative to make gluten-free products is the use of chestnut flour. Cookie dough from chestnut flour is characterized by the presence of small and large starch granules, which are enmeshed in a partially formed protein matrix (Sarabhai and Prabhasankar, 2015). With the same goal to improve nutritional values, Park et al. (2015) proposed the use of okara (by-product of tofu manufacturing) to develop okara cookies. Those were made by adding starch, soy flour, and HPMC, and micrographs of the okara cookie doughs showed a disrupted matrix structure.

11.5 Crust microstructure and changes due to processing and specific treatments

Crust refers to the part of the bread near its surface, where the density is significantly higher than elsewhere (Jefferson et al., 2006). When dough is placed into the oven, water of the surface evaporates very fast, resulting in much lower water content than inside the product. The temperature in the crust can exceed 100°C, and then Maillard reactions, responsible for the development of color and some flavors, take place (Vanin et al., 2009). There are many bakery products with crispy crust that are sought by consumers for their appealing texture at the first bite. That sensory perception due to the low water content is felt when consuming cookies or crispy bread. A study carried out on the bread crust indicates that both cell size and shape as observed by SEM are significantly related to crust crispiness (Altamirano-Fortoul et al., 2013). The structure of the bread crust indicates a continuous gluten network with embedded nongelatinized large and small starch granules (Fig. 11.5A), maybe because the rapid water evaporation reduces the required water for starch gelatinization (Primo-Martín et al., 2006). Furthermore, the size of gas cells in the crust is lower than in the crumb, because of the rapid loss of the extensibility of dough (Vanin et al., 2009).

Pursuing the goal of extending the crispiness characteristics of the bread crust, some authors proposed the use of enzymes to change the crust microstructure.

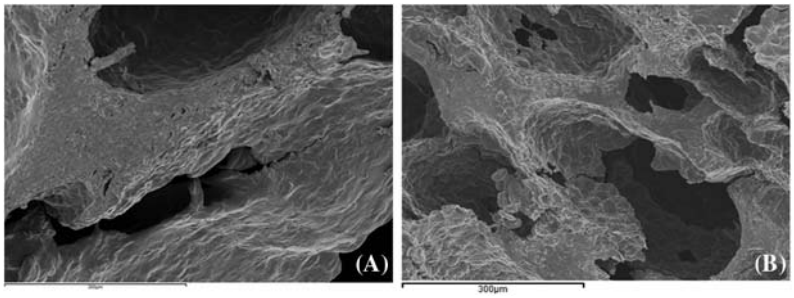

Figure 11.5 Scanning electron micrographs of bread crust (A) and that resulting after spraying amyloglucosidase onto the dough surface (B).

Primo-Martín et al. (2006) sprayed protease on the dough surface, and the resulting bread crust showed significantly lower water content, indicating the presence of fractures that favored water diffusion. Other enzymes reported for increasing the crispness is amyloglucosidase (Altamirano-Fortoul et al., 2014). When this enzyme was sprayed onto the surface of partially baked bread, the resulting bread had lower water activity and moisture content in its crust, and SEM micrographs revealed a more disordered structure with small irregular voids and great cracks (Fig. 11.5B).

To understand the action of different additives on the crust texture, and thus on its microstructure, a crust model was proposed by Altamirano-Fortoul et al. (2015). These authors tested the effects of glycerol, gluten, protease, DATEM, citric acid, linoleic acid, beeswax, and HPMC on the mechanical properties and water vapor permeability of the model crust. The crust layer containing protease exhibited a compact structure, more disrupted gluten network, and high deformation of starch granules; addition of HPMC revealed irregular starch granules within a disrupted and discontinuous protein network. Glycerol functionality was greatly dependent on its concentration, with 1% glycerol the crust structure resembled a continuous and smooth gel, but with 10% glycerol microstructure was rather compact; crust layer with DATEM showed starch granules covered with alternate continuous veil-like film and some cracks. Finally addition of lipids gave smooth and nonporous structure without phase separation (Altamirano-Fortoul et al., 2015).

11.6 Digital image analysis of bakery and confectionary products

In the last decades, great attention has been paid to gas cell distribution in the cross-section of baked products, owing to the relationship between that and volume or texture (Zghal et al., 1999). At a macroscopic level crumb is composed of two phases: a fluid (air) and a solid (cell wall material) (Scanlon and Zghal, 2001). Crumb structure exhibits a gelatinized starch network associated with a protein

network (Mondal and Datta, 2008) and after baking shows an open cell structure of medium- and small-sized air cells with homogeneous distribution (Hayman et al., 1998; Primo-Martín et al., 2006). Study of gas cell size, shape, and distribution can be made by image digital analysis of cross-sections of the products. For that purpose, it is necessary to capture images of the cross-section and then use image software to binarize the images using appropriate algorithm (Fig. 11.6).

Different algorithms have been proposed for better understanding and quantification of the gas cells in bakery products (Gonzales-Barron and Butler, 2006), which could be very useful for assessing the effects of process, ingredients, and additives. Digital image analysis has been used for determining differences in the gas cell distribution of different commercial types of breads, allowing discrimination among them regarding the number of cells, their surface, diameter, and circularity

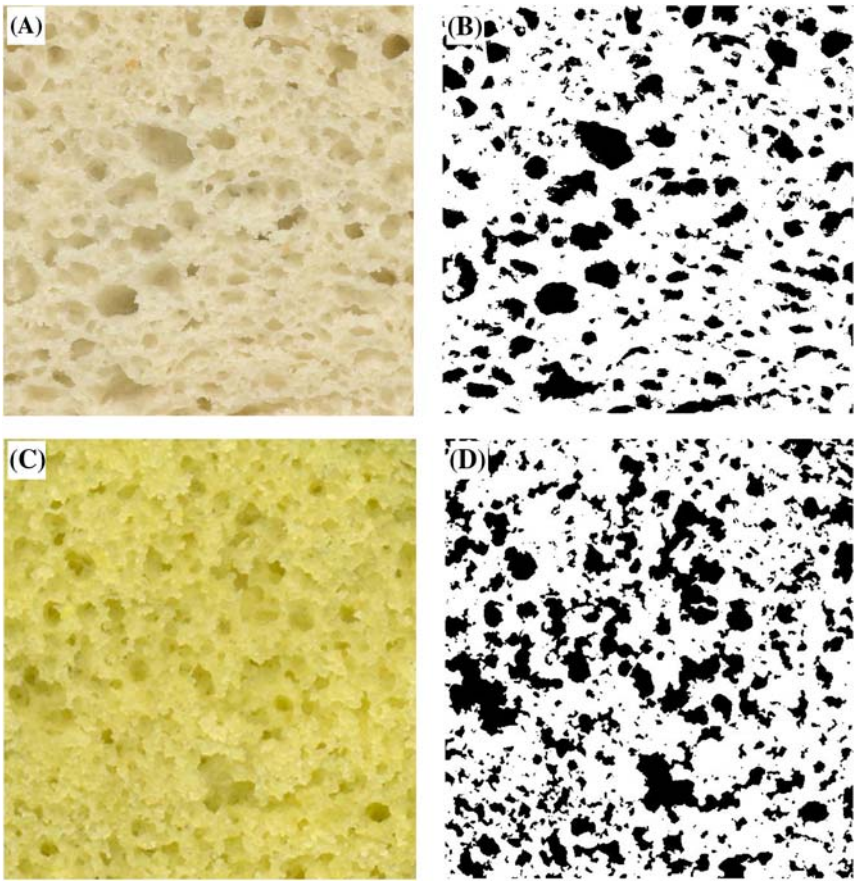

Figure 11.6 Cellular structure of the crumb of the products. Left column: scanned crumbs of gluten-free bread and muffin, respectively (A, C). Right column: binarized images of gluten-free bread and muffin (B, C).

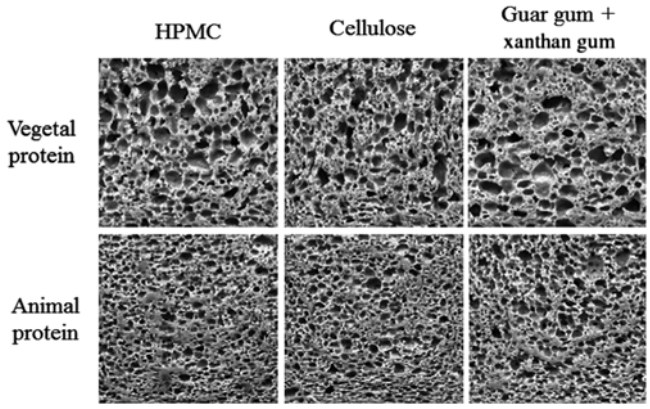

Figure 11.7 Digital image analysis of crumb cross-sections from different gluten-free breads. A comparison of the effects of protein origin (vegetal, animal) and the use of different hydrocolloids (HPMC, cellulose, xanthan gum, and guar gum blend) are shown.

(Altamirano-Fortoul and Rosell, 2011). The use of different flours could result in different crumb structure. The use of buckwheat and quinoa results in the differences in the number of cells, cell volume, and wall thickness; particularly buckwheat flour yields the larger number of cells than quinoa (Alvarez-Jubete et al., 2010). Garzon and Rosell (2013) studied the effect of rice-based gluten-free bread formulation and the role of vegetal protein, animal protein, and different gums like xanthan and guar gum, Ultracel and HPMC on the structure of the bread crumb (Fig. 11.7).

The digital image analysis of the bread crumbs allows confirmation that casein and albumin lead to more homogeneous and smaller air cells than proteins from vegetal sources. Image digital analysis can be useful to study different parameters during the breadmaking process. Nevertheless, no relationships have been reported between quality parameters and values derived from digital image analysis. An attempt for finding that type of correlation is presented in Table 11.2. The digital image analysis of the cross-section of breads shown in Fig. 11.7 was correlated with some quality parameters, namely crumb hardness and color. Only significant correlations were observed between gas cell number per square centimeter and hardness with color parameters, specifically luminosity (L^*) and a^*. Gas cell number per square centimeter was positively correlated with L^* and negatively with a^*, and the opposite trend was observed with the hardness. It has indeed been previously described that crumb structure has a great influence on the texture and luminosity of the crumb of gluten-free breads (Aguilera, 2005, Matos and Rosell, 2012).

Marston et al. (2016) used this tool to determine the effect of heat treatment of sorghum flour on the crumb structure, and observed that heat treatment of flour increased the number of crumb cells, resulting in an irregular crumb structure compared with that of bread from nontreated flour.

Table 11.2 Correlation matrix between bread quality (hardness and color) and parameters from crumb digital image analysis

	L^*	a^*	b^*	Hardness (N)	Gas cells/cm^2	Cell average size (mm^2)	Total gas cell area (cm^2)	Circularity
L^*	–	−0.8909***	0.5335***	−0.3532**	0.7342**	−0.5829*	–	–
a^*	–	–	−0.6400***	0.3172**	−0.581*	–	–	0.6226*
b^*	–	–	–	–	–	–	–	−0.6156*
Hardness (N)	–	–	–	–	–	–	–	–
Gas cells/cm^2	–	–	–	–	–	−0.9477***	–	–
Cell average size (mm^2)	–	–	–	–	–	–	–	–
Total gas cell area (cm^2)	–	–	–	–	–	–	–	–
Circularity	–	–	–	–	–	–	–	–

Correlations indicated by r values. ***$P < .001$, **$P < .01$, *$P < .05$.

11.7 Future trends

Microstructure analysis of bakery and confectionary products has been carried out for confirming purposes, but rarely as a main subject of research. Although powerful microstructure techniques have been developed and applied in different areas, they have been scarcely applied to bakery and confectionary products. Therefore, use and analysis of microstructure information of bakery and confectionary products should be exploited to a greater extent, mainly with the purpose of better understanding of the role of ingredients and additives, and even of optimizing process conditions, and further defining rapid predictors of product quality.

11.8 Further sources of information

ImageJ program for image analysis can be downloaded at http://rsb.info.nih.gov/ij/download.html.

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RESEARCH ARTICLE

Rapid assessment of starch pasting using a rapid force analyzer

Raquel Garzon | **Cristina M. Rosell**

Institute of Agrochemistry and Food Technology (IATA-CSIC), Valencia, Spain

Correspondence
Cristina M. Rosell, Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain.
Email: crosell@iata.csic.es

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Abstract

Background and objectives: The falling number is a rapid method developed for assessing amylase level on flours, based on viscosity changes. This measurement records the time required for a stirring rod to travel through a flour paste. The Chopin Amylab FN, developed for assessing the FN, allows working as a rapid force analyzer (RFA), recording the force changes of a starch/flour slurry under controlled mixing/heating conditions. The objective of this research was to underline the starch changes occurring along 90 s under continuous mixing and heating.

Findings: Four different starches (corn, rice, wheat, and potato) were analyzed using the RFA, evaluating step-by-step the structural and textural modifications. Scanning electron micrographs revealed the progressive gelatinization process, that was specific for each type of starch. Nevertheless, the 90-s procedure was sufficient to ensure complete gelatinization of all starches. Parameters recorded from the RFA showed strong significant correlations with onset and peak gelatinization temperature, besides gelatinization enthalpy.

Conclusions: Rapid force analyzer could be used as a rapid method for starch pasting assessment, being valid for discriminating among different types of starches.

Significance and novelty: Study shows the potential of Amylab as RFA that records starches pasting performance in 90 s. A test of 90 s allows determining starch paste performance using a rapid force analyzer.

KEYWORDS

Amylab, gel, microstructure, rapid force analyzer, starch, texture

1 | INTRODUCTION

Starches are common ingredients for numerous industrial food applications, which explain why their quality control have been the objective of much researches. Gelatinization is a primary physicochemical property of the starches, because of that viscosity measurements are widely applied for assessing the starch quality, particularly consisting of heating–cooling cycles to follow gelatinization and gelling phenomena (reviewed by Ai & Jane, 2015). Different systems (Brabender amylograph and viscograph, Rapid Viscoanalyzer) have been developed for assessing the starch viscosity. The majority of them are empirical methods based on recording the relative or apparent viscosity, derived from a torque, of a starch paste subjected to thermal and mechanical constraints (Suh & Jane, 2003). Those systems record the starch gelatinization in an excess of water, going from granular state to pastes, because of that it is widely referred as pasting performance. Differences on those systems rely on the amount of sample (2–80 g), the rotational speed, temperature gradient, and so on. As consequence, differences in the pasting parameters obtained from each instrument have been reported, which have been attributed to differences in the spindle structure (Suh & Jane, 2003). One of the most successful devices for assessing apparent viscosity of starches has been the rapid viscoanalyzer (RVA) designed back in 1987 (Walker

et al., 1988), owing to the lower amount of sample required and the short-time assay (within minutes), besides the versatility of the operational conditions (Balet et al., 2019; Batey & Curtin, 2000). Nowadays, similar versatility and benefits can be obtained with the micro-visco-amylograph (Wang & Shi, 2020). In addition, the change of operational conditions is continuously extending those instruments' applications, for instance to assess ingredients impact (Abdel-Aal et al., 2019), batter characteristics (Rios et al., 2018), breeders selection (Gil-Humanes et al., 2012), or even more fundamental information like proteins impact on starch pasting properties (Li et al., 2020), among others. All those applications reveal the endless interest for assessing pasting performance of starches using rapid methods.

Apart from starch/flour quality assessment, viscosity has been the basis for predicting α-amylase activity and cereal sprouting in a rapid test (measurement in seconds), widely known as falling number (FN) parameter (Best & Muller, 1991). The Hagberg falling number method approved by AACC International (AACCI) consists in a rapid heating of a starch suspension, with simultaneous stirring during the first 60 s, till reaching a point where the stirrer falls following the gravity force, and it is recorded the time required for the rod to cross the viscous suspension (Chang et al., 1999). This methodology is extensively used, and the FN is one of the most extended parameters in wheat quality surveys, although its repeatability and precision have been lately investigated (Delwiche et al., 2015). Therefore, there is extensive use of instruments for assessing the pasting properties of starches. Likewise, the falling number methodology is also based on apparent viscosity, recording the traveling time of the stirring rod through a starchy paste.

Recently, Chopin Technologies launched the Amylab FN to measure the Hagberg FN. Although this device was originally design to determine the falling number index of the flours, which is related to the α-amylase activity, such a rapid test might be very helpful for assessing starch performances if provided enough comprehensive information for understanding the process. This research aims to study the starch changes underlining during a short (90 s) heating cycle with simultaneous stirring. The rheological properties of different starches during their rapid force analysis (RFA) carried out in the Chopin Amylab have been related to the changes undergone on their microstructure and textural features.

2 | MATERIALS AND METHODS

2.1 | Materials

Commercial native starches of food grade for wheat starch (ADM, Chicago, US), corn starch (Tate & Lyle, London, UK), and potato starch (Tereos, Zaragoza, Spain) were directly used in the study. Rice starch was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Other reagents were of analytical grade.

2.2 | Starches characterization

Water (WBC) and oil (OBC) binding capacities were determined to evaluate the hydration properties of the different starches. Those properties were assessed as previously described (Cornejo & Rosell, 2015). Briefly, starch (100 mg) was suspended into one milliliter of water, in the case of WBC, or vegetable oil for the OBC. Suspensions were vortexed for 5 min and then centrifuge at 5,000 *g* for 5 min. The sediment was weighed after draining the tubes. Results were expressed as grams of water or oil adsorbed by gram of starch. Five replicates were made for each experimental result.

Thermal properties were determined using a differential scanning calorimeter (DSC-TAQ2000, TA Instruments Ltd., New Castle, DE, USA). Starch samples (8 mg) were weight in stainless steel pans, and distilled water was added in the proportion 1:4 (starch: water, w/w). Pans were heated at 10°C/min from 30 to 120°C, and an empty pan was used as reference. Onset temperature (To), heat of transition (ΔH, in joules per gram of starch), and peak temperature (Tp) were determined. All measurements were done at least in triplicate.

2.3 | Rapid force analyzer of different starches

The Chopin Amylab® (Chopin Technologies, Villeneuve-la-Garenne, Cedex, France) in its testogram mode was used as a rapid force analyzer (RFA) to record the gelatinization of the different starches. This device operates with a continuous up and down motion of the stirrer rod during 90 s at a constant temperature of 100°C. A slurry containing 7 g (14% mb) of starch and 25 ml of distilled water were placed into the precision test tubes of the device and manually shaken vigorously for 30 s. After immersing the stirring rod into the slurry, the tube was capped with a plunger and placed into the holder of the device. An insulated thermocouple (type K) was inserted and the wire leads attached to the bottom of the rod to record the temperature changes during measurements with a Comark N2014 multisensor temperature data logger (Comark Instruments, Norwich, Norfolk, UK). Temperature readings were recorded every second. Plot recorded shows the force, expressed in Newtons, of the slurry/gel under continuous heating/shearing (Figure 1). Parameters defined include the following: onset force (F0) before force increase due to gelatinization, onset time (s) at which F0 is detected, 50% (t1) and 75% (t2) of time to onset force, maximum force

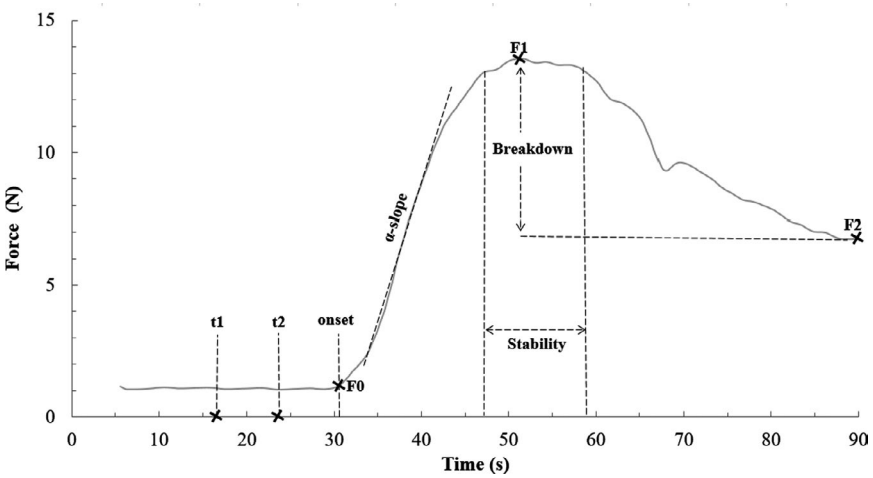

FIGURE 1 Typical plot recording force versus time along starch gelatinization using a Rapid Force Analyzer (Amylab). The main parameters used to evaluate slurries/gels are detailed in the drawing. Parameters defined include the following: onset force (F0) or starting force before gelatinization, onset time (s) at which F0 is detected, 50% (t1) and 75% (t2) of time to onset force, maximum force (F1), final force at 90 s (F2), α (slope between F0 and F1), gel stability as the elapsed time in which force was kept ± 10% of the maximum force (F1) and breakdown (force difference between F1 and F2)

(F1), and final force at 90 s (F2). Other parameters calculated from the previously identified forces included the following: α (slope between F0 and F1), gel stability as the elapsed time in which force was kept ±10% of the maximum force (F1), and the force difference between F1 and F2 was associated to starch breakdown. To understand starch changes along the gelatinization carried out with this device, the equipment was stopped to allow sampling at different times (t1, t2, onset, time to reach F1 and F2) as indicated in Figure 1. Three replicates were carried out for each sampling point, and type of starch and results showed the average of experimental data.

At each point, samples obtained from the RFA were immediately poured into cylindrical containers of 25 mm internal diameter, covered with lids, and kept at room temperature to cool down up to 25°C in the center of the gel. Simultaneously, the slurry/gel samples obtained from each stage were immediately frozen in liquid nitrogen and then freeze-dried to avoid microstructure alteration during freezing.

2.4 | Gel texture and degree of gelatinization

In each stage, gel firmness analysis was conducted in a TA-XT2 Texturometer fitted with a 5 kg load cell (Stable Microsystems, Surrey, UK). Then, gels were measured through a single compression test using a 10 mm diameter aluminum round probe. Measurements were carried out at 1 mm/s crosshead speed and 50% of strain. Firmness was considered as the maximum penetration force and adhesiveness defined as the area required to remove the probe from the gel. All textural analyses in each stage were done at least four times.

The degree of gelatinization (DG %) for samples from the different stages was assessed by running DSC test as previously described for native starches, but using the freeze-dried powder of the samples taken from rapid force analyzer. DG was calculated using the equation suggested by Ozge Sakiyan et al. (2011):

$$DG(\%) = (1 - (\Delta Hsample)/(\Delta Hnative)) \cdot 100$$

2.5 | Scanning electron microscopy (SEM)

Native starches and freeze-dried samples, from slurries/gels taken at different times from the rapid force analyzer, were observed using SEM (Hitachi S-4800, Tokyo, Japan). All samples (native and gels) were coated with gold using a vacuum evaporator (JEE 400; JEOL, Tokyo, Japan) for 5 min. The images taken at 10 kV acceleration voltage were captured using 900x magnification. Four micrographs captured at each stage were analyzed by digital image analysis using ImageJ software (ImajeJ 1.52p, National Institutes of Health, Bethesda, Maryland, US) to characterize microstructure. All micrographs were modified as 8-bit color and improved in contrast and brightness as reported by Espinosa-Ramirez et al. (2018). Threshold was assessed by adapting the software algorithm to each micrograph. Finally, sample analysis was carried out and mean cell area (μm^2) and gel porosity (%) were calculated.

2.6 | Statistical analysis

Each quality parameter was subjected to a one-way analysis of variance (ANOVA) using Statgraphics Centurion XVII V 17.2 (Statgraphics Technologies, Inc., The Plains, Virginia,

USA). Fisher least significant difference test was used to assess significant differences ($p < .05$) among samples that might allow discrimination among them. Additionally, Pearson correlation analysis was applied to establish possible relationships among experimental variables extracted from the different analysis.

3 | RESULTS AND DISCUSSION

3.1 | Starches characterization

To understand the starch changes when subjected to a rapid pasting procedure (90 s), different starches were selected, three from cereals (corn, rice and wheat) and one tuber starch from potato. Hydration properties and thermal parameters of those starches were determined because they might be potentially related to the pasting performance in this rapid procedure. Specifically, water and oil binding capacities reflect the hydrophilic and hydrophobic surface of the starch granules, which might affect the water uptake during granule swelling. Likewise, thermal properties have been related to changes in granular structure and endothermic gelatinization. Cereal starches showed much higher values for OBC than for WBC (Table 1), indicating greater superficial hydrophobicity, likely their A-type polymorphs with the double helixes closely packed are responsible of that behavior (Waterschoot et al., 2015). Wheat starch exhibited the lowest WBC compared to the other starches. Conversely, potato starch showed similar values for WBC and OBC, likely its B-type polymorphs that allow more water within the loosely packed helices could be responsible of that result.

The DSC parameters confirmed the significantly lower onset temperature of wheat starch, whereas the highest one was exhibited by corn starch. The peak temperature of the endothermic peak showed the same tendency described for the onset temperature. Nevertheless, potato starch required greater enthalpy for gelatinization, and the opposite behavior was observed in the rice starch. Results are within the range of gelatinization properties previously reported for those starches (Ai & Jane, 2015).

3.2 | Starch gelatinization recorded with a Rapid Force Analyzer

The Chopin Amylab has been designed for inducing starch gelatinization in a rapid test (90 s) applying heating to a starch slurry subjected to continuous stirring (Figure 1). The device records the force changes during the starch gelatinization that occurs within the 90 s, acting as a rapid force analyzer (RFA), although there is no previous information about changes occurring during the assay. A brief explanation of the plot recorded is following. In the present

TABLE 1 Characterization of raw starches regarding hydration properties, calorimetric parameters, and performance during rapid force analysis assessed with the Chopin Amylab

	Corn	Potato	Rice	Wheat
Hydration properties				
WBC	2.09 ± 0.23^{a}	2.05 ± 0.17^{ab}	2.16 ± 0.18^{a}	1.85 ± 0.05^{b}
OBC	2.56 ± 0.09^{a}	2.17 ± 0.06^{c}	2.60 ± 0.06^{a}	2.44 ± 0.08^{b}
Calorimetric properties				
To (°C)	69.90 ± 0.11^{a}	62.90 ± 0.54^{c}	65.62 ± 0.23^{b}	60.66 ± 0.23^{d}
ΔH (J/g)	12.76 ± 0^{b}	14.46 ± 0.25^{a}	6.56 ± 0.04^{d}	11.42 ± 0.11^{c}
Tp (°C)	74.37 ± 0.21^{a}	67.77 ± 0.60^{c}	72.13 ± 0^{b}	65.29 ± 0.04^{d}
RFA-Amylab				
t1 (s)	19 ± 0^{a}	15 ± 0^{b}	20 ± 0^{a}	15 ± 0^{a}
t2 (s)	28 ± 1^{a}	23 ± 1^{b}	30 ± 0^{a}	23 ± 0^{a}
F0 (*N*)	2.45 ± 0.07^{b}	3.76 ± 0.16^{a}	3.76 ± 0.01^{a}	2.92 ± 0.36^{b}
Onset (s)	36 ± 0^{c}	30 ± 0^{b}	36 ± 0^{c}	29 ± 0^{a}
α-slope	1.83 ± 0.01^{b}	2.07 ± 0.04^{a}	0.38 ± 0.02^{d}	0.65 ± 0.01^{c}
F1 (*N*)	11.51 ± 0.07^{c}	21.04 ± 0.30^{a}	11.34 ± 0.29^{c}	15.33 ± 0.20^{b}
Time F1 (s)	47 ± 0^{c}	38 ± 0^{d}	67 ± 0^{a}	57 ± 3^{b}
Stability (s)	12 ± 2^{b}	4 ± 1^{c}	22 ± 1^{a}	22 ± 1^{a}
F2 (*N*)	7.70 ± 0.04^{c}	12.78 ± 0.34^{a}	9.56 ± 0.05^{b}	12.21 ± 0.47^{a}
Breakdown (*N*)	3.81 ± 0.10^{b}	8.26 ± 0.64^{a}	1.78 ± 0.24^{c}	3.12 ± 0.28^{b}

Note: Means within the same row followed by different letters indicate significant differences by LSD multiple range test $p < .05$.

study, 7 g of starch (adapted to 14% moisture content) suspended on 25 ml water was used, which corresponds to the best precision for measuring the FN (Chang et al., 1999). Firstly, the starch or flour slurry is in a liquid form that does not require any force for stirring. As the heating progresses, swelling of starch granules increases viscosity, and in consequence, the force requires for keeping homogenous shearing increases significantly. The initial force (F0) of the slurry, before granules swelling, indicates the slurry consistency and might be related to the rapid starch water uptake on the surface besides the potential impact of granules size. The time at which F0 is detected is referred

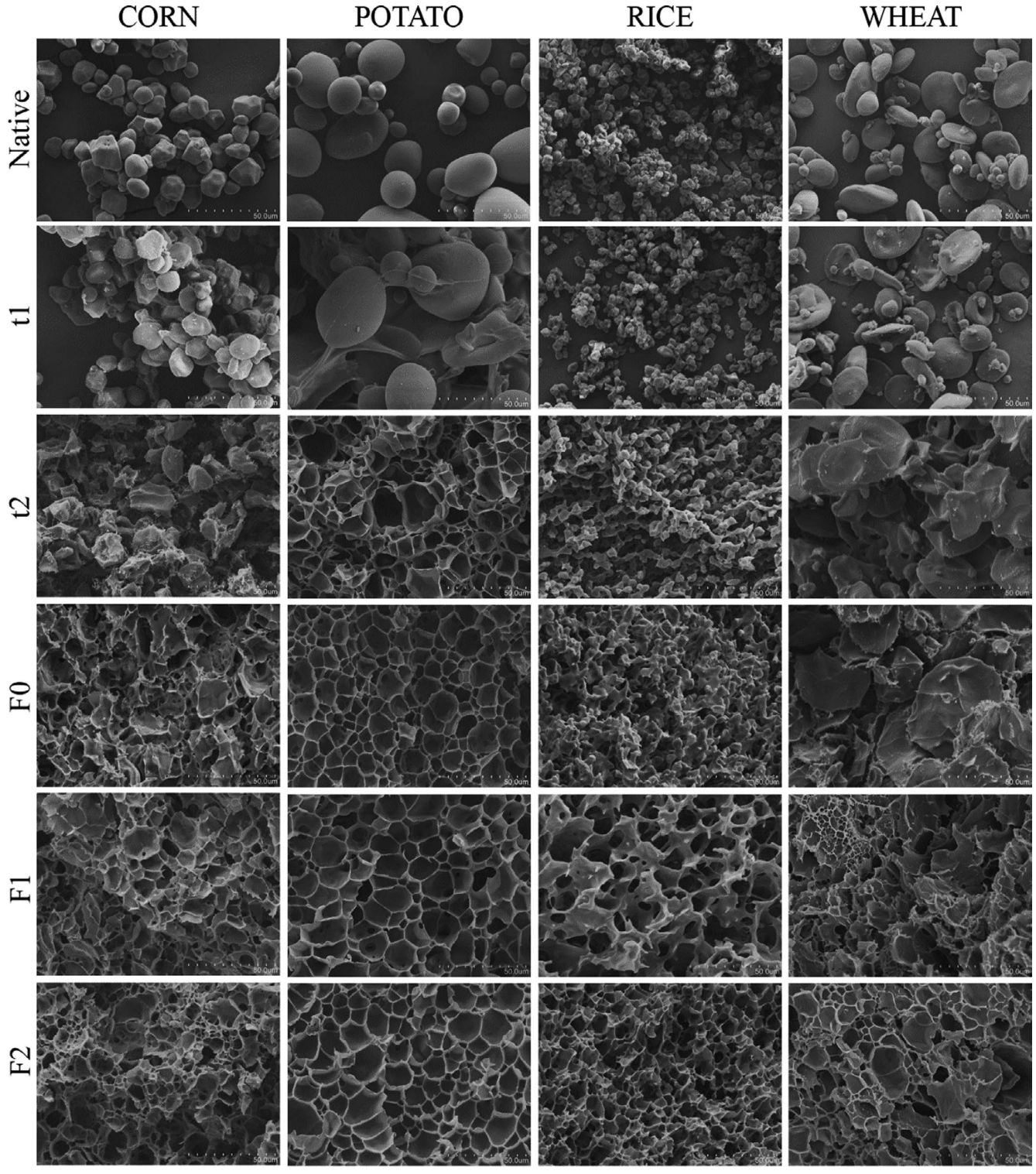

FIGURE 2 SEM micrographs captured along the rapid force analyzer (RFA) cycle showing the starch changes along heating and stirring. Micrographs were taken at 900x magnification. Micrographs were taken at the different RFA stages described in Figure 1

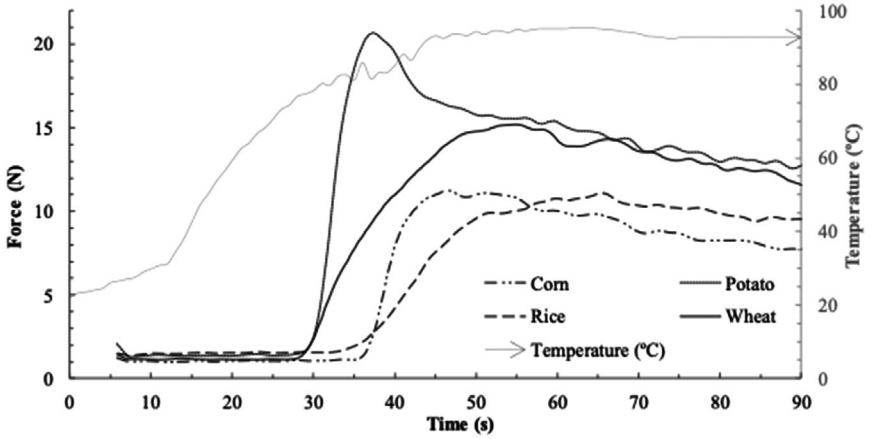

FIGURE 3 Plots from the rapid force analyzer for corn, potato, rice, and wheat starches assessed with the Amylab. Temperature was simultaneously recorded with a multisensor temperature data logger (plot with secondary y-axis) [Correction added on 11 February 2021, after first online publication: Figure 3 has been updated to include a missing sample and to distinguish between plotlines.]

as the gelatinization onset, and it could be related to the gelatinization temperature. The slope (α-slope) was also quantified to evaluate whether the rate of starch swelling could be related to granules morphology. It is well known that granules swelling continues till their disintegration or breaking down that leads to a force decrease, which lasted until an even gel is formed (Figure 1). The maximum force detected was referred to F1, and the time of holding force was defined as stability. To understand morphological and textural changes during the gelatinization process, the assay was stopped at the points (t1, t2, onset, time at F1, and at 90 s) where major changes were expected (Figure 1). Microstructure on those points was compared with the native granular structure of each starch (Figure 2).

Micrographs of corn and rice starches at t1 were rather similar to the native starches, showing intact granules that kept their polyhedral shape forming agglomerates (Figure 2). Therefore, t1 was not enough to alter those starches structure and initiate the gelatinization, which agrees with their higher onset temperature (Table 1). Conversely, potato and wheat starches even at t1 exhibited signals of either distortion (wheat) or leaching (potato). In wheat starch, the two granules populations were detected but the bigger A-type displayed flat morphologies and some small B-type granules showed a deep groove in the center. In the case of potato starch, swollen granules together with some amylose leaching were observed, although it seems that those changes were not sufficient to modify the stirring force (Figure 3). Despite potato has a B-type starch, its early gelatinization has been attributed to the negative charges of the phosphate-monoester derivatives that destabilize double helical structure (Ai & Jane, 2015). Considering the different thermal properties of the tested starches (Table 1), significant correlations were detected between t1 recorded in the RFA with DSC parameters, To ($r = .78$) and Tp ($r = .89$). In t2, which corresponded to 75% of the total time required for the onset, potato starch was completely gelatinized and a network was already observed, although it was not completely homogenous, showing irregular cavities sizes. At that point, wheat starch was even more distorted, with flat structures that were held together by the leached molecules. Wheat granules perimeter was still defined in some granules, and granules were interacting one to another. Similarly, corn and rice starches started their gelatinization with the deformation of the structures; thus, deformed granules were surrounded by leaching material and granules fragments resulting in an irregular mass. At the onset force (F0), the potato gel was completely formed, exhibiting an uniform network structure, whereas granules deformation progressed in cereal starches. At this point, some gel network was envisaged in corn and rice starches, but wheat granules were even thinner adopting flakes-like structures, with still defined perimeters.

Micrographs captured at the maximum force (F1) confirmed the gels formation in all the starches although some irregularities were observed in wheat gel. Considering that the maximum force is the inflection force point corresponding to the balance between swollen granules and fragmented ones, all starches lost their integrity and the granules interaction led to the gel mass. Further heating and gel shearing (till 90 s) did not provoke additional changes in the gel mass that could be visually detected, with the exception of the rice starch that exhibited more packed network at the end of the assay (90 s), likely better organization was achieved with the continuous stirring motion that allowed air bubbles removal. Similar structural changes were described for corn starch when gelatinization was sufficiently extended with the RVA and sampling was carried out at different times (Nelles et al., 2003). Therefore, considering the micrographs information, the force plot recorded with the RFA reproduced the rheological changes that has been reported to describe starch gelatinization (Singh et al., 2003).

The plots recorded by the rapid force analyzer (Figure 3) confirmed differences among the different starches. The temperature profile shows that 40 ± 3 s was required to reach 95 ºC, which is much faster than the over 150 s reported for the Perten FN (Chang et al., 1999). Initially, all slurries required low force for stirring, but after variable heating time (30–40 s) a fast increase of the recorded force was detected

due to starch gelatinization. At that moment, the rapid heating rate underwent a decrease due to the energy required for the endothermic gelatinization, which agrees with reported results for the Perten FN (Chang et al., 1999). Cereal starches showed some minor decay after reaching the maximum force. In opposition, potato starch showed a well-defined peak of gel force with a large force decrease when heating-stirring progressed.

Parameters defined from the plots are included in Table 1. Potato and rice starches required significantly ($p < .05$) higher force (F0) for keeping homogenous slurries, which might be related to their WBC. In fact, a significant correlation ($r = .89$) was identified between F0 and WBC, in accordance with the relationship reported among the capacity to hydrate and swell and the starch viscosity (Cornejo-Ramírez et al. 2018). The lower onset observed for potato and wheat starches indicated that their gelatinization started at lower temperature, which agrees with data from DSC. In fact, a very strong positive correlation was encountered between the RFA onset time and the DSC To ($r = .90$) and Tp ($r = .97$). Potato starch displayed the faster gelatinization (α-slope), and rice starch had the lowest rate of gelatinization, with higher time (Time F1) to reach the maximum gel force (F1). Interestingly, a significant positive correlation ($r = .87$) was detected between the α-slope and the gelatinization enthalpy (ΔH).

As expected, potato starch exhibited the highest force (F1) followed by wheat starch, whereas corn and rice starches did not differ significantly ($p < .05$) on their maximum force. A negative correlation ($r = .88$) was observed between the OBC and the F1. To assess behavior of gels after complete granule disintegration, gels stability was defined as the time holding the maximum force, which confirmed the short stability of the potato gel, and the longer stability of rice and wheat gels. Potato and wheat gels required higher forces indicating their higher viscosity. The force decay indicated by the breakdown reflected the granules resistance to physical rupture, which has significantly higher for potato starch, supporting that starches exhibiting higher swelling are less resistant to breakdown on cooking (Singh et al., 2003). In fact, a highly significant correlation ($r = .90$) was observed between F1 and breakdown.

3.3 | Starch gels properties along gelatinization in RFA

The texture, RFA parameters, gelatinization degree (GD), and microstructure of the samples taken along the gelatinization carried out in the RFA were evaluated (Table 2). The statistical analysis of the variance showed that the starch factor significantly ($p < .05$) affected all the parameters tested, with exception of the degree of gelatinization. Likewise, samples taken at the different stages of the gelatinization (different times along RFA analysis) showed significantly ($p < .05$) different properties.

The RFA provokes a rapid gelatinization of the different starches, which was confirmed with the 100% gelatinization degree determined with the DSC. As it was previously described for the microstructure changes, the GD was reached at different times depending on the type the starch. Corn and wheat starches required 28 s and 23 s (Force time column in Table 2), respectively, to reach 100% GD. Conversely, potato and rice starches reached 100% GD at 30 s and 36 s, respectively. Surprisingly, according to the GD, corn and wheat starches were rapidly gelatinized, when the *SEM* micrographs still revealed granular structures at those times. Considering that the gelatinization degree was calculated based on the transition enthalpy, it might be that the changes required to obtain a complete lattice structure do not require additional energy, and in consequence, no enthalpy was detected when evaluated those samples.

Potato gel had the highest firmness (5.5 N), which agree with the highest force value recorded with the RFA. But those maxima values were reached at different times, that is the firmest potato gel was obtained after 30 s, whereas its maximum force recorded with the RFA was observed at 38 s. This result agrees with the SEM observation that indicated smaller air voids in the potato gel at F0 than at F1 (Figure 3), leading to firmer gels. On the contrary, for cereal-based gels the highest firmness was in accordance to the maximum force. Rice gel was the softer one with the lowest firmness. Nonetheless, in cereal-based gels, there was not a direct trend between the firmness of the gels and the maximum force (F1) detected with the RFA. Previous studies carried out with potato and wheat starches stated the linear relationship between macroscopic and microscopic viscosity determined with creep and rotational measurements, respectively, within the temperature range 30–50°C (Yamano et al., 1996). Nevertheless, with this rapid analysis carried out at higher temperatures, no linearity was observed between F1 and firmness. The firmness of the gels obtained after the 90 s (at F2) tended to decrease, although no significant differences were observed. Presumably, the molecular order of the gels was kept till a point where the thermal and mechanical constraints caused their weakening, and that effect was more noticeable in firmer gels like potato. Similarly, a significant ($p < .05$) decrease in the force values was observed in all starches as gelatinization progresses, which might be related to the thixotropic (shear thinning) behavior of the starch pastes with respect to time (Sikora et al., 2015).

From the image analysis of the gels structure, it was calculated the porosity of the network and the median cell area, the former to avoid the misrepresentation that using the average value could create. Porosity observed in the micrographs corresponded to the regions initially occupied by water that was removed by freeze-drying. Those parameters were identified

TABLE 2 Characterization of the starchy gels obtained along the gelatinization process including texture, RFA parameters, gelatinization degree (GD), and microstructure

Starch	RFA-Stage	Force time (s)[a]	Force (*N*)	Firmness (*N*)	Adhesiveness (*N*·s)	GD (%)	Porosity (%)	Median cell area (μm^2)
Corn	t1	19 ± 0e	1.1 ± 0d	n.d.*	n.d.	40 ± 4	n.d.	n.d.
	t2	28 ± 1d	1.1 ± 0d	2.3 ± 0.9b	1.4 ± 0.9b	100 ± 0	n.d.	n.d.
	F0	36 ± 0c	2.5 ± 0.1c	4.7 ± 0.5a	3.0 ± 0.7a	100 ± 0	37.8 ± 0.3b	24 ± 8a
	F1	47 ± 0b	11.5 ± 0.1a	4.8 ± 0.6a	2.6 ± 0.7ab	100 ± 0	45.5 ± 2.8ab	18 ± 4ab
	F2	90 ± 0a	7.7 ± 0.0b	4.4 ± 0.4a	3.4 ± 0.3a	100 ± 0	52.2 ± 3.7a	6 ± 1b
Potato	t1	15 ± 0e	1.3 ± 0.1d	n.d.	n.d.	49 ± 4	n.d.	n.d.
	t2	23 ± 1d	1.4 ± 0.2d	4.8 ± 0.8b	1.4 ± 0.3a	84 ± 3	49.3 ± 3.5b	27 ± 9b
	F0	30 ± 0c	3.8 ± 0.2c	5.5 ± 0.5a	1.3 ± 0.3a	100 ± 0	52.4 ± 3.0b	28 ± 2b
	F1	38 ± 0b	21.0 ± 0.3a	2.5 ± 0.3c	0.9 ± 0.2b	100 ± 0	67.8 ± 2.0a	62 ± 5a
	F2	90 ± 0a	12.8 ± 0.3b	2.1 ± 0.1c	0.7 ± 0.1b	100 ± 0	64.6 ± 3.8a	53 ± 3a
Rice	t1	20 ± 0e	1.5 ± 0.1d	n.d.	n.d.	22 ± 2	n.d.	n.d.
	t2	30 ± 0d	1.5 ± 0.1d	0.5 ± 0.1b	0.5 ± 0.2b	96 ± 1	n.d.	n.d.
	F0	36 ± 0c	3.8 ± 0.0c	0.9 ± 0.1a	1.0 ± 0.1a	100 ± 0	15.0 ± 2.5c	8 ± 1b
	F1	67 ± 0b	11.1 ± 0.3a	1.0 ± 0.1a	1.1 ± 0.1a	100 ± 0	35.0 ± 2.5b	29 ± 5a
	F2	90 ± 0a	9.5 ± 0.1b	1.0 ± 0.1a	0.7 ± 0.2b	100 ± 0	51.0 ± 2.8a	18 ± 3b
Wheat	t1	15 ± 0_e	1.1 ± 0.1d	n.d.	n.d.	73 ± 1	n.d.	n.d.
	t2	23 ± 0d	1.1 ± 0.2d	1.4 ± 0.1c	1.5 ± 0.2b	100 ± 0	n.d.	n.d.
	F0	28 ± 0c	2.9 ± 0.4c	2.7 ± 0.6b	1.9 ± 0.9b	100 ± 0	n.d.	n.d.
	F1	57 ± 3b	15.3 ± 0.2a	4.4 ± 0.4a	3.5 ± 0.8a	100 ± 0	16.7 ± 2.1b	12 ± 2a
	F2	90 ± 0a	12.2 ± 0.5b	4.2 ± 0.2a	3.2 ± 0.5a	100 ± 0	64.9 ± 0.5a	16 ± 1a
p-Value								
Starch		.0006	.0016	.0000	.0000	.0506	.0000	.0000
RFA-stage		.0000	.0000	.0000	.0006	.0000	.0000	.0157

Abbreviation: RFA, Rapid force analyzer (Chopin Amylab working in its testogram mode).

[a]Force time: time to reach sampling point as displayed in Figure 1.*n.d. (not detected)Means within the same column in each starch followed by different letters indicate significant differences by LSD multiple range test $p < .05$.

as soon as a gel was detected, which was reached in the early stages in the case of potato starch. From the porosity results, it was evident in all the gels the progressive increase in the porosity as the RFA progresses, indicating the gradual formation of an even gel structure, as was observed in the micrographs. The continuous stirring might help to obtain a more uniform structure since the shear force favors the alignment of the molecules within the lattice structure of the gels (Nelles et al., 2003). The higher median area of the lattice voids of potato gel indicated a more open structure of this gel with bigger cavities than those obtained for cereal-based gels. Moreover, in the last stages it could envisage a decrease in the median area of the holes, with exception of wheat gel. Although the network structure visualized in the micrographs resulted from the removal of water leading to voids, changes in the last stages after complete gelatinization might be associated with the removal of air bubbles entrapped within the gel forced by the stirring motion.

A correlation matrix within the measured parameters confirmed the significant relationship among parameter recorded from the RFA and resulting gel features (Table 3), allowing better understanding of the changes undergone in the RFA along gelatinization. Only a very strong positive correlation ($r \geq .7$) was observed between the force measured in the RFA and the porosity of the gels, indicating that higher force gels would lead to more porous gels. Positive moderate ($.4 < r < .7$) correlations were observed between force and the gelatinization degree and median cell area, confirming the total gelatinization of the starches and higher force resulted from gels with thicker walls and big holes. There was a highly significant ($p < .0000$) correlation ($r = .66$) between force and time to reach those forces, which suggested longer time in the RFA was required for the stronger gels, like it was observed in the case of potato. The correlation between force and gel firmness, although statistically significant, was rather weak ($r < .3$). In the same sense, no correlation was found by Gaines et al. (2000) between pasting properties and gel hardness. Force time was also positively correlated with the GD and gel porosity. Other important correlations were detected among the gel firmness with

TABLE 3 Correlation matrix among texture properties, RFA parameters, gelatinization degree (GD), and microstructure obtained from the different starches

	Firmness (*N*)	Adhesiveness (g·s)	Force (*N*)	Force time (s)	GD (%)	Porosity (%)
Adhesiveness (g·s)	**0.66**					
	(0.0000)					
Force (*N*)	**0.33**	0.13				
	(0.0371)	(0.4885)				
Force time (s)	0.27	0.20	**0.66**			
	(0.0869)	(0.3041)	(0.0000)			
GD (%)	**0.52**	0.16	**0.47**	**0.50**		
	(0.0005)	(0.3947)	(0.0022)	(0.0010)		
Porosity (%)	**0.62**	0.08	**0.70**	**0.68**	**0.48**	
	(0.0000)	(0.6656)	(0.0000)	(0.0000)	(0.0016)	
Median cell area (μm^2)	−0.14	**−0.49**	**0.44**	−0.15	−0.05	**0.55**
	(0.5089)	(0.0248)	(0.0298)	(0.5587)	(0.8312)	(0.0052)

Note: Upper row = *Pearson* correlations, bold values indicate significant correlations.
In parenthesis *p*-values for statistical significance of estimated correlation.

adhesiveness ($r = .66$; $p = .0000$), GD ($r = .52$; $p = .0005$), and porosity ($r = .62$; $p = .0000$). The moderate negative correlation observed between the adhesiveness and median cell area suggested that gels with smaller voids were more adhesive.

4 | CONCLUSIONS

The underlying mechanism occurring during a rapid starch gelatinization carried out with a rapid force analyzer was investigated by stopping the measurement at different stages. Different parameters have been defined from the RFA plots to characterize starch performance during pasting. The microstructural changes observed with four different starches (corn, potato, rice, and wheat) confirmed the complete starch gelatinization within the 90 s test, although time to reach gelatinization was dependent on the starch source. The force plots obtained from the RFA allowed the discrimination among the different starches. Significant correlations were detected between the maximum force (F2) recorded by RFA with the gelatinization degree and gel microstructure (porosity and median cell area).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Cristina M. Rosell https://orcid.org/0000-0001-7197-5874

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Interaction of dough acidity and microalga level on bread quality and antioxidant properties

Raquel Garzon [a], Adriana Skendi [a,b], Marco Antonio Lazo-Velez [a,c], Maria Papageorgiou [b], Cristina M. Rosell [a,*]

[a] Institute of Agrochemistry and Food Technology (IATA-CSIC), C/ Agustin Escardino, 7, Paterna 46980, Valencia, Spain
[b] Department of Food Science and Technology, International Hellenic University, POB 141, GR-57400 Thessaloniki, Greece
[c] Universidad del Azuay, Grupos Estratégicos de investigación en Ciencia y Tecnología de Alimentos y Nutrición Industrial (GEICA-UDA), Av. 24 de mayo 7-77, Apartado01.01.981, Cuenca, Ecuador

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ABSTRACT

Microalgae nutritional and healthy dietary pattern might be affected by processes like breadmaking when used as ingredient. This study aims to determine the role of dough acidification on the nutritional pattern of *Chlorella vulgaris* enriched breads. Different levels of microalga (1%, 2% and 3%) were incorporated in the recipe in the presence of either 10% sourdough or chemically acidified doughs. Dough and bread characteristics were evaluated. Addition of microalga reduced the slice area and increased the crumb hardness, but it could be counteracted by increasing dough hydration and adapting proofing time. Doughs and breads enriched with microalga had green color. Dough acidification led to softer breads and enhanced the antioxidant activity of enriched breads. Microalgae incorporation increased the protein and ash content of the breads. Microalgae enriched breads made with chemically acidified doughs or sourdoughs had higher Total Phenolic Content and antioxidant activity as assessed by FRAP and ABTS methods.

1. Introduction

Bread, as one of the most consumed bakery products worldwide (with average per capita consumption 24.5 kg in 2020 and an expected market growth annually by 2.8% (CAGR (Compound Annual Growth Rate) 2020–2025)) has gained the researchers' interest to be used as a vehicle for delivering bioactive compounds to the consumers (Statista, 2020). To this end, a great number of plant materials rich in bioactive compounds have been incorporated in a bread recipe such as aromatic plants (Skendi, Irakli, Chatzopoulou, & Papageorgiou, 2019), fruits and vegetables (Betoret & Rosell, 2020a), microalgae (Graça, Fradinho, Sousa, & Raymundo, 2018) etc., returning breads with an enhanced nutritional profile. Microalgae have received increased consideration due to the high content of pigments, favourable protein and polyunsaturated fatty acid profiles, phenolics, vitamins, and minerals (Ferreira et al., 2020; Gopal et al., 2019; Yun, Kim, & Yoon, 2020). The microalgae *Chlorella luteoviridis*, *Chlorella pyrenoidosa*, and *Chlorella vulgaris*, used prior to May 1997 in Europe, are authorized as food (European Union, Novel Food catalogue). Among the genus *Chlorella*, in the USA, only *Chlorella protothecoides* is included in the GRAS list (FDA, GRAS Notices) and in Australia, all *Chlorella* species (including *Chlorella sorokiniana*) and derivatives for which a novel food application was submitted have been so far considered as traditional food (FSANZ, 2016). *Chlorella* sp. is a rich source of proteins (51–58%), lipids (14–22%) and carbohydrates (2–17%) (Batista et al., 2011; Niccolai, Chini Zittelli, Rodolfi, Biondi, & Tredici, 2019) Those facts have prompted the research of its incorporation in different bakery products. Incorporation of 2% *C. vulgaris* resulted in "high in selenium" cookies (Uribe-Wandurraga, Igual, García-Segovia, & Martínez-Monzó, 2020) whereas the addition level of both 2% and 6% resulted in higher protein and total phenolic contents as well as in higher *in vitro* antioxidant capacity while maintaining texture stability and sensory quality of cookies (Batista et al., 2017). According to Batista et al. (2019) crackers with 6% *C. vulgaris* could be claimed to be a "source of protein". Fradique et al. (2010) added *C. vulgaris* (up to 2% w/w) in a pasta recipe rendering pasta with high levels of protein and minerals, without interfering with sensorial quality, cooking and textural properties of the final product. Graça et al. (2018) studied the effect of adding *C. vulgaris* (up to 5% in

* Corresponding author.
E-mail address: crosell@iata.csic.es (C.M. Rosell).

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wheat flour base) on dough and bread quality. Up to 3% addition level they observed a strengthening of the gluten network and thus a positive impact on dough rheology and viscoelastic characteristics. Nevertheless, to fully exploit their potential derived from the pigments and phenolic compounds, the impact of process conditions might be considered, since pH and temperature affect antioxidant activity (Betoret & Rosell, 2020b).

It is already stated the importance of food processing on the bioavailability of functional compounds owing to the impact of process conditions on their physicochemical structure (Dima, Assadpour, Dima, & Jafari, 2020). In the particular case of phenolic compounds and their antioxidant capacity, the processing conditions might play a crucial role modulating bioavailability through chemical modifications or cleavage of phenolic compounds-matrixes bonds (Ribas-Agustí, Martín-Belloso, Soliva-Fortuny, & Elez-Martínez, 2018). Breadmaking is a very complicated process where mechanical constraints are entwined with the microbial action during fermentation encompassing dough acidification and activation of endogenous enzymes (Gobbetti, Rizzello, Di Cagno, & De Angelis, 2014). Both endogenous and bacterial enzymes are responsible for the solubilisation of dough components thus increasing their bioavailability and digestibility and/or produce through specific synthetic pathways new bioactive compounds thus improving antioxidative activity (Hur, Lee, Kim, Choi, & Kim, 2014). Actually, an increase in the amount of free and bound polyphenols was obtained by fermenting KAMUT® khorasan and durum wheat flours with sourdoughs (Saa, Di Silvestro, Dinelli, & Gianotti, 2017). The same trend was observed by Ferri, Serrazanetti, Tassoni, Baldissarri, and Gianotti (2016) for fermented doughs with sourdoughs containing different lactic acid bacteria strains. Those authors confirmed the positive correlation between short fatty acids with the polyphenol content and antioxidant activity in wheat breads.

The beneficial effect of *C. vulgaris* addition has been related with its nutritional content and antioxidant properties, but when used as ingredient, those could be affected during the process. The aim of this research was to evaluate the impact of breadmaking (dough acidification) on microalga enriched dough and bread. To this end, three different levels of *C. vulgaris* were used (1%, 2% and 3%) aiming to establish the maximum acceptable content of *C. vulgaris* that could be used in industrial applications while maintaining acceptable bread quality. To unravel the effect of simultaneous addition of microalgae and dough acidification on dough properties and bread quality, two sets of the same experiment were performed. The first set contained 10% sourdough and in the second set chemically acidified dough was prepared by adjusting the pH with organic acids so as to reach the pH of the sourdough.

2. Materials and methods

2.1. Materials

Wheat flour for breadmaking (12.07% of protein (N × 5.7), 12.9% moisture) was purchased from Harinera la Meta S.A. (Valencia, Spain). Commercial *Chlorella vulgaris* microalga flour was purchased from Allmicroalgae (Lisbon, Portugal). The DPPH (2,2-diphenyl-1-picrylhydrazyl) was from Sigma Aldrich, USA, whereas TPTZ (2,4,6-tripyridyl-s-triazine) was obtained from Alfa Aesar, GmbH & Go KG, Karlsruhe, Germany. ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox ((S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and gallic acid were from J&K Scientific GmbH, Pforzheim, Germany. Folin-Ciocalteu reagent, and methanol (HPLC grade) were obtained from Chem-Lab NV, Zedelgem, Belgium. Iron (III) chloride hexahydrate was from Merk, KGaA, Darmstadt, Germany. All the other chemicals used were of analytical grade.

2.2. Chemical composition

The proximate composition of raw materials and breads was determined using the standard methods of the International Organization for Standardization (ISO): moisture (712:2009), protein (16634-2:2016), ash (2171:2007) and total fat (11085:2015). Carbohydrates content was calculated by difference.

2.3. Sourdough preparation and cell count

Sourdough of type IV (Papadimitriou et al., 2019) was prepared without the use of baker's yeast. Wheat flours (200 g) were mixed with tap water (126 mL) to obtain a final dough yield (DY) [dough weight × 100/flour weight] of 163. Fermentation and daily back-slopping (refreshments) were conducted according to Barber, Baguena, de Barber, and Martinez-Anaya (1991). To initiate fermentation, 60% of water was substituted with previously macerated wheat bran (that contains endogenous microorganisms) with tap water at a ratio of 20:80 for at least 20 min. Daily refreshments were carried out for the 3 successive days by mixing 50% of the previously fermented dough with flour and water (final dough yield of 163). Dough was incubated at 30 °C and RH 80% under anaerobic conditions. The resulting sourdoughs were freeze-dried, pulverized, packed in plastic bags and stored at 4 °C for further analysis.

Microbial plate counts were determined by plating successive dilutions of sourdough and dough samples onto selective media. Man, Rogosa and Sharpe agar (MRS) and Potato Dextrose Agar (PDA) and standard medium glucose, yeast extract and calcium carbonate (GYC) were used to determined lactic acid bacteria (LAB) and yeasts and acetic bacteria, respectively. MRS plates were anaerobically incubated at 32 °C for 48–72 h, while PDA plates were incubated at 37 °C for 24–48 h and GYC at 30 °C for 72 h, both under aerobic conditions.

2.4. Breadmaking procedure

Reference bread doughs (W) conventionally prepared (by using compressed baker's yeast), bread doughs containing sourdough (SW) and bread doughs chemically acidified (AW) containing different levels of microalgae powder (0, 1, 2 or 3%) were prepared. A basic recipe, based on white wheat flour (f.b.) was used: 2% compressed baker's yeast, 1.5% salt (corrected based on the salt present in the microalgae powder added), and the amount of water that dough needs to reach optimum consistency (1.1 Nm) in Mixolab (Chopin, Paris, France). Sourdough containing doughs were prepared using the basic recipe but replacing 10% wheat flour by freeze-dried sourdough. After preliminary assays for checking the final pHs of the sourdough containing doughs, chemically acidified doughs with pH value of 4.90 ± 0.05 were prepared with the basic recipe and adding 0.15% of a mixture 1:4 of acetic acid (100% w/w) and lactic acid (85% w/v) (Xu, Tang, Hu, Xu, & Gänzle, 2018). Ingredients were mixed in a Farinograph 300 g-bowl (Brabender, Germany) at 30 °C for 10 min. The dough was allowed to rest for 10 min after which, it was divided in portions of 40 g, shaped manually, placed into silicon pans (100 mm length × 50 mm width × 50 mm height) and allowed to ferment at 30 °C till the volume doubles itself. Finally, fermented doughs were baked at 180 °C for 15 min and then cooled down at room temperature for 45 min. Samples codes indicated the type of bread followed by a number that described the level of microalgae, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3). Two batches in different days were made for each sample.

2.5. Dough characterization during breadmaking

The water absorption (WA) or the amount of necessary water to reach dough optimum consistency (1.1. Nm) for each formulation (without added yeast) was calculated using a Mixolab (Chopin, Paris,

France) equipment following the method S defined by the supplier. Consistency of doughs was registered during 30 min at 30 °C in the Mixolab. Proofing performance of the doughs was monitored by recording the increment of dough volume (ΔV) every 10 min during 100 min. To evaluate the fermentation kinetics, the slope (mL/min) of the volume increase, the maximum volume (mL) and the time to duplicate initial dough volume (2 V (min)) were calculated. Samples were analyzed in duplicate.

2.6. pH, total titratable acidity (TTA) and acid content in doughs

The pH of fermented doughs was potentiometrically determined using a penetration electrode. The pH of breads was measured after vortexing one gram of bread diluted with 9 mL deionized water. Total Titratable Acidity (TTA) was measured in 10 g of doughs or breads blended with 100 mL distilled water that were titrated with 0.01 N NaOH and expressed as mL NaOH/10 g.

Concentrations of D-lactic acid and acetic acid were analyzed using a specific kit Acetic Acid Assay Kit (Acetate Kinase) and D-Lactic Acid Assay Kit (D-Lactate) (Megazyme International, Ireland), respectively, according to the manufacturer's instructions. The results were expressed as g of D-lactic or acetic acid per kilogram of sourdough, fermented dough or bread. Three replicates were averaged for each sample.

2.7. Technological characterization of breads

Breads were automatically cut it in 15 mm slice thickness and then characterized. Color of bread crumb and crust was measured using a chroma meter CR-400 (Konica Minolta, Japan). The color was recorded using CIE-L^* a^* b^* scale, where L^* indicates lightness, a^* indicates hue on a green (−) to red (+) axis, and b^* indicated hue on a blue (−) to yellow (+) axis. Total color difference (ΔE^*) was calculated with the following equation:

$$\Delta E^* = \left((L_r^* - L_i^*)^2 + (a_r^* - a_i^*)^2 + (b_r^* - b_i^*)^2\right)^{1/2}$$

where L_r^*, a_r^*, b_r^* are color parameters of reference bread without microalgae, and L_i^*, a_i^*, b_i^* are color parameters of breads containing microalgaes and/or acidified doughs. Crumb hardness (N) was determined using a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) applying a TPA test with a double compression. A piece of crumb (20 mm diameter) was taken from the center of each slice and was compressed up to 50% using a 36 mm aluminum probe. The following parameters were calculated from the compression curve: hardness, springiness, cohesiveness, chewiness, and resilience.

The slice 2D area and crumb structure were evaluated by digital image analysis using the ImageJ software (ImageJ 1.52p, National Institutes of Health, USA) following the method described by Espinosa-Ramírez, Garzon, Serna-Saldivar, and Rosell (2018). Briefly, images of bread slices were captured using a flatbed scanner (Epson V550, Japan) at 600 dpi of resolution. Then images were improved by splitting channels, taking the green channel for the analysis and enhancing the contrast; for segmentation method a predefined algorithm ("Otsu") was applied. Finally, the 2D slice area (cm^2) was measured in order to analyze the crumb cell distribution, mean cell area (mm^2) and porosity (%). At least four bread loaves for each recipe were used for the above measurements.

2.8. Extraction of bioactive compounds

Bioactive compounds from the freeze-dried breads were extracted in 70% aqueous methanol as reported in Skendi et al. (2019) with some modifications. Freeze-dried bread (1.5 g) was first homogenized in 4 mL of 70% methanol on a vortex for 30 s and then the extraction was performed in an ultrasonic bath for 15 min. Following, the sample was centrifuged at 2264×g for 15 min and the supernatant was collected. The precipitate was re-dissolved into 3 mL of 70% methanol and extracted for a second time as reported previously. The supernatants were pooled together and centrifuged once more at 8400×g for 10 min and finally frozen stored until analysis. Extraction of the bioactive compounds from the bread samples was carried out at least in triplicate.

2.9. Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method reported by Singleton, Orthofer, and Lamuela-Raventós (1998). For the determination the extract (200 mL) was first oxidized with 800 mL of the Folin-Ciocalteu reagent diluted with water (1:10 v/v) for 2 min. Following, 2 mL sodium carbonate 7.5% w/v was added to the mixture and allowed to react for 2 min before adding 8 mL of distilled water. Finally, the mixture was incubated in the dark for one hour at room temperature. The absorption of the mixtures was measured at 725 nm and the results were expressed as milligrams of gallic acid equivalents per gram of dry weight basis (mg GAE/gdw).

2.10. Determination of antioxidant activity

Three assays were utilized to determine the antioxidant activity of breads based on antiradical activities: a) radical scavenging capacity activity (DPPH), b) radical cation scavenging activity (ABTS) and c) ferric reducing antioxidant power (FRAP). Radical scavenging activity (DPPH) was measured according to Yen and Chen (1995). The extract (150 μl) was mixed with 2.85 mL of 100 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent and allowed to rest for 5 min before reading the absorbance at 516 nm.

The radical cation scavenging activity (ABTS) was determined by mixing 100 μl extract with 3.9 mL ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) reagent with an absorbance of 0.70prepared according to Re et al. (1999). The absorbance of the mixture was measured at 734 nm after reacting for 4 min at room temperature.

The ferric reducing antioxidant power (FRAP) assay was carried out by mixing 100 μl of extract with 3 mL of FRAP reagent for 4 min at 37 °C under dark conditions as reported from Benzie and Strain (1999). The FRAP assay consisted of a mixture of three solutions: 20 mM ferric chloride solution, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 0.3 mM (pH 3.6) acetate buffer in a proportion of 1:1:10, respectively. The absorbance of the mixture was measured at 593 nm against a blank.

For all the three assays the results were expressed as mg Trolox ((S)-(−)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)equivalents per gram of sample on a dry weight basis (mg TE/g dw). All the measurements for determination of TPC and antioxidant activity were performed at least in duplicate.

2.11. Statistical analysis

The results are reported as the average and standard deviation of measurements. Analysis of variance (ANOVA) was applied using Statgraphics Centurion XVII (Statistical Graphics Corporation, UK) and SPSS Statistics 25.0 software (SPSS Inc., Chicago, USA). The Duncan test was used to assess significant differences ($P < 0.05$) among samples. The statistical analysis of TPC and antioxidant activity includes the application of general linear model for multiple variables as well as correlation analysis among the variables. Additionally, a principal component analysis (PCA) was carried out to detect possible discrimination among samples.

3. Results and discussion

Different levels of *C. vulgaris* powder (0, 1, 2 or 3%) were incorporated into breads. Based on the optimum amount reported by Graça et al.

(2018), 3% was selected as the maximum incorporation level. To evaluate the potential impact of acidification, doughs fermented with a spontaneous sourdough and doughs chemically acidified were carried out and the resulting breads evaluated. The final yeast, LAB and acetic acids counts (CFU/g) in the lyophilized sourdough were 2×10^3, 3×10^8; and 1×10^5, respectively.

3.1. Dough characteristics

Dough rheological properties during mixing were determined using the Mixolab, which also allowed defining the water absorption to be used in the breadmaking for obtaining doughs with constant consistency. The addition of microalgae powder resulted in a steady increase of the water absorption required to reach constant consistency of 1.1 Nm (Table 1). This increase might be expected considering that microalgae are a source of hydrocolloids, which are generally recognized for their ability to enhance water absorption in breadmaking (Rosell, Rojas, & Benedito de Barber, 2001). Specifically, in *C. vulgaris*-based crackers, Batista et al. (2019) reported that the addition of high concentrations of microalgae biomass could lead to a weaker gluten network, unable to efficiently trap gas bubbles and water molecules. Batista et al. (2011) also reported that *A. platensis* biomass (commercially known as spirulina) impairs starch gelatinization in model gel systems, namely by increasing the gelatinization temperature, likely due to competition for water binding zones during the hydration of starch granules. In fact, during dough mixing, the available free water is partitioned between microalgae and wheat flour hydrophilic sites, which might also impair gluten protein network development. It is possible that the formation of a weaker gluten network might result in the collapse of small gas cells into larger cavities, which might influence gas and water trapping during baking (Batista et al., 2019). Likewise, sourdough containing doughs required significantly higher water absorption compared to their counterparts (reference and chemically acidified dough). The presence of microalgae at different levels did not significantly modify the doughs' stability during mixing (Figure S1). All doughs showed a good performance during mixing for 30 min, although those containing sourdough showed some decay after 20 min of mixing. The effect was more pronounced in samples with added microalgae powder, showing that the interaction between microalgae and sourdough resulted in a slight weakening of the dough when overmixed. *C. vulgaris* effect on dough mixing agrees with results reported by Graça et al. (2018) within the level range used in the present study, showing the increase in water absorption but without affecting dough stability, effects that these authors ascribed to the supplemented proteins from the microalga. Same absorption trend has been reported with red and brown seaweeds powder when added up to 8% to wheat flour (Arufe et al., 2018; Mamat, Akanda, Zainol, & Ling, 2018), due to their high water retention capacity.

The fermentation of the doughs was monitored to compare breads obtained with the same level of fermentation. Doughs were baked when dough volume doubled itself and to reach that level, doughs containing sourdough (SW0) showed the faster fermentation (50 min) compared to reference dough (W0) (60 min) and chemically acidified dough (AW0) (60 min) (Fig. S2 and Table S1). Addition of the microalga in the recipe accelerated the fermentation of the chemically acidified doughs (40 min) but not of the reference dough and sourdough. Therefore, *C. vulgaris* addition up to 3% did not affect the fermentation kinetics by bakers' yeast as reported by Graça et al. (2018), but in the presence of acids an underlying more complex mechanism than the obvious "lowering of pH" might affect fermentation, thus speeding the process. The compounds comprising *C. vulgaris* could represent an attractive media for yeast growth. It is recognized that it is rich in amino acids, fatty acids and minerals (Niccolai et al., 2019). The presence of these compounds in certain amounts could represent the right combination that possibly boosts the yeast metabolism and increase the fermentation rate. Nevertheless, that effect was not observed in the presence of

Table 1
Impact of the microalgae level and type of dough on water absorption (WA), pH, TTA and acids content in doughs and breads. Samples codes indicated the type of bread followed by a number that described the level of microalgae, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Sample	WA (g/100 g)	pH		TTA (ml NaOH/10 g)		D-Lactic acid (g/kg)		Acetic acid (g/kg)	
		Dough	Bread	Dough	Bread	Dough	Bread	Dough	Bread
W0	56.6	5.48 ± 0.00^{e}	5.82 ± 0.02^{f}	4.2 ± 0.1^{a}	0.4 ± 0.0^{a}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	1.86 ± 0.17^{a}	0.30 ± 0.07^{abcd}
W1	56.8	5.38 ± 0.01^{d}	5.77 ± 0.01^{e}	5.3 ± 0.2^{bc}	0.6 ± 0.1^{b}	0.07 ± 0.02^{a}	0.04 ± 0.00^{a}	2.06 ± 0.16^{abcd}	0.27 ± 0.03^{abc}
W2	58.5	5.39 ± 0.01^{d}	5.81 ± 0.01^{ef}	5.6 ± 0.2^{cd}	0.6 ± 0^{c}	0.03 ± 0.01^{a}	0.06 ± 0.01^{ab}	1.98 ± 0.13^{abcd}	0.26 ± 0.07^{ab}
W3	60.4	5.40 ± 0.01^{d}	5.83 ± 0.04^{f}	5.3 ± 0.9^{bc}	0.7 ± 0^{d}	0.06 ± 0.00^{a}	0.06 ± 0.02^{ab}	1.93 ± 0.19^{ab}	0.20 ± 0.06^{a}
AW0	56.6	4.88 ± 0.02^{ab}	5.38 ± 0.01^{c}	4.8 ± 0^{ab}	0.7 ± 0^{d}	0.05 ± 0.00^{a}	0.05 ± 0.01^{a}	2.30 ± 0.09^{e}	0.52 ± 0.07^{d}
AW1	56.8	4.86 ± 0.01^{a}	5.34 ± 0.01^{b}	6.3 ± 0.2^{de}	0.8 ± 0^{f}	0.06 ± 0.01^{a}	0.09 ± 0.04^{ab}	1.97 ± 0.04^{abc}	0.49 ± 0.14^{cd}
AW2	58.5	4.88 ± 0.03^{abc}	5.44 ± 0.01^{d}	6.9 ± 0.1^{e}	0.8 ± 0^{ef}	0.06 ± 0.01^{a}	0.07 ± 0.03^{ab}	2.01 ± 0.05^{abcd}	0.44 ± 0.13^{bcd}
AW3	60.4	4.88 ± 0.01^{ab}	5.45 ± 0^{d}	8.2 ± 0.0^{f}	0.9 ± 0^{g}	0.04 ± 0.00^{a}	0.12 ± 0.03^{b}	1.89 ± 0.06^{a}	0.37 ± 0.11^{abcd}
SW0	61.5	4.90 ± 0.01^{bc}	5.27 ± 0.03^{a}	4.9 ± 0.1^{ab}	0.6 ± 0^{b}	0.48 ± 0.06^{b}	0.51 ± 0.04^{c}	2.15 ± 0.04^{bcde}	0.27 ± 0.08^{abc}
SW1	63.9	4.91 ± 0.01^{c}	5.32 ± 0.02^{b}	6.3 ± 0.4^{e}	0.7 ± 0^{d}	0.49 ± 0.05^{b}	0.54 ± 0.09^{c}	2.17 ± 0.21^{cde}	0.51 ± 0.18^{d}
SW2	64.1	4.89 ± 0.01^{abc}	5.38 ± 0.01^{c}	7.0 ± 0.4^{e}	0.8 ± 0de	0.44 ± 0.06^{b}	0.56 ± 0.03^{c}	2.13 ± 0.01^{bcde}	0.30 ± 0.09^{abcd}
SW3	64.6	4.88 ± 0.01^{ab}	5.35 ± 0.01^{bc}	9.0 ± 0.4^{g}	0.7 ± 0de	0.49 ± 0.04^{b}	0.68 ± 0.03^{d}	2.20 ± 0.03^{de}	0.31 ± 0.02^{abcd}
P-value									
Type of dough		**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0000**	**0.0072**	**0.0010**
Chlorella vulgaris level		0.0562	**0.0008**	**0.0000**	**0.0000**	0.4078	**0.0008**	0.5900	0.1169

Means with different letters within the same column denote significant differences ($P \leq 0.05$) among the means, as determined by the Duncan test.

sourdough due to the LAB competition with yeast for nutrients.

As expected, the presence of the sourdough significantly ($P < 0.05$) decreased the dough pH and increased the TTA, which was similar to that of chemically acidified doughs (Table 1). Addition of the microalga decreased only the pH of the reference dough without affecting significantly that of other types of dough that already had lower pH due to the presence of the sourdough or acidification process. The TTA values in the reference dough were affected by the presence of the microalga but not by the addition level, whereas a progressive increase with the fortification level was observed for the other two types of dough.

The type of dough significantly ($P < 0.05$) affected the content of lactic and acetic acids in fermented doughs (Table 1). Sourdough containing doughs showed higher content of lactic acid than their counterparts without sourdough, whereas, chemically acidified dough exhibited the highest concentration of acetic acid (Table 1). The ANOVA statistical analysis indicated that microalga addition did not affect significantly the amount of lactic and acetic acids of the doughs. Lactic and acetic acids are the main metabolites of the sourdough processes; lactic acid bacteria being a part of the stable biota of the mature sourdoughs (Gobbetti et al., 2014). In consequence, compared to conventionally produced breads with baker's yeast, sourdough fermentation increased lactic and acetic acid values of doughs, increasing acidity and reducing pH, whereas the presence of microalga, no related to the level of incorporation, increased the acidity and reduced the pH in minor extent possibly due to the presence of short fatty acids diverse than lactic and acetic acids.

3.2. Bread technological characteristics and composition

The presence of microalga to doughs and respective breads was readily visible from the change in their color (Fig. 1), showing a greenish color that was intensified with the amount of C. *vulgaris* powder. Nevertheless, a nice crumb was obtained from all the recipes. At first glance it was observed that acidified breads and sourdough breads had lighter crumb than their reference counterparts. As it was visually detected, microalga powder significantly ($P < 0.05$) altered the color of both the crust and crumb of the breads (Table S2). The crust showed a steady reduction of the luminosity when increasing the amount of microalga, independently of the type of dough. Likewise, b^* parameter decreased with the amount of microalga in all the breads, thus reducing the yellowish tone of the crust. The total color difference (ΔE^*) confirmed the drastic color change, since values were higher than 3.0 (Arufe et al., 2018). Similarly, the luminosity of the crumb was reduced when augmenting the level of microalga powder. Conversely to the trend observed in the crust, b^*values of the crumbs increased in the presence of microalga, with a steady decrease when augmenting the level of microalga powder, although in sourdough breads, no trend was detected. Again, the total color difference (ΔE^*) of the crumb confirmed the substantial change in the color of the crumbs containing the microalga but also the very different color induced by the type of dough (AW0, SW0).

Breads prepared with sourdough had significantly ($P < 0.05$) lower pH followed by breads obtained from chemically acidified doughs. Breads containing *C. vulgaris* had significantly higher pH than the

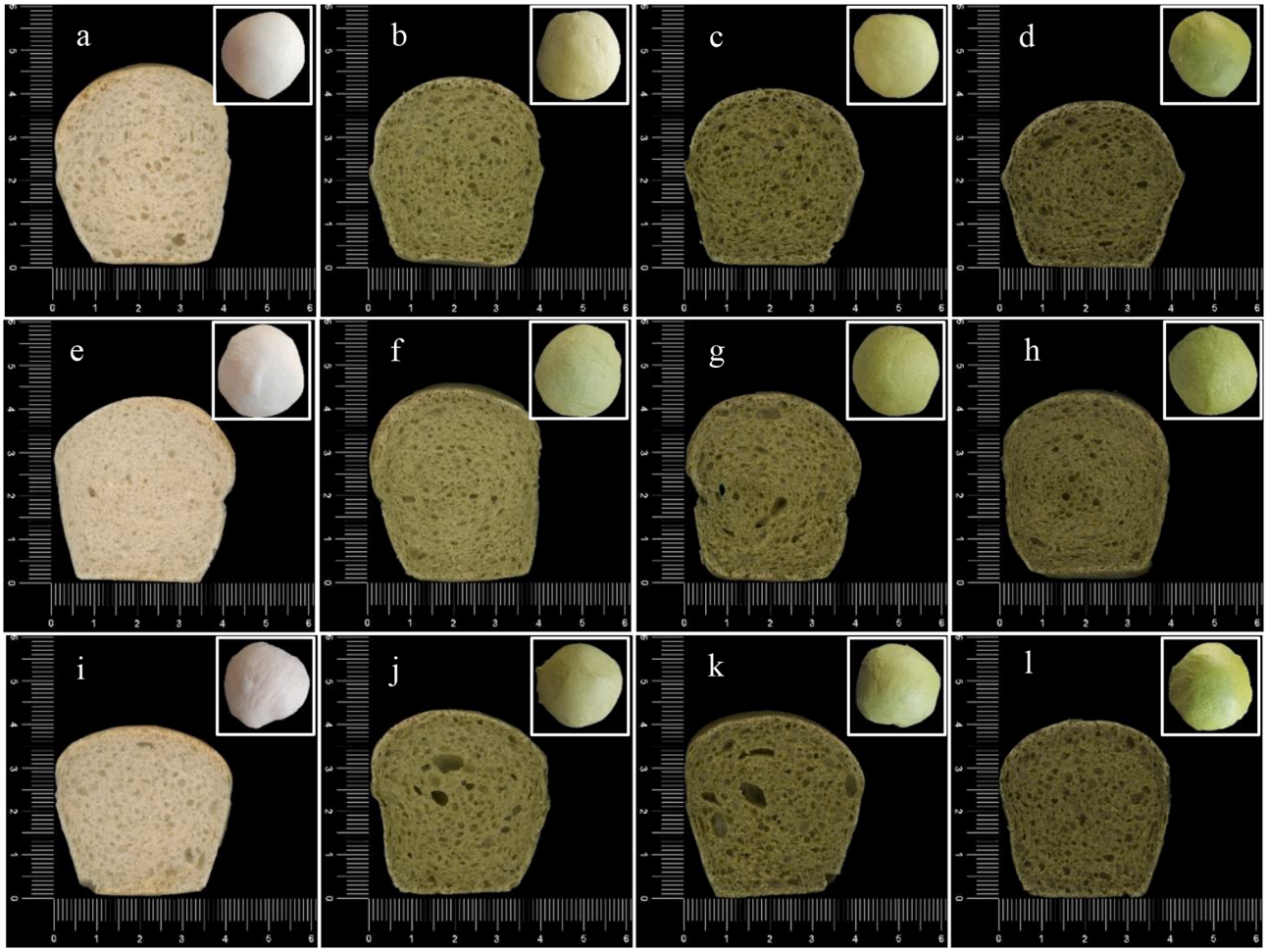

Fig. 1. Captured images of doughs and bread slices. Reference (W): a - d, Chemically acidified (AW): e - h and Sourdough (SW): i - l, with 0, 1, 2 or 3% of *C. vulgaris* powder from left to right.

respective controls, with exception of the reference recipe, in which no trend was observed when increasing the level of the microalga. In regard to concentration of organic acids in breads, D-lactic acid enhanced its concentration compared to its dough counterparts, either by type of dough and microalgae levels, with exception of the reference recipe (Table 1). Meanwhile, acetic acid was significantly lower in breads than in doughs (Table 1). In a similar way, Brandt (2019), noticed that, during baking or drying, acetic acid is volatilized, while lactic acid is more stable, due to the non-volatility of this compound.

When the bread technological characteristics were statistically analyzed considering the type of dough and the microalgae level, it was observed that the type of dough significantly affected the bread hardness and the mean cells area of the crumb, whereas the level of microalgae significantly affected the bread 2D slice area (related to bread volume) and the porosity of the crumb, as well (Table 2). The textural properties of the breads are included in Table S1. The 2D crumb area decreased when increasing microalgae level, with exception of sourdough-bread, where no significant direct trend was observed. In spite of reference and acidified doughs were fermented to the same level, the oven rise during baking seems to be affected by microalgae incorporation, leading to a reduction of the bread expansion and in consequence the bread volume. Arufe et al. (2018) described a decrease in the dough porosity during fermentation, in the presence of brown seaweeds, which was associated to the high elongational viscosity provided by the seaweeds, but that effect was more ascribed to proofing operation than baking. Similar bread volume reduction was reported by Graça et al. (2018), who described noticeable changes in the crumb structure, with bigger gas cells due to dough weakening when adding *C. vulgaris*. The same trend was observed in the present study, microalgae addition led to an increase in porosity and the size of gas cells showed a steady increase, but the effect was only significant ($P < 0.05$) at the higher microalgae level tested. Probably, the adapted fermentation time limited the bubbles coalescence during fermentation, minimizing the microalgae impact on crumb structure, and only the highest level tested impacted on the dough viscoelasticity.

Breads containing sourdough showed softer crumbs, although slight increase was observed when increasing the microalgae level. Taking into account the crumb image analysis, in general those breads displayed higher porosity and bigger gas cells that suggests a more open crumb structure due to dough bubble coalescence, likely derived from the weakening of gluten matrix due to both microalgae addition and lactic acid bacteria metabolites.

The chemical composition of the breads was significantly affected by the microalgae level, with exception of the fat content, and no significant differences were induced with the type of dough (Table 3). Despite the different water absorption of the diverse doughs, no pattern was identified among the samples. As expected, the protein as well as the ash contents were significantly increased with the microalga's addition due to the higher presence of these two components in *C. vulgaris*. This is also reported in the literature for other bakery products (Batista et al., 2019). On the other hand, addition of *Chlorella vulgaris* did not significantly affected the fat content of the resulting breads.

3.3. Variation of total phenolics and antioxidant activity among bread samples

The TPC and antioxidant activity of breads were significantly increased with increasing level of the microalgae (Fig. 2). The application of General Linear Model revealed that only microalgae levels equal or greater than 2% were able to increase significantly the TPC. Furthermore, it was found that the type of dough plays an important role in the amount of TPC. The TPC increases in the following order: reference dough < sourdough < acidified dough. It is recognized that low pH values facilitate the extraction of phenolic compounds. Thus, sourdough and acidified breads theoretically should have more extracted phenolics in the free form compared to the control dough that has higher pH among the doughs. Since in the case of acidified dough the dough matrix is exposed for longer time to lower pH than in the sourdough, it is probable that this could explain the greater TPC content of acidifies dough.

FRAP antioxidant activity was also significantly increased with increasing of the microalgae level compared to the reference (bread without *C. vulgaris*) regardless the type of dough used (Fig. 2b). It seems that type of dough used for bread preparation played also a significant role in the amount of increase in the FRAP values (reference dough ≤ sourdough ≤ acidified dough).

DPPH and ABTS activity of breads augmented with increasing of the microalgae level for all the types of dough (Fig. 2c, d), indicating higher levels of hydrophilic compounds like ascorbic acid and hydrophobic compounds like pigments, respectively. Again, the type of dough plays an important role in the increase observed. Sourdough seemed to increase ABTS values at a greater extent than acidified dough and reference dough, that showed a similar magnitude of increase. The opposite was observed when comparing the effect of dough type on the increase of DPPH values, with greater DPPH values in the reference dough followed by acidified dough and then sourdough. It was surprising the significant increase observed in DPPH values in breads made with 3% microalga and acidified dough. Likely, the combination of high amount of acetic acid and the low lactic acid content might be responsible of that response. Considering that the ABTS method has higher sensitivity towards hydrophobic compounds, results could be related with such compounds released by lactic acid bacteria, whereas DPPH with higher

Table 2
Technological characteristics of breads supplemented with different levels (0, 1, 2, 3%) of *C. vulgaris* powder. Reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3) and sourdough breads (SW0, SW1, SW2, SW3) were evaluated.

Sample	2D Slice Area (cm^2)	Hardness (N)	Mean cell area (mm^2)	Porosity (%)
W0	15.6 ± 0.4^{ef}	2.6 ± 0.2^{g}	0.61 ± 0.12^{bc}	26.96 ± 4.03^{abcd}
W1	14.4 ± 0.4^{abc}	2.3 ± 0.2^{f}	0.61 ± 0.08^{bc}	31.96 ± 3.99^{de}
W2	13.8 ± 0.1^{abc}	2.8 ± 0.2^{h}	0.60 ± 0.05^{bc}	31.41 ± 0.72^{cde}
W3	13.4 ± 0.3^{a}	2.6 ± 0.2^{gh}	0.88 ± 0.04^{ef}	36.31 ± 2.07^{ef}
AW0	16.0 ± 0.5^{f}	1.9 ± 0.1^{cd}	0.43 ± 0.05^{a}	22.46 ± 1.08^{ab}
AW1	15.4 ± 0.2^{def}	1.7 ± 0.1^{abc}	0.48 ± 0.08^{ab}	24.82 ± 2.42^{ab}
AW2	14.5 ± 0.1^{bcd}	2.1 ± 0.2^{def}	0.50 ± 0.03^{ab}	22.04 ± 2.20^{a}
AW3	13.8 ± 1.0^{abc}	2.2 ± 0.2^{ef}	0.65 ± 0.05^{c}	35.19 ± 1.11^{ef}
SW0	13.6 ± 0.0^{ab}	1.6 ± 0.3^{ab}	0.55 ± 0.01^{abc}	25.40 ± 1.99^{abc}
SW1	14.4 ± 0.7^{abc}	1.4 ± 0.2^{a}	0.69 ± 0.02^{cd}	28.58 ± 4.17^{bcd}
SW2	14.7 ± 0.1^{cde}	1.8 ± 0.1^{bc}	0.80 ± 0.09^{de}	32.03 ± 1.57^{de}
SW3	13.9 ± 0.1^{abc}	1.9 ± 0.2^{cde}	0.97 ± 0.02^{f}	38.80 ± 2.52^{f}
P-value				
Type of dough	0.0839	**0.0000**	**0.0000**	**0.0014**
Chlorella vulgaris level	**0.0202**	**0.0000**	**0.0000**	**0.0002**

*Means with different letters within a column were significantly different ($P < 0.05$).

Table 3
Chemical composition in g /100 g (as is) of breads supplemented with seaweed powder. Samples codes indicated the type of bread followed by a number that described the level of seaweed, thus reference breads (W0, W1, W2, W3), acidified breads (AW0, AW1, AW2, AW3), sourdough breads (SW0, SW1, SW2, SW3).

Type of dough	Moisture			Protein			Ash			Total Fat			Carbohydrates
W0	35.39	±	0.51^{bc}	12.17	±	0.02^{a}	0.84	±	0.05^{a}	0.24	±	0.06	51.36
W1	35.16	±	0.13^{b}	12.67	±	0.03^{c}	0.99	±	0.03^{b}	0.25	±	0.05	50.94
W2	35.41	±	0.29^{bc}	13.27	±	0.00^{e}	1.10	±	0.05^{cde}	0.22	±	0.06	50.00
W3	35.49	±	0.96^{bcd}	13.75	±	0.06^{f}	1.12	±	0.08^{def}	0.25	±	0.07	49.38
AW0	35.30	±	0.70^{b}	12.25	±	0.03^{a}	0.86	±	0.02^{a}	0.21	±	0.04	51.28
AW1	33.89	±	0.10^{a}	12.93	±	0.06^{d}	0.99	±	0.05^{bc}	0.25	±	0.02	51.94
AW2	34.79	±	0.32^{ab}	13.31	±	0.11^{e}	1.04	±	0.08^{bcd}	0.25	±	0.02	50.60
AW3	35.01	±	0.06^{b}	13.90	±	0.03^{g}	1.21	±	0.07^{f}	0.28	±	0.03	49.61
SW0	35.68	±	0.55^{bcd}	12.18	±	0.10^{a}	0.83	±	0.04^{a}	0.26	±	0.10	51.05
SW1	36.46	±	0.17^{cd}	12.50	±	0.11^{b}	0.97	±	0.05^{b}	0.26	±	0.05	49.81
SW2	35.02	±	0.70^{b}	13.22	±	0.02^{e}	1.04	±	0^{bcd}	0.27	±	0.03	50.45
SW3	36.57	±	0.06^{d}	13.66	±	0.04^{f}	1.17	±	0.02^{ef}	0.24	±	0.09	48.36
P-value													
Type of dough	0.0043			0.3802			0.6067			0.8118			0.1112
Seaweed quantity	**0.3172**			**0.0000**			**0.0000**			0.9610			**0.0106**

*Means with different letters within a column were significantly different ($P < 0.05$).

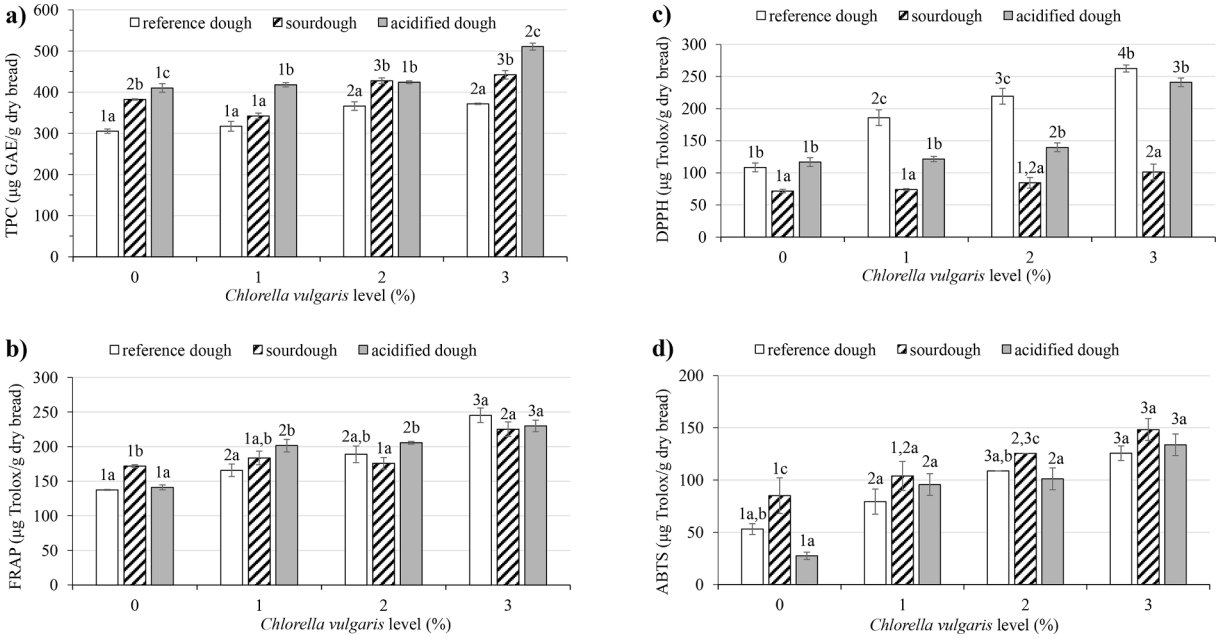

Fig. 2. Total phenolic content (TPC, expressed as µg GAE/g of bread dw, a), and antioxidant activity (ferrous reducing capacity (FRAP); b), DPPH free radical scavenging activity; c), and ABTS scavenging activity; d), expressed as µg Trolox equivalent (TE)/g of bread dw, for the different breads fortified with *C. vulgaris* at three addition levels. Different numbers above error bars within the same type of bread indicate significant differences ($P \leq 0.05$) among the means, as determined by the Duncan's multiple range test. Different letters above error bars within the same microalgae concentration indicate significant differences ($P \leq 0.05$) among the means, as determined by the Duncan's multiple range test.

sensitivity towards hydrophilic compounds were closely related to reference dough.

In the present study, the total phenolic content showed significantly moderate correlation with antioxidant activity of breads determined by FRAP ($r = 0.541$) and ABTS ($r = 0.486$) assays. Nevertheless, no correlation was observed between the antioxidant activity measured by DPPH assay and the TPC. FRAP antioxidant activity correlated very well with ABTS ($r = 0.816$) and moderately with DPPH ($r = 0.520$), while the data obtained from DPPH assay did not correlate with those gained from ABTS assay.

Phenolic compounds must be first released from the bread matrix during extraction in order to be detected for their antioxidant activity. Their chemical structure as well as the food matrix interactions (such as with carbohydrates, proteins and lipids) are factors that impede bioaccessibility and bioavailability of phenolics (Dima et al., 2020; Ribas-Agustí et al., 2018). This could explain the small increase in the amount of phenolic compounds with increasing of microalgae level as well as the different patterns among the three assays. The literature cited several possible processing conditions (such as mechanical processing, enzymatic and chemical treatments, thermal processing, pasteurization and sterilization, extrusion cooking, and bioprocessing, drying, ultrasounds, high pressure, pulsed electric fields, etc.) that affect the release of phenolics from the food matrix (Ribas-Agustí et al., 2018; Wang, He, & Chen, 2014). It is obvious that the type of fermentation plays an important role not only in the amount of phenolics but also in the antioxidant activity of the bread. Although the amount of free phenolic compounds detected in the acidified dough bread was higher than in sourdough and reference bread only FRAP assay data follow the same trend. However, with reference to DPPH and ABTS, reference dough and sourdough bread showed the highest antioxidant activity in each respective assay. According to Skendi, Irakli, and Chatzopoulou (2017), factors such as mechanisms of action of the applied assays, presence of

different compounds with different antioxidant activity, synergistic/antagonistic effects between phenolic compounds have an important effect on the overall antioxidant activity of the extracts. Thus, fermentation type affects not only the amount of bioactive compounds (Ferri et al., 2016) but also their profile since other compounds may be synthesized resulting in an alteration of the antioxidant activity (Hur et al., 2014).

3.4. Principal component analysis

A principal component analysis (PCA) was carried out with all the variables evaluated to identify possible discrimination among samples containing microalgae and the impact of the dough type (Fig. 3). Two components were able to explain 63.3% of the samples' variability. Component 1 explained 37% whereas Component 2 described 26.3% of the variation. The score plot revealed that samples were grouped based on the dough type, with clear discrimination between reference breads and those containing acidified doughs (sourdough or chemically acidified). Component 1 allowed discrimination of the breads obtained from different type of doughs, whereas Component 2 discriminated between reference breads and acidified breads (sourdough or chemically acidified). Breads containing microalgae were located in the positive axis of Component 1, with exception of AW1, being discriminated by the lactic acid content and TTA of doughs and breads, the crumb structure (porosity and gas cells size), the textural properties, breads composition and the total phenolic content and antioxidant activities. However, Component 2 allowed better discrimination of the acidified breads that were grouped in the negative axis of PC2, with exception of AW3, owing their textural properties, acids content, total phenolic content and ABTS activity. Furthermore, reference breads were grouped in the positive axis of PC2, mainly due to their higher pHs, longer fermentation, higher crumb hardness and DDPH activity. Therefore, the PCA confirmed the role of acid doughs for promoting the antioxidant activity and TPC when incorporating *Chlorella vulgaris* into breadmaking process.

4. Conclusions

Incorporation of *C. vulgaris* into wheat breads allowed obtaining breads with innovative technological characteristics but also enriched in bioactive compounds. However, its supplementation to acidified doughs permitted to enhance the antioxidant activity. The incorporation of *C. vulgaris* into bread recipes required the water absorption adjustment but no further impact on dough stability was observed. Breads enriched with *Chlorella vulgaris* up to 3% were mainly characterized by intense green hue, slightly lower slice 2D area and higher crumb hardness that was associated to the thicker gas cell walls compared to the control bread. Particularly, in microalgae enriched breads, the use of chemically acidified doughs or sourdoughs allows enhancing the TPC and antioxidant activity in the resulting breads. Nevertheless, antioxidant assays suggested differences in the profile and amount of phenolics released since the increase in the antioxidant activity was different among the prepared breads. Thus, to optimize the health benefits of *Chlorella vulgaris* in breads, it would be advisable to use acidic doughs, prepared chemically or by applying sourdough fermentation.

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CRediT authorship contribution statement

Raquel Garzon: Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **Adriana Skendi:** Investigation, Data

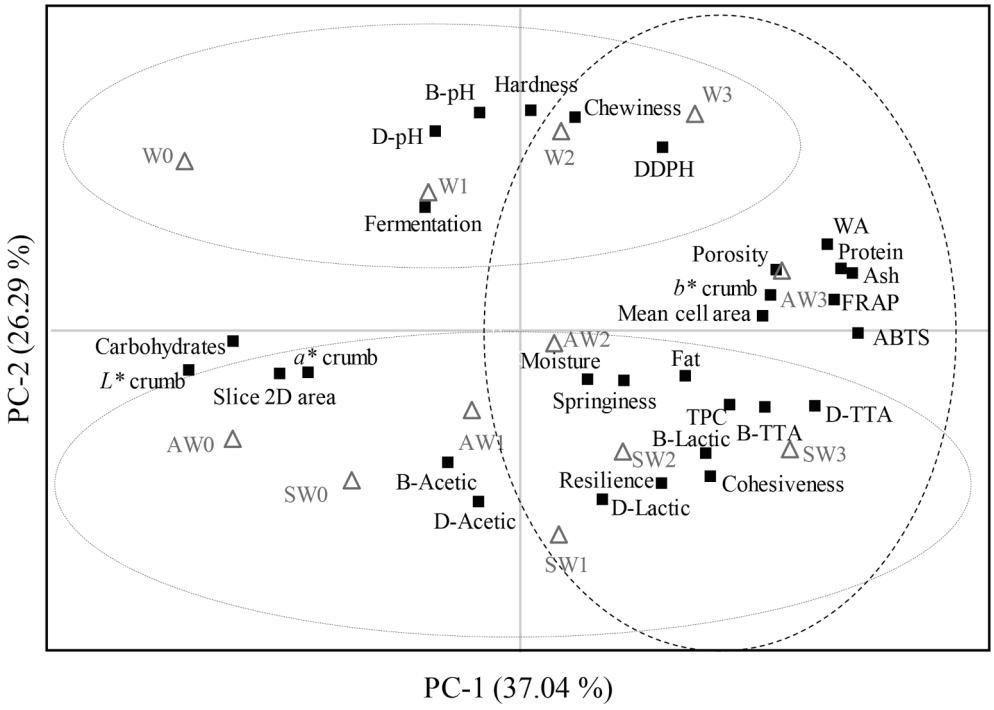

Fig. 3. Plot (PC 1 × PC 2) of the set of bread samples and evaluated variables obtained by principal component analysis (PCA). Samples are labelled as in the text. pH, TTA and acids content preceded by D or B are referred to dough or bread, respectively. Dotted lines are grouping the samples obtained from different type of doughs.

curation, Formal analysis, Writing - original draft. **Marco Antonio Lazo-Velez:** Investigation, Methodology, Data curation, Writing - review & editing. **Maria Papageorgiou:** Funding acquisition, Writing - review & editing. **Cristina M. Rosell:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128710.

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Functional and nutritional replacement of gluten in gluten-free yeast-leavened breads by using β-conglycinin concentrate extracted from soybean flour

Johanan Espinosa-Ramírez[a,b], Raquel Garzon[a], Sergio O. Serna-Saldivar[b], Cristina M. Rosell[a,*]

[a] Institute of Agrochemistry and Food Technology (IATA-CSIC), C/ Agustin Escardino, 7, Paterna 46980, Valencia, Spain
[b] Centro de Investigación y Desarrollo de Proteínas (CIDPRO) and Centro de Biotecnología FEMSA. Escuela de Ingeniería y Ciencias, Tecnológico de Monterrey, Av. Eugenio Garza Sada 2501 Sur, C.P. 64849 Monterrey, NL, Mexico

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ABSTRACT

The search of proteins that could act as both structural agents and nutritional enhancers in gluten free bread is still a challenge for the food industry. The present study evaluated the inclusion of 10% β-conglycinin concentrate (βCC) extracted from defatted soybean flour to rice flour to improve the structure and protein quality of gluten-free yeast-leavened bread. βCC breadmaking performance was compared to vital gluten functionality. Batter pasting properties and bread characterization in terms of color, texture, image analysis, microstructure a, nd protein quality were assessed. βCC and gluten led to batters with higher peak viscosity and breakdown, although only βCC was able to increase the setback. The inclusion of 10% βCC in rice flour formulations led to breads with improved color parameters and protein quality. The texture properties of βCC breads did not present significant differences compared to vital gluten breads. The crumb analysis showed that βCC led to lower cell density with highest mean cell area. The micrographs showed that βCC was able to create a net-like structure similar to the one created by gluten, confirming that βCC is capable of acting as structuring agent and protein quality improver in gluten-free formulations.

1. Introduction

The baking properties of wheat flour are determined by the unique characteristics of its proteins, namely gluten. After hydration and kneading of wheat flour, gluten proteins form a network responsible of the cohesiveness, viscosity and elasticity of the dough, making it capable of holding the gas produced during fermentation and leading to the characteristic foam structure of bread (Veraverbeke & Delcour, 2002). However, these proteins are not considered safe for patients with celiac disease, which is the most common food intolerance of the world population (Wieser & Koehler, 2008). In these patients, gluten ingestion triggers an immune mediated response that causes villous atrophy in the small intestine and subsequent malabsorption. Therefore, celiac disease is frequently associated with deficiencies of macro and micronutrients (Theethira, Dennis, & Leffler, 2014). The most efficient treatment for celiac is a strict withdrawal of gluten containing foods from the diet (Wieser & Koehler, 2008). However, gluten-free baked goods are generally produced with refined starch, leading to products with low amounts of proteins, dietary fiber and micronutrients in comparison with their gluten product counterparts (Matos Segura & Rosell, 2011; Taylor & Rosell, 2016). Hence, the nutritional deficiencies of the celiac patients cannot be mitigated with the current gluten-free products unless those are nutritionally closer to their gluten containing equivalents.

Rice flour is one of the most common ingredients in the production of gluten-free foods including bread (Deora, Deswal, & Mishra, 2014) because it has bland taste, is colorless, inexpensive, easily digested, and present hypoallergenic properties (Capriles & Areas, 2014). However, due to the differences in its storage proteins, rice flour proteins are unable to develop a network with properties similar to gluten (Marco & Rosell, 2008a). To improve the quality of the final products, several additives including hydrocolloids, enzymes, emulsifiers and proteins are supplemented in gluten-free formulations to increase the acceptability of consumers (Houben, Höchstötter, & Becker, 2012). In this backdrop, proteins have been evaluated in rice-flour formulations to overcome the problems associated to the lack of structure, mimic the characteristics that gluten provides to bread and also to enhance their nutritional value (Aprodu, Alexandra Badiu, & Banu, 2016; Marco & Rosell, 2008a; Patrascu, Banu, Vasilean, & Aprodu, 2017; Shin, Gang, & Song, 2010; Storck et al., 2013). Soybean has been one of the most

* Corresponding author.
E-mail address: crosell@iata.csic.es (C.M. Rosell).

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evaluated sources of proteins for breadmaking due to their high lysine content, improving the essential amino acids content when combined with cereals (Tsen & Hoover, 1973), and more recently due to its potential to serve as structure-forming agent in gluten-free formulations (Marco & Rosell, 2008a, 2008b; Matos, Sanz, & Rosell, 2014; Miñarro, Albanell, Aguilar, Guamis, & Capellas, 2012; Sciarini, Ribotta, León, & Pérez, 2010; Ziobro, Witczak, Juszczak, & Korus, 2013). Nevertheless, in these studies the inclusion of additives such as hydrocolloids and enzymes was necessary to obtain the desired gluten-like network structure, since hitherto, no other protein has been able to fully replace gluten functionality.

In a recent model study, a β-conglycinin concentrate (βCC) proved to be an adequate structuring agent for yeast leavened breads produced with native corn starch (Espinosa-Ramírez, Garzon, Serna-Saldivar, & Rosell, 2018). The βCC was evaluated in a lean system where no other additives were used, which resulted in breads with higher 2D area, height, softness and cohesiveness compared to vital gluten breads. These results showed the potential of the βCC to improve the rise and quality properties of other gluten-free formulations acting as structure-forming agent. Moreover, since this βCC was obtained after the fractionation of soybean proteins (Qi, Venkateshan, Mo, Zhang, & Sun, 2011), it could also contribute to improve the nutritional quality of rice flour breads.

The objective of this study was to evaluate the potential of βCC to mimic the gluten functionality in rice flour gluten-free leavened breads and to enhance the nutritive value of breads in terms of protein content and quality. Other structuring agents such as hydrocolloids or enzymes were not used during the process. Doughs and breads produced with rice flour and βCC or vital gluten were compared.

2. Material and methods

2.1. Materials

The rice flour was purchased from Harinera La Meta S.A (Lleida, Spain) (7.4% protein; 12.5% moisture). Vital gluten (77.4% protein; 8.3% moisture) was donated by Puratos (Groot-Bijgaarden, Belgium), whereas the β-conglycinin concentrate (βCC) was obtained according to the method proposed by Qi et al. (2011) with modifications reported by (Espinosa-Ramírez et al., 2018). The protein content of the lyophilized βCC containing 6.3% moisture was 87.5% (N*6.25).

Three formulations were tested for bread production: 1) 100% rice flour (negative control), 2) 90% rice flour + 10% vital gluten (positive control), and 3) 90% rice flour + 10% βCC (experimental treatment). Vital gluten and βCC were blended with rice flour in a proportion 90:10 based on the results obtained in a previous study, where the substitution of corn starch with 10% βCC improved the height and texture of gluten-free breads (Espinosa-Ramírez et al., 2018). Gluten was substituted in the same concentration for comparison purposes.

2.2. Flours characteristics

The water binding capacity (WBC) of the rice flour and two composite flours was determined according to Method 44–15.02 (AACC, 1999). This value was selected as the optimal hydration level for the breadmaking process. Results were expressed as percentage in flour basis (g water/100 g flour) in accordance to baker's formulations. Five replicates were made for each sample.

The pasting and apparent viscosity properties of the batters were determined by Rapid Visco Analyzer (RVA 4500, Perten Instruments SA, Stockholm, Sweden) following the method reported by Xing, Niu, Su, and Yang (2016). Batters were prepared according to baker's formulation with 100% flour, 1.5% salt, 1.0% sugar and the hydration selected from the WBC value for each flour. For the analysis, batters were diluted (8 g of bread batter and 12 g of water) and held for 60 s at 50 °C, then heated from 50 to 95 °C at 12 °C min^{-1} and held at 95 °C for 60 s. Thereafter, the batters were cooled to 50 °C at 12 °C min^{-1} and held at 50 °C for 2 min. The mixing speed was 600 rpm and the apparent viscosity was recorded during the heating-cooling cycle using Thermocline software for Windows (Perten Instruments SA). The onset temperature, peak viscosity at 95 °C, breakdown (difference between peak viscosity and trough viscosity), final viscosity and setback (difference between final viscosity and trough viscosity) were evaluated. The RVA determination was conducted twice for each batter.

2.3. Breadmaking process

For breadmaking, the lean baker's formulation consisted of 100% flour, 1.5% salt, 1.0% sugar and 1.0% dry yeast. The water hydration used was selected from the WBC value determined for each flour. Breadmaking was carried out following the mini-scale procedure described by Garzon, Rosell, Malvar, and Revilla (2017). Briefly, doughs were kneaded at 100 rpm for 90 s in a stirrer with a turbine accessory (IKA Eurostar 40, Staufen, Germany). After kneading, 2 g of dough were set in greased glass pans (diameter, 1.8 cm; height, 3 cm) and proofed for 40 min at 35 °C and 65% relative humidity. After fermentation, 100 μL of distilled water were added to the surface of the proofed dough to avoid dough surface dryness and then baked in an oven set at 130 °C for 10 min. Two batches were run for each formulation.

2.4. Bread characterization

Resulting breads were characterized in terms of color, texture and internal bread crumb structure. The color of the crumb was determined using a colorimeter (Chroma Meter CR-400/410, Konica Minolta, Tokyo, Japan) after white calibration ($L^* = 96.9$, $a^* = -0.04$, $b^* = 1.84$). Results were means of 4 replicates and expressed using the CIE-$L^*a^*b^*$ scale, where L^* indicates lightness, a^* redness to greenness, and b^* yellowness to blueness.

The texture parameters were determined according to Garzon et al. (2017) in a Texture Analyzer TA-XT2i (Stable Micro Systems, Surrey, UK) using a texture profile analysis (TPA) double compression test. Bread slices of 10 mm thickness were compressed to 50% of their original height at a test speed of 1 mm s^{-1} with a stainless-steel probe of 25 mm diameter. Texture was assayed in four slices of each batch. The parameters evaluated were hardness, springiness, cohesiveness, chewiness, and resilience.

The 2D area of breads and their crumb cell structure were evaluated by digital image analysis. For that, eight breads of each formulation were selected and cut with a scalpel to obtain longitudinal and cross section images captured at 1200 ppi using a flatbed scanner HP Scanjet G3110 (Hewlett-Packard, USA). A 10 × 10 mm field of view was evaluated for each image. Image were improved by Image J software (National Institutes of Health, Bethesda, MD, USA) using the Otsu's algorithm for assessing the threshold (Gonzales-Barron & Butler, 2006). Data derived from the crumb structure analysis included: cell density (u/cm^2), mean cell area (mm^2) and cell circularity.

2.5. Protein quality of breads

The protein content of breads was determined according to the official method AOAC 920.87 (AOAC, 1990). The *in vitro* protein digestibility (IVPD) of lyophilized breads was assayed by the methods of Hsu, Vavak, Satterlee, and Miller (1977) and Bilgiçli, Ibanoglu, & Herken (2007) with some modifications. Briefly, 1 mL of aqueous protein suspension containing 6.25 mg protein/mL was prepared. Samples were placed in a 37 °C water bath and the pH was adjusted to 8.00 using 0.1 N NaOH and/or 0.1 N HCl, while stirring. Trypsin at a concentration of 1.6 mg/mL was maintained in an ice bath and the pH was adjusted to 8.00 with 0.1 N NaOH and/or 0.1 N HCl. Then, 0.1 mL enzyme solution was added to the protein suspension, which was continuously stirred at 37 °C. The trypsin had an activity of 13,766 BAEE units/mg proteins.

The pH drop was recorded at 15 s after the addition of the enzymatic solution and at 1-min intervals during 10 min. The IVPD percentage was calculated using Eq. (1) (Hsu et al., 1977), where x is the change in pH after 10 min.

$$y = 210.464–18.1\ x \quad (1)$$

Amino acids analysis of the flours used for breadmaking was performed using the official method AOAC 982.30 (AOAC, 1990) coupled to an HPLC system. Protein digestibility corrected amino acid scores (PDCAAS) were calculated according to WHO/FAO/ONU (2007) using the formulas:

$$\textit{Limiting amino acid score} = \frac{\textit{Limiting essential amino acid content of test protein}}{\textit{Amino acid requirement pattern}} \quad (2)$$

$$\text{PDCAAS} = (\%\ \text{protein digestibility} * \text{amino acid score})/100 \quad (3)$$

2.6. Scanning electron microscopy

For scanning electron microscopy (SEM), fermented dough and bread samples were frozen in liquid nitrogen immediately after production and freeze-dried. Doughs and breads were fractured and placed on a metal stub. Then samples were sputter-coated with gold and palladium by ion sputter (Bio-Rad SC-500, Aname, Madrid, Spain). Sample images were recorded with a Hitachi S-4800 (Ibaraki, Japan) scanning electron microscope and observed at 10 kV accelerating voltage with magnification level of 700x.

2.7. Statistical analysis

Analysis of variance (ANOVA) was used for the statistical analysis of the results. Means were compared using Tukey's test. Analyses were performed using Minitab 17 statistical software and 95% confidence.

3. Results and discussion

3.1. Flours and batters' characteristics

Due to the important role of water in the quality of gluten-free bread products (Aprodu et al., 2016), the water binding capacity (WBC) of rice flour and blends was determined to select the optimum water absorption for the breadmaking process. The formulation containing 10% βCC presented the lowest WBC (Table 1). According to a previous work, the WBC of βCC was significantly lower compared to vital gluten (0.98 g/g vs 1.45 g/g) (Espinosa-Ramírez et al., 2018). Therefore, the lower WBC of the blends containing βCC was attributed to the lower quantity of water bound by the βCC in comparison to rice flour and vital gluten. Crockett, Ie, and Vodovotz (2011) added higher amounts of water in breadmaking formulations of rice flour supplemented with a soybean protein isolate. The WBC of a soybean protein isolate could be up to 5 times higher (Paredes-Lopez, Ordorica-Falomir, & Olivares-Vazquez, 1991) when compared to the βCC used herein. Differences in the WBC according to the ingredients used in the formulation, highlight the importance of its assessment for each breadmaking system.

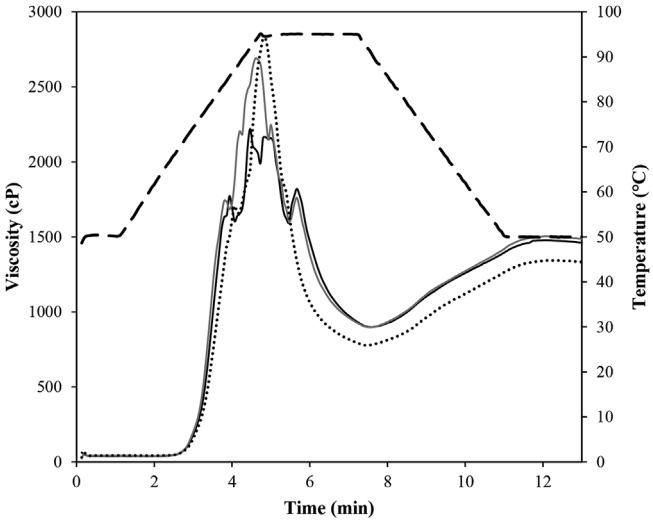

Fig. 1. Viscosity profile determined by Rapid Visco Analyser of batters produced with 100% rice flour (black line); 90% rice flour + 10% gluten (dotted line); and 90% rice flour + 10% β-conglycinin concentrate (gray line). Dashed line represents the temperature profile. Values are means of two replicates.

The RVA parameters and curves were obtained to evaluate the effect of the replacement of rice flour with proteins on the pasting properties of batters (Table 1; Fig. 1). The onset pasting temperature did not show significant differences among flours ranging from 66.4 °C to 68.1 °C. The onset pasting temperature was governed by the starch type and coincided with the pasting temperature of rice flour (Garzon et al., 2017; Heo, Jeon, & Lee, 2014). Peak viscosity was higher for batters containing 10% proteins. When the final viscosity was evaluated, the batter composed of rice flour and gluten showed the lowest value. Other authors found that the rice flour replacement with protein isolates (5%) resulted in lower peak and final viscosity (Marco & Rosell, 2008b; Storck et al., 2013). These authors related the lower final viscosity of protein-containing batters with the lower amount of gelatinized starch resulting from the partial replacement of the rice flour. Nevertheless, present results indicated that batters' performance is different than when only rice flour was tested. Conversely, batters containing 10% βCC presented higher values for final viscosity compared to those for gluten. Marco and Rosell (2008b) observed higher final viscosities when rice flour was supplemented with whey and egg proteins when compared to batters produced with other sources of protein, stating the

Table 1
Effect of gluten and βCC (10% flour replacement) on the rice flour hydration and batter properties.[a]

Parameter	Formulation											
	100% Rice flour				90% Rice flour + 10% Gluten				90% Rice flour + 10% βCC			
WBC (g water/100 g flour)	134	±	2	**a**	130	±	2	**a**	121	±	5	**b**
Pasting properties												
Onset pasting temperature (°C)	67.3	±	0.7	**a**	68.1	±	0.5	**a**	66.4	±	0.5	**a**
Peak viscosity (mPa)	2221	±	16	**c**	2839	±	175	**a**	2554	±	81	**b**
Breakdown (mPa)	1323	±	1	**b**	2062	±	185	**a**	1936	±	89	**a**
Final viscosity (mPa)	1462	±	7	**b**	1333	±	4	**c**	1747	±	11	**a**
Setback (mPa)	564	±	8	**b**	556	±	14	**b**	849	±	1	**a**

[a] Values are means and standard deviation of at least two replicates; means with different letters within the same parameter differ significantly ($P < 0.05$). WBC = Water binding capacity; βCC = β-conglycinin concentrate; mPa = millipascal.

different behavior of the proteins depending their nature. These authors associated this behavior to the heat-induced gelation capacity of whey and egg proteins. Soy proteins also form heat-induced gel structures, which get stiffer after cooling (Bainy, Tosh, Corredig, Woodrow, & Poysa, 2008), that could explain the high viscosity of βCC batters despite the replacement of rice starch. When breakdown was compared, higher values were observed for batters containing added proteins, thus batters' stability during cooking was lowered with the proteins. Conversely, the setback values were only significantly modified when βCC was present in the batter, likely, this protein favored the amylose retrogradation.

The RVA curves (Fig. 1) for the batters showed plots similar to native rice flour pasting pattern. This pattern consists in an increment in viscosity to reach a peak viscosity during heating and breakdown on holding at 95 °C followed by setback during cooling (Heo et al., 2014). However, even when the same trend was obtained for all batters, irregular lines and multiple peaks before and after the peak viscosity were observed especially in RVA curves from rice flour (Fig. 1). These irregularities were attributed to destabilization of the batter during the heating stage. When proteins were blended with rice flour, the shape of the RVA curves became more regular, especially in gluten batters. The vanishing of irregular peaks in RVA curves of batters that contained gluten and βCC could be associated to a higher water absorption of the proteins in comparison to the rice flour components leading to the thickening of the suspension and thus to less destabilization. The higher stability in the RVA curves of βCC and gluten batters could also be associated to the formation of network structures during heating among proteins and rice flour components. Matos et al. (2014) associated the changes in the rheological behavior during heating of gluten-free batters supplemented with proteins, to the formation of a more rigid protein network promoted by the protein denaturation compared to the rice-based control. On the other hand, the small irregular peaks in batters containing βCC could be related to the dissociation of proteins due to the heating treatment causing protein aggregation (Bainy, Corredig, Poysa, Woodrow, & Tosh, 2010; Matos et al., 2014). The aggregation of soybean proteins at temperatures lower than 95 °C where the peak viscosity of the rice flour is obtained has been reported before (Bainy et al., 2010; Marco & Rosell, 2008a).

3.2. Bread characterization

The effect of the replacement of rice flour with 10% proteins in the quality of breads was evaluated. The crumb color, textural parameters, 2D area and cell structure features are summarized in Table 2. The color of the crumb was affected by the addition of proteins. Breads that contained βCC or wheat gluten presented lower lightness and higher yellowness compared to rice flour counterparts. The differences in breads color were associated to the color of the proteins powders. Gluten ($L^* = 85.9 \pm 0.3$, $a^* = -0.4 \pm 0.0$, $b^* = 13.8 \pm 0.4$) and βCC ($L^* = 88.3 \pm 0.1$, $a^* = -1.2 \pm 0.0$, $b^* = 14.1 \pm 0.6$) used herein had lower L^* and higher b^* values compared to the values for rice flour ($L^* = 92.8 \pm 0.1$, $a^* = -1.0 \pm 0.0$, $b^* = 5.4 \pm 0.1$). Previous reports have shown the same behavior in the color of breads produced with rice flour and supplemented with proteins and associated these changes to the color of raw materials (Marco & Rosell, 2008a; Shin et al., 2010). Ziobro, Juszczak, Witczak, and Korus (2016) also found that the addition of proteins modified the color of gluten-free breads by increasing the darkening and yellowness of crumbs. Moreover, according to these authors, the color changes were advantageous resulting in higher organoleptic acceptability by consumers. The a^* parameter presented values ranging from −1.53 to −1.11. These values were similar to those reported for rice flour breads (Garzon et al., 2017). The differences in the color of the raw materials compared to breads were associated to the baking process which enhanced non-enzymatic browning reactions, favored by the supplemented proteins (Marco & Rosell, 2008a).

Hardness and chewiness were increased due to the addition of gluten and βCC proteins (Table 2). Previous reports also found an increment in the crumb hardness caused by the addition of proteins to rice flour formulations (Marco & Rosell, 2008a; Storck et al., 2013). Although no significant differences were detected, breads containing βCC presented the highest hardness values. This could be associated to the low water binding capacity of the rice flour/βCC bread formulation (Table 1). Aprodu et al. (2016) found a negative relationship between the water absorption and the hardness of the crumb of gluten-free breads. Although, it must be stressed that the increment in the crumb hardness of breads that contained gluten or βCC could be also attributed to the formation of more rigid structures among the supplemented proteins and rice flour components during the thermal treatment of baking.

Interestingly, bread springiness, which is positively related to crumb elasticity, increased in breads that contained gluten or βCC (Table 2). Cohesiveness and resilience also increased due to the protein addition, but compared to rice flour breads, the increment was only significant when the supplemented protein was wheat gluten. High values of cohesiveness, springiness and resilience are desirable since they represent

Table 2
Effect of the substitution of gluten and βCC in the color, texture, 2D area and crumb structure features of yeast-leavened rice flour breads.[a]

Parameter	Formulation											
	100% Rice flour				90% Rice flour +10% Gluten				90% Rice flour +10% βCC			
Color												
L^*	79.2	±	1.2	**a**	72.5	±	2.1	**b**	72.9	±	1.7	**b**
a^*	−1.37	±	0.03	**ab**	−1.11	±	0.29	**a**	−1.53	±	0.18	**b**
b^*	7.8	±	0.5	**c**	13.7	±	0.8	**a**	10.1	±	1.2	**b**
Texture												
Hardness (g)	96.0	±	5.0	**b**	126.8	±	22.0	**a**	138.2	±	15.7	**a**
Springiness	0.95	±	0.06	**b**	1.00	±	0.00	**a**	1.00	±	0.01	**a**
Cohesiveness	0.68	±	0.04	**b**	0.75	±	0.06	**a**	0.71	±	0.03	**ab**
Chewiness (g)	61.6	±	6.4	**b**	94.0	±	12.8	**a**	98.3	±	8.3	**a**
Resilience	0.34	±	0.04	**b**	0.39	±	0.03	**a**	0.37	±	0.02	**ab**
2D Area (cm^2)	2.98	±	0.23	**b**	2.88	±	0.21	**b**	3.37	±	0.26	**a**
Crumb cell structure												
Cell density (u/cm^2)	13	±	2	**a**	12	±	1	**ab**	11	±	2	**b**
Mean cell area (mm^2)	1.99	±	0.92	**b**	2.31	±	0.44	**b**	3.12	±	0.64	**a**
Cell circularity	0.37	±	0.04	**a**	0.36	±	0.03	**a**	0.39	±	0.04	**a**

[a] Values are means and standard deviations of at least three replicates; means with different letters within the same parameter differ significantly ($P < 0.05$). βCC = β-conglycinin concentrate.

lower disaggregation during mastication and adequate crumb elasticity (Cornejo & Rosell, 2015; De La Hera, Rosell, & Gomez, 2014). It must be remarked that no significant differences were found in these texture parameters when breads containing βCC were compared to those containing vital gluten. Moreover, βCC values were higher than those obtained for gluten-free formulations that used other non-gluten proteins (Krupa-Kozak, Baczek, & Rosell, 2013; Marco & Rosell, 2008a; Shin et al., 2010; Storck et al., 2013) and most of the commercial gluten-free breads analyzed by Matos and Rosell (2012).

The 2D area and crumb cell features were evaluated since they are related to the CO_2 binding ability during both proofing and baking. Breads produced with 10% βCC had the highest 2D area (Table 2). This is consistent with a previous report where βCC and corn starch were blended to produce model breads which showed higher 2D area in comparison to gluten-corn starch (Espinosa-Ramírez et al., 2018). Authors attributed this significant improvement to higher retention of carbon dioxide by βCC proteins during proofing. The cell density, which represents the number of cells in one cm^2, presented lower values when proteins were blended with rice flour, being significant in βCC breads (Table 2). Likewise, βCC breads presented the highest mean cell areas among formulations. Ziobro et al. (2016) also found that the addition of non-gluten proteins to gluten-free bread formulations resulted in crumbs with lower cell density and in most cases, higher cell or loci sizes. Marco and Rosell (2008a) found that the formation of networks during heating in gluten-free formulations was favored by the addition of proteins and hydrocolloids. This network gave strength to the expanding cells of the dough and led to an improved crumb structure with less number and larger size of gas cells (Marco & Rosell, 2008a). In the present study no hydrocolloids were used. Therefore, the results support the assumption that βCC forms a strong network able to retain the gas released during proofing. The cells shape was similar for all breads, as the cell circularity did not show significant differences among formulations (Table 2).

3.3. Protein quality of breads

The protein content, amino acid profile, PDCAAS and IVPD of breads are depicted in Table 3 and Fig. 2. Breads produced with composite flours containing βCC or gluten had almost twice the protein content of the rice flour breads. As expected, breads supplemented with βCC contained the highest protein content because the βCC contained higher protein purity compared to the vital wheat gluten. Matos Segura and Rosell (2011) analyzed eleven commercial gluten-free breads and found a protein content ranging from 0.9 to 15.1 g/100 g (dry basis, db) with a mean value of 4.8 g/100 g. The protein content reported for white wheat breads was around 13 g/100 g (db) (Villarino et al., 2015). Therefore, the bread produced with a composite flour containing 10% βCC and rice flour contained higher protein content compared to commercial gluten-free breads and wheat breads.

The amino acid profiles of breads are presented in Table 3. Breads

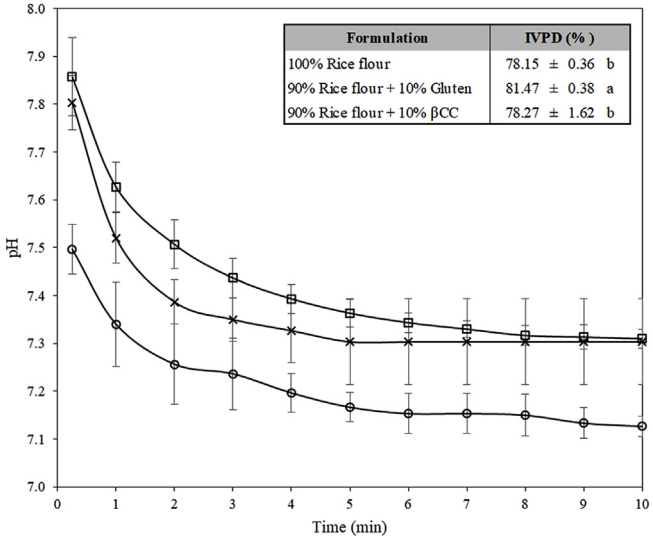

Fig. 2. *In vitro* protein digestibility (IVPD) and pH drop in yeast leavened breads produced with 100% rice flour (□); 90% rice flour + 10% gluten (◯); and 90% rice flour + 10% β-conglycinin concentrate (×).

Table 3
Effect of the substitution of gluten and βCC in the protein quality characteristics of rice flour yeast-leavened breads.[a]

Parameter	Formulation			WHO/FAO/ONU suggested requirement for essential amino acids[c]
	100% Rice flour	90% Rice flour + 10% Gluten	90% Rice flour + 10% βCC	
Protein (%, db)[b]	7.81 ± 0.15 c	15.31 ± 0.11 b	16.10 ± 0.15 a	
Amino acid content (g/100 g protein)				
Alanine	5.78	4.45	4.66	
Arginine	8.06	5.70	8.40	
Aspartic Acid + Asparagine	9.14	6.01	10.78	
Glutamic Acid + Glutamine	17.88	26.83	20.36	
Glycine	4.70	3.97	3.79	
Histidine	2.42	2.26	2.42	1.8
Isoleucine	4.44	4.15	4.77	3.1
Leucine	8.60	7.73	8.24	6.3
Lysine	4.30	2.99	5.91	5.2
Methionine + Cysteine	2.82 + 2.42	2.15 + 2.27	1.72 + 1.52	2.6
Phenylalanine + Tyrosine	5.65 + 3.23	5.51 + 3.41	5.85 + 3.31	4.6
Proline	4.30	8.57	4.74	
Serine	4.84	4.25	4.36	
Threonine	3.63	3.05	2.96	2.7
Tryptophan	1.08	1.06	0.85	0.74
Valine	6.32	5.19	5.10	4.2
Limiting amino acid score	0.83 (lys)	0.57 (lys)	1.14[d]	
PDCAAS	0.65	0.47	0.78	

[a] βCC = β-conglycinin concentrate; PDCAAS = Protein digestibility corrected amino acid score.
[b] Values are means and standard deviations of three replicates; means with different letters within the same parameter differ significantly ($P < 0.05$).
[c] Suggested requirements for 1–2 years infants. Values are expressed in g/100 g protein.
[d] Value was truncated to 1.0 for the calculation of PDCAAS as recommended by WHO/FAO/UNU, 2007.

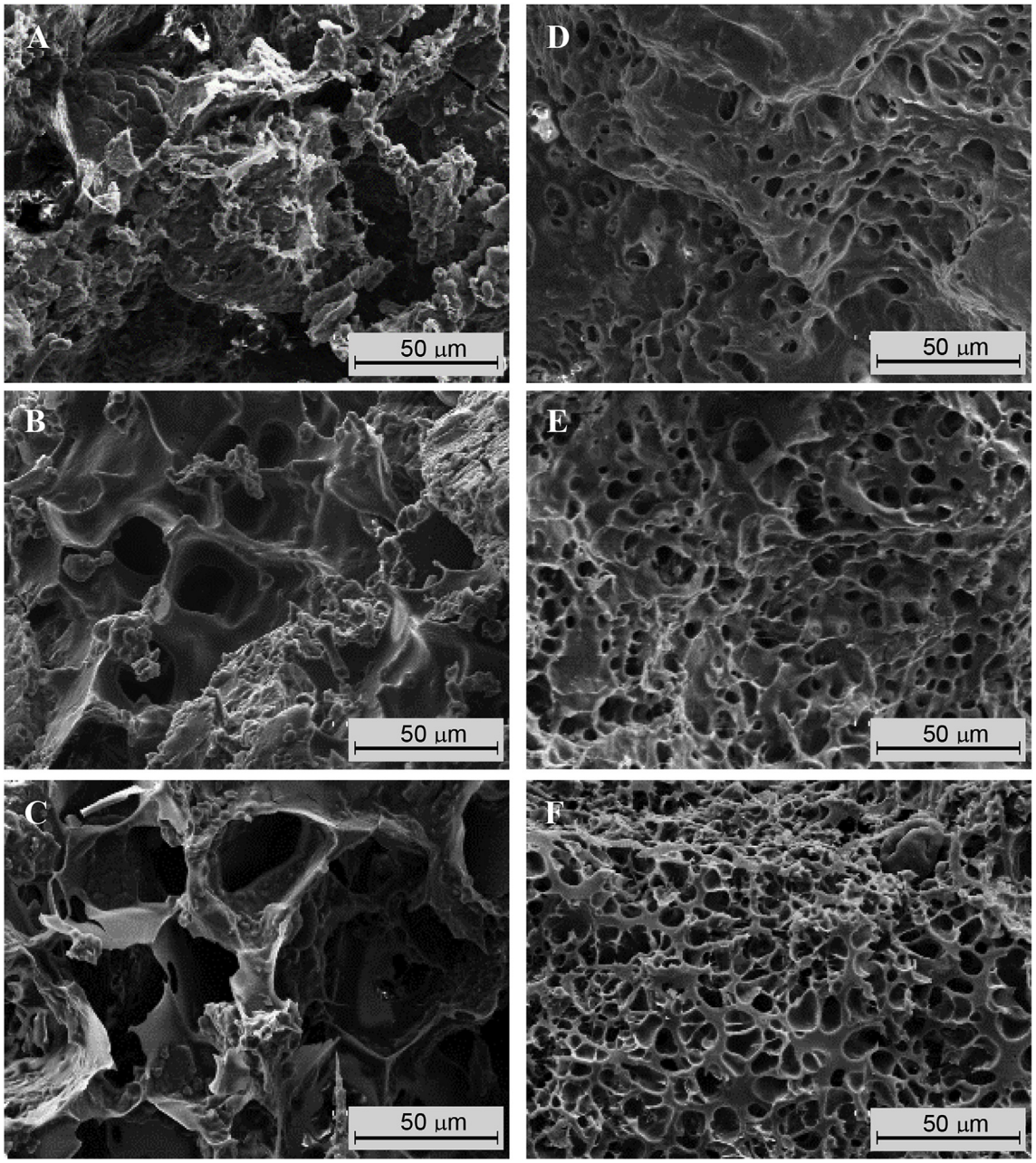

Fig. 3. Scanning electron microscope images of the fermented doughs (A–C) and yeast-leavened breads (D–F) of formulations with 100% rice flour (A and D), 90% rice flour + 10% gluten (B and E), and 90% rice flour + 10% β-conglycinin concentrate (C and F) at 700× magnification.

containing gluten presented up to 99% and 50% more proline and glutamic acid + glutamine, respectively, compared to the other bread formulations. In contrast, gluten breads had the lower concentrations of the amino acids: aspartic acid + asparagine, arginine and lysine, among breads. This is consistent with the amino acid profile of gluten proteins, which is characterized by high levels of proline and glutamine and low contents of charged amino acids (Wieser, 2007). The unique amino acid composition and its sequence determine the type of inter- and intra-chain interactions which promote the molecular weight distribution of gluten fractions, especially its polymeric nature, which is recognized as a determinant factor of dough properties and baking performance (Veraverbeke & Delcour, 2002). On the other hand, the amino acids that had a similar content in both gluten and βCC breads were alanine, glycine, threonine and valine. In contrast, 100% rice flour breads presented higher concentrations of these amino acids. The non-covalent interactions formed by the agglomeration of hydrophobic amino acids or electrostatic interactions and hydrogen bonds are necessary to stabilize the gluten network (Ortolan & Steel, 2017). The amino acids that were similar among gluten and βCC formulations but were different to rice breads, could be related to important non-covalent interactions that lead to net-like structures. In this backdrop, the concentration of these amino acids would be essential to favor those net-forming interactions. Breads containing 10% βCC had the lowest content of sulfur amino acids among breads (Table 3) since soybean proteins have low concentrations of these sulfur-containing amino acids (Karr-Lilienthal, Grieshop, Spears, & Fahey, 2005). Disulfide bonds formed by the crosslinking of cysteine residues are the most important structural element of the gluten network (Ortolan & Steel, 2017). However, the similar content of cysteine in breads produced with 100% rice flour or substituted with 10% gluten, proves that the presence of this amino acid is not enough to favor these interactions.

To evaluate the protein quality, the limiting amino acid was

determined based on the FAO/WHO/ONU requirement of a two-year infant (Table 3). In most instances, lysine is the limiting amino acid in wheat bread and other cereal-based foods (Villarino et al., 2015). However, when rice flour was blended with βCC, the lysine content increased 37% in breads. This led to an increment from 0.83 to 1.14 in the amino acid score of lysine. Lysine was the essential amino acid present in the lowest concentration compared to the requirement for infants. Therefore the limiting amino acid score was calculated for lysine, being rice flour and rice flour +10% gluten the breads, which did not fulfill the WHO/FAO/ONU (2007) requirements for 1–2 years infants (which covers the range appropriate for human adults) (Table 3). Even when the replacement of rice flour with 10% βCC led to lower levels of cysteine + methionine, the suggested requirement for sulfur amino acids (WHO/FAO/UNU, 2007) was still fulfilled by this composite bread. This proved that the selected level of substitution of rice flour with 10% βCC led to the improvement of the amino acid profile of gluten-free breads based on rice flour.

The digestibility of proteins is an indicative of their amino acids availability (Hsu et al., 1977). The IVPD of breads containing 10% gluten was the highest among bread formulations (Fig. 2). Villarino et al. (2015) found a lower IVDP value for wheat bread (78.0%) compared to that obtained herein for breads containing wheat gluten (Fig. 2). The higher IVDP could be associated to a lower interaction between gluten proteins and rice starch in comparison to those occurring with wheat starch, which led to a higher accessibility of these proteins to hydrolysis. On the other hand, the addition of 10% βCC did not induce significant differences in the IVPD values compared to 100% rice flour breads (Fig. 2). However, βCC proteins seemed to be more rapidly accessible to proteases since the drop of pH, which is related to the release of the amino acids carboxyl groups (Hsu et al., 1977), was faster for βCC breads in comparison to rice flour proteins (Fig. 2). The IVDP values for βCC gluten-free breads were comparable to those obtained for wheat bread (Villarino et al., 2015) and rice flour gluten-free cakes supplemented with legumes (Gularte, Gómez, & Rosell, 2012).

The PDCAAS is a quality index for food protein sources that considers their digestibility and limiting amino acid score (WHO/FAO/UNU, 2007). In the present study, the scores for lysine were used for the PDCAAS calculation (Table 3). Breads containing βCC presented the highest PDCAAS value, which was 20% higher compared to the 100% rice flour breads. The improvement in the PDCAAS was associated to the complementary effect of the rice proteins and the βCC rich in lysine as well as its adequate IVPD. Moreover, PDCAAS of βCC breads was 3.4 times higher than that reported by Villarino et al. (2015) for wheat bread. This proved that the substitution with 10% βCC led to the improvement of the protein quality of rice flour gluten-free formulations.

3.4. Microstructure of doughs and breads

Scanning electron microscopy was used to evaluate the effect of the addition of βCC on the microstructure of fermented doughs and breads (Fig. 3). A reduction of pore geometry was observed from dough to bread microstructure, shifting to smaller closing size, as previously reported by Bajd and Serša (2011), when following the pore size dependency with proofing and baking of doughs and breads obtained from different mixed flours. Micrograph of the 100% rice flour fermented dough showed an amorphous structure where starch granules were held together by the proteins but no gas cells were observed (Fig. 3A). On the other hand, the structure of fermented doughs containing gluten or βCC was significantly different. These doughs presented a continuous protein network, where gas cells were trapped covered by a veil of rice starch granules (Fig. 3B–C). Compared to gluten, a higher size of gas cells in βCC doughs and also more uniform distribution of the starch granules over the entire protein network was observed.

In bread micrographs, a continuous structure resulted from gelatinized starch and denatured proteins in which air cells were visible (Fig. 3D–F). Therefore, the use of the water binding capacity as an indicator of the water required for forming the gluten free structure allowed an adequate interaction of the components after heating However, several differences were noticed in the microstructure depending on the proteins added. For 100% rice flour breads continuous zones with no air cells were observed, and wherever pores, those were smaller (Fig. 3D), in comparison to the formulations with proteins. Conversely, βCC and gluten breads presented the same net-like structure, but βCC displayed higher number of larger pores and thinner lamellae. Previous reports highlighted the importance of the addition of proteins to gluten-free formulations to form web-like structures and stabilize gas cells (Ahlborn, Pike, Hendrix, Hess, & Huber, 2005; Marco & Rosell, 2008a). However, these authors used several additives including hydrocolloids and crosslinking enzymes to obtain improved aerated structures similar to those produced in wheat bread. The breads produced herein had lean formulations and no hydrocolloids/enzymes were used. Notwithstanding, βCC breads produced a web-like structure similar to those produced with gluten-rice flour and to wheat bread (Ahlborn et al., 2005).

The development of the net-like structures observed in SEM images after breadmaking could be the reason of the increased hardness and chewiness observed in breads containing wheat vital gluten or βCC, but also of their improved values of springiness, cohesiveness and resilience (Table 2). Moreover, these results confirmed the high capacity of βCC proteins to give the strength needed to hold the expanding CO_2 during fermentation and baking, which led to the observed higher 2D bread crumb area and mean cell areas (Table 2). Therefore, βCC serves as structuring agent and has the potential to form net-like structures similar to gluten during breadmaking.

4. Conclusion

Rice-based yeast-leavened breads with adequate features (volume and texture) were produced from batters using the water binding capacity of the flours as the parameter to set the optimum amount of water. βCC (10%) when incorporated to rice flour for the production of gluten-free bread provided the required network for holding the carbon dioxide during proofing and also for allowing expansion during baking. In fact, SEM micrographs reveal that βCC brought about a net matrix similar to the one obtained in the presence of wheat gluten, and without the addition of any hydrocolloid. Breads containing 10% βCC showed improved quality in terms of color, texture, 2D area, crumb structure and protein quality in comparison to the bread counterpart elaborated with 100% rice flour. It is noteworthy to remark that a concentration of 10% βCC allowed to reach a complete balance of essential amino acids in rice flour formulations. Overall, the use of this ingredient is useful to fulfill both technological and nutritional challenges in gluten-free breads.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2018.06.021.

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Understanding the effect of emulsifiers on bread aeration during breadmaking

Raquel Garzón,[a] Isabel Hernando,[b] Empar Llorca[b] and Cristina M Rosell[a*]

Abstract

BACKGROUND: Much research has been done to explain the action of emulsifiers during breadmaking, but there is still plenty unknown to elucidate their functionality despite their diverse chemical structure. The aim of the present study was to provide some light on the role of emulsifiers on air incorporation into the dough and gas bubbles progress during baking and their relationship with bread features. Emulsifiers like diacetyl tartaric acid ester of monoglycerides (DATEM), sodium stearoyl lactylate (SSL), distilled monoglyceride (DMG-45 and DMG-75), lecithin and polyglycerol esters of fatty acids (PGEF) were tested in very hydrated doughs.

RESULTS: Emulsifiers increase the maximum dough volume during proofing. Emulsifiers increase the number of bubbles incorporated during mixing, observing higher number of bubbles, particularly with PGEF. Major changes in dough occurred at 70 K when bubble size augmented, becoming more heterogeneous. DMG-75 produced the biggest bubbles. As a consequence, emulsifiers tend to increase the number of gas cells with lower size in the bread crumb, but led to greater crumb firmness, which suggested different interactions between emulsifiers and gluten, affecting protein polymerization during baking.

CONCLUSION: The progress of the bubbles during baking allowed the differentiation of emulsifiers, which could explain their performance in breadmaking.

Keywords: emulsifier; image analysis; bubble; dough aeration; bread; crumb

INTRODUCTION

Bakery products are extensively consumed worldwide due to their nutritional and physical characteristics.[1] Among the diversity of bakery products obtained from either different raw ingredients or making processes, the most appreciated products are the sponge baked wheat bread, with low density and soft crumb. In the course of flour and water mixing, gluten formation and aeration brought about during kneading is responsible for the subsequent cellular structure of the baked bread.[2] Air incorporated into the dough during mixing must be kept through the breadmaking process to attain low density breads. Bread contains about 70% gas that comes from the initial aeration and fermentation, both are important stages to take into account during the making process.[3] Therefore, air bubble incorporation during mixing has been the focus of many studies that stated the influence of mixer type and mixing time,[4,5] besides the important role of ingredients.[6,7] Certainly, the progress of those initial nuclei bubbles throughout fermentation when carbon dioxide is generated[8] and final expansion of the gases occluded into the bubbles during baking determines the diversity of cellular structures encountered on bread crumbs.[3] Bubbles are very fragile and whatever changes their number and size will have a direct impact on the internal crumb structure.[9]

Nowadays, large-scale production and consumers demand higher quality, homogeneity and longer shelf life that have been achieved with the use of processing aids such as enzymes, hydrocolloids, emulsifiers, etc. to adjust dough properties. These additives are essentials for improving dough properties and final quality of the fresh product.[10] Specifically, emulsifiers are active surfactant composites used in breadmaking for their ability to stabilize dough, a thermodynamically unstable system, through their interactions with gluten proteins.[11] During mixing, the use of emulsifiers increases the strength and the extensibility of the dough; in the fermentation stage they improve gas retention and avoid dough collapse,[11,12] leading to softer bread crumbs,[13] although their effect is greatly dependent on the wheat flour protein content[14] and proofing duration.[15] Studies have confirmed the effect of different emulsifiers on the breadmaking processes, specifically on improving the internal structure of bread.[16] In spite of the knowledge acquired on the action of emulsifiers during breadmaking, further research is still required as there is much unknown about their functionality despite their chemistry diversity. For instance, despite the impact of dough aeration into bread crumb features, there is no information about the role of emulsifiers on dough aeration and the number of bubbles and size along the process. To understand the role of emulsifiers on determining the cellular structure of bread crumb, the main objective of this study was to assess the amount of gas occluded into the dough

* *Correspondence to: CM Rosell, C/Agustin Escardino, 7. Paterna-46980 (Valencia), Spain. E-mail: crosell@iata.csic.es*

a *Department Food Science, Institute of Agrochemistry and Food Technology (IATA-CSIC), Paterna, Spain*

b *Food Microstructure and Chemistry Research Group, Department of Food Technology, Universitat Politècnica de València, Valencia, Spain*

and bread during breadmaking and how several emulsifiers with diverse chemical structure affect the bubble size distribution.

MATERIALS AND METHODS

Breadmaking wheat flour was supplied by Harinera La Meta (Lleida, Spain) and compressed yeast by (DHW Europe, Dessau, Germany). The selected emulsifiers included: diacetyl tartaric acid ester of monoglycerides (DATEM), sodium stearoyl lactylate (SSL) and distilled monoglyceride (with potassium citrate added) with two different particle sizes 45 µm (DMG-45) and 75 µm (DMG-75), which were provided by Danisco (Grindsted, Denmark), defatted hydrolyzed sunflower lecithin (Tricalcium phosphate) from Lasenor (Barcelona, Spain), and polyglycerol esters of fatty acids containing polysorbate 80 (PGEF) from Palsgaard (Juelsminde, Denmark).

Gas bubbles during fermentation and baking

A very hydrated dough recipe containing wheat flour, water (900 mL kg^{-1} based on wheat flour weight) and 10 g kg^{-1} of compressed yeast was used. Emulsifiers were added at 5 g kg^{-1} (flour basis) whenever tested. Ingredients were mixed for 3 min at 328 rpm in a mixer (RZR-1 Heidolph, Schwabach, Germany).

The gas released and dough development characteristics during fermentation were recorded using the Rheofermentometer F3 (Chopin, France), slightly modifying the instructions given by the supplier. Briefly, hydrated dough (315 g) was confined in a glass recipient. The tests were performed on dough at 30 K for 3 h, with a slight cylindrical weight. Registered parameters included: Hm (in millimeters), maximum dough fermentation height; T1, the time (in minutes) at which Hm is attained; H′m (in millimeters) maximum height of gaseous release; T′1, the time (in minutes) at which H′m is reached; Tx, the time (in minutes) at which gas starts to escape from the dough, thus when porosity of dough develops. All determinations were made at least in duplicate, and the average values were adopted.

A microscope was used to follow bubble changes of dough during baking as previously described by Rodríguez-García, Salvador and Hernando[17] For that purpose, doughs were prepared as described earlier but without the addition of yeast to follow behavior of bubbles from air incorporation. Microbaking was performed using a system controller unit for heating and freezing stages (Analysa-LTS350, Linkam, Tadworth, UK) mounted under the lens of a light microscope (Nikon ECLIPSE 80i, Nikon Co., Ltd, Tokyo, Japan). The temperature profile settings were from 30 K to 105 K increasing at 1.5 K min^{-1}. Samples were captured at ×4 magnification (objective lens ×4/0.13∞/– WD 17.1, Nikon). During microbaking, a video film was recorded with an attached camera (ExWaveHAD, model no. DXC-190) and images were acquired every 10 K. The analysis software (Linksys 32, Linkam) was directly interfaced with the microscope, enabling temperature control and image recording control. Duplicates were recorded. The number, size and distribution of the bubbles in the dough were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Breadmaking and characterization

A scale-down breadmaking method was carried out[18] to identify the effect of the emulsifiers. Recipes were prepared as described earlier, and then 4 g of dough were placed in previously oiled cylindrical glass molds (17 mm × 300 mm, diameter × height). They were fermented for 100 min at 30 K and finally baked at 130 K for 11 min. Two batches were run for each sample.

Texture profile analysis of crumbs was carried out in a TA-XTPlus (Stable Micro Systems, Godalming, UK). Hence, 10 mm thick slices were compressed twice with a 0.6 mm diameter probe up to 50% at 1 mm s^{-1} speed. The registered parameters were crumb hardness (in grams), springiness, cohesiveness, chewiness (in grams) and resilience. In order to study cell crumb distribution and morphogeometric characteristics of the loaves, both cross-section and longitudinal section of breads were captured using a scanner (HP Scanjet G3110, Hewlett-Packard, Palo Alto, CA, USA) with 600 dpi resolution. The two-dimensional (2D) area and perimeter of longitudinal section was assessed using ImageJ software. The same software was used to analyze the cell crumb distribution in 10 mm × 10 mm crumb cross-sections. Image section was improved by splitting RGB (red, green, and blue) channels and selecting the channel with greater contrast between background and object. Finally, Otsu algorithm (predefined by the software) was applied to convert image into a binary image and particle analysis of the image was carried out. The parameters assessed were cell cm^{-2}, mean area (mm^2) and circularity (from 0, rectangle, up to 1, perfect circle). Six slices were used for each determination.

Statistical analysis

Experimental data were statistically analyzed by analysis of variance (ANOVA) using Statgraphics Centurion XVI.I 16.1 software (Statistical Graphics Corporation, Rockville, MD, USA), to identify significant differences among them. Cluster analysis and principal component analysis (PCA) were also performed to discriminate among emulsifiers with the tested variables.

RESULTS AND DISCUSSION

Dough development and gaseous release characteristics

To evaluate the action of diverse emulsifiers on dough performance during breadmaking, very hydrated doughs were used. The effect of emulsifiers on gas retention during dough fermentation was recorded in the rheofermentometer plots (Fig. 1). After an initial elapsed time, a steady increase of dough volume was displayed, but certain variation was observed in the presence of emulsifiers (Fig. 1(a)). Lecithin and PGEF delayed the onset of volume increase compared to the control and the other emulsifiers. All emulsifiers increased the proofing rate, calculated as the initial slope of dough volume increase (Table 1). The maximum dough development (Hm) reached in the presence of the emulsifiers was higher than the one observed in the control dough, being greater in the case of PGEF (34.6 mm), followed by SSL and DMG-75 (33.0 and 32.4 mm, respectively). The presence of polysorbate blended with the PGEF might contribute to the high volume obtained due to its action as dispersing agent. This result agrees with those obtained by Gómez, del Real, Rosell, Ronda, Blanco and Caballero.[15] where polysorbate addition to low hydrated doughs led to higher dough volumes than other emulsifiers as DATEM and SSL. Nevertheless, the time (T1) required to reach the maximum dough development was higher in the presence of emulsifiers than in the control, confirming that emulsifiers are much more effective when longer dough fermentations are applied.[15] This improvement has been ascribed to the emulsifiers ability for strengthening the gluten network, increasing dough extensibility[15] and dough volume,[16] which in turn was attributed to the formation of aggregates with gluten proteins.[19] However,

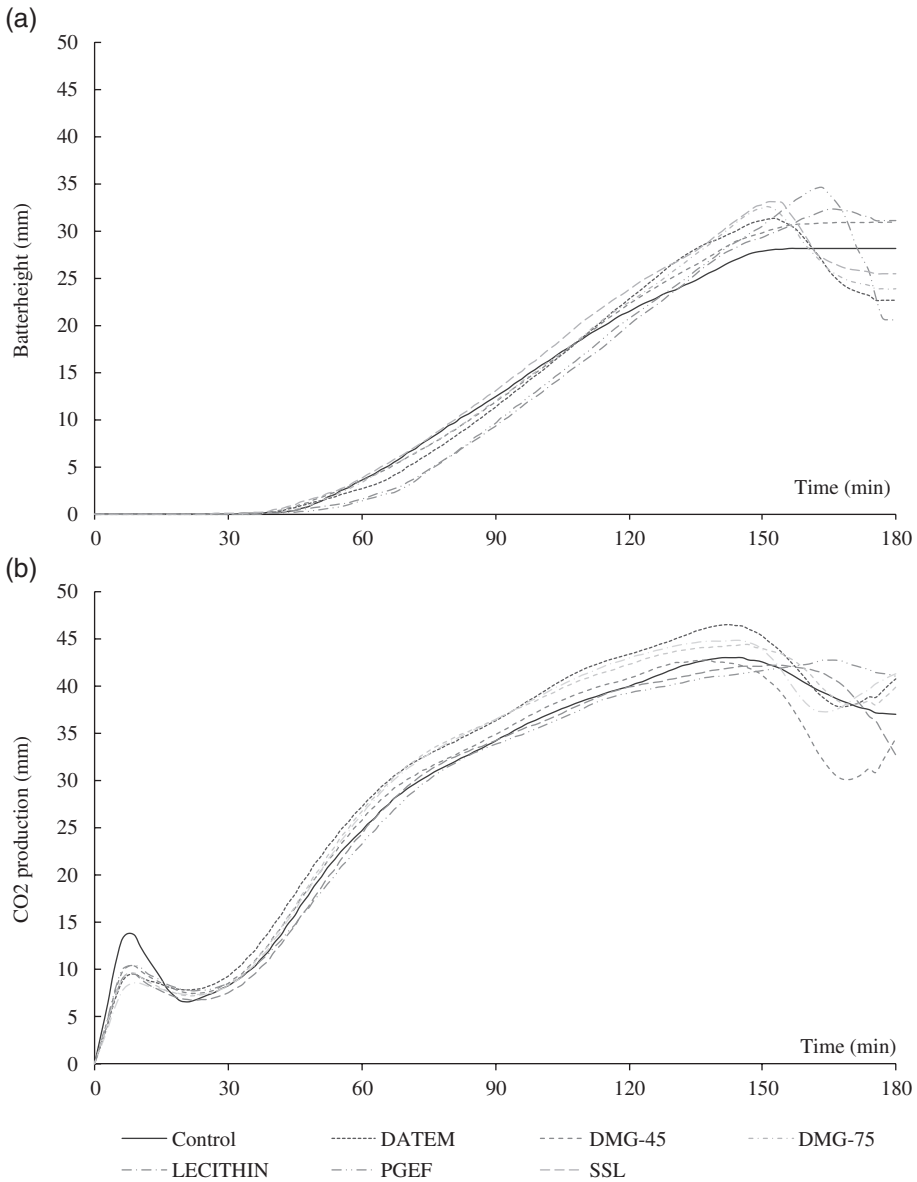

Figure 1. Rheofermentometer curves consisted of dough development time curves (a) and gas release curves (b).

Table 1. Analysis of fermentation stage of batters containing emulsifiers by rheofermentometer

	Dough development			Gas behavior		
	Hm (mm)	T1 (min)	Proofing rate (%)	H'm (mm)	T'1 (min)	Tx (min)
Control	28.5 ± 0.5^{a}	145 ± 0^{a}	30.79	43.7 ± 1.0^{a}	146 ± 3^{a}	140 ± 1^{b}
DATEM	31.4 ± 1.0^{b}	167 ± 9^{c}	33.44	47.0 ± 0.8^{c}	161 ± 7^{c}	145 ± 2^{c}
DMG-45	31.0 ± 1.0^{b}	169 ± 4^{c}	31.13	43.6 ± 0.1^{a}	140 ± 6^{a}	136 ± 5^{a}
DMG-75	32.4 ± 0.8^{b}	150 ± 2^{b}	31.62	45.8 ± 1.3^{b}	161 ± 8^{c}	134 ± 0^{a}
Lecithin	32.3 ± 0.6^{b}	165 ± 3^{c}	33.77	43.4 ± 0.8^{a}	165 ± 4^{c}	136 ± 3^{a}
PGEF	34.6 ± 0.9^{d}	164 ± 6^{c}	34.77	44.0 ± 0.5^{ab}	176 ± 7^{d}	175 ± 5^{d}
SSL	33.0 ± 0.8^{c}	153 ± 4^{b}	31.46	44.5 ± 0.9^{b}	150 ± 3^{b}	148 ± 4^{c}

Mean ± standard deviation. Different letters within the same parameter differ significantly ($P < 0.05$).

that effect cannot be explained only by the emulsifier chemical structure, given that distilled monoglycerides with different particle size (DMG-45 and DMG-75) produced different responses. Dough stability during fermentation was greatly dependent on the emulsifier tested, and only lecithin and DMG-45 extended the stability of the dough longer than the control.

Regarding the gas production during fermentation (Fig. 1(b)), the most evident effect was the decrease in the initial carbon dioxide (CO_2) production when emulsifiers were present. It seems that emulsifiers, independent of their chemical structure, affected the initial release of CO_2. Taking into account that no sugar was added in the recipe, possible explanations could be related to either some interactions between emulsifiers and the free sugars, available in the flour for proofing, that decrease their readiness for the yeast or due to physical constraints derived of the more ordered and stronger protein structure in the presence of emulsifiers.[20] As the proofing progresses, the main difference was observed during the last hour of fermentation when a decrease in the CO_2 production was observed, due to dough permeability to gas in some of the doughs. Doughs with DMG-45, DMG-75 and lecithin showed greater permeability than the control, which resulted in a decrease of the ability to retain CO_2 at the end of the fermentation. The highest CO_2 production was with DATEM addition.

Microscopy and analysis image of simulated microbaking

The ability of the emulsifiers to stabilize the gas bubbles, incorporated into the doughs during mixing, was continuously monitored under a microscope. Very hydrated doughs were subjected to a steady temperature increase to simulate the baking process and consequently the capacity of the dough to hold the gas. Turbin-Orger, Boller, Chaunier, Chiron, Della Valle and Réguerre[21] suggested that the liquid fraction present in the dough influences the cellular structure by affecting the connectivity of bubbles and their possible coalescence. In this study, very hydrated doughs were used to discard the possible interference of liquid effect. The captured images of doughs along temperature increase are shown in Fig. 2. Initially, differences in the structure of the doughs were barely visible. Junge, Hoseney and Varriano-Marston[22] reported that emulsifiers increase the incorporation of gas bubbles during mixing, but they did not find modifications during the baking stage. However, dough images (Fig. 2) showed progressive changes with the temperature increase and major changes were observed when reaching 70 K. Babin et al.[23] reported that the cell structure stabilization occurs with the temperature range 50–70 K when main changes associated to starch granule swelling and gluten crosslinking are produced. In all cases, the bubble size augmented as the temperature increased and their number and size were dependent on the type of emulsifier. The most important differences were observed when using DMG-75: bigger bubbles were observed at low temperature (40 K) if compared to the doughs prepared with the other emulsifiers, and these bubbles were very large at 70 K, giving place to the largest bubbles at 100 K.

Quantitative analysis of bubble distribution and size is shown in Fig. 3, where distributions were ordered from smaller to larger bubble width when temperature increased. In all the samples, the addition of emulsifiers increased the number of bubbles incorporated during mixing if compared to control, which may be due to the lower surface tension induced by the addition of emulsifiers. Kokelaar, Garritsen and Prins[24] showed that addition of some emulsifiers as SSL and DATEM originated more and smaller bubbles during mixing, because of the lower surface tension of dough inducing the subdivision of the entrapped air bubbles. When comparing the doughs prepared with the different emulsifiers (Fig. 3), the dough formulated with PGEF presented greater incorporation of air bubbles during mixing, as the diagram corresponding to this emulsifier shows greater frequency of bubbles at the beginning of the microbaking process. Through temperature rise, all the samples, including control, showed an increase in the amount of detected bubbles, and bubbles size distribution became more heterogeneous due to expansion and interaction of the bubbles. The doughs prepared with DATEM, lecithin and DMG-45 presented a frequency distribution similar to that obtained for the control dough; in fact, the size of the bubbles increased in a uniform, controlled way (Fig. 2). All these doughs showed small bubbles at low temperatures and a tendency to regular distribution of bubbles during heating; moreover, bubbles exceeding 120 000 μm^2 were not generally detected regardless of the heating temperature. Nevertheless, the samples prepared with SSL, PGEF and DMG-75 exhibited larger bubbles, over 120 000 μm^2. Specifically, DMG-75 dough diagram presented very large bubbles, which continued interacting and coalescing even at 100 K. When temperature reached 100 K the samples containing SSL, PGEF and DMG-75 presented coarser distribution of bubbles, while DATEM, DMG-45 and lecithin had more bubbles but smaller ones. When baking temperature rises, the bubbles expand increasing the coalescence due to Ostwald maturation,[25] where large bubbles grow at the expense of small ones, consequently there is an increase in the size of the bubble. With the addition of the emulsifiers, this phenomenon often decreased, due to the stabilization of the interface.[26] However, in the present work, it can be observed that depending on the emulsifier used in the dough formulation, the expansion of bubbles is controlled in a different way, being DATEM, DMG-45 and lecithin more effective for controlling this mechanism. It must be stressed that besides the different chemical structure of the emulsifiers, their physical structure must be considered, since DMG-45 and DMG-75 induced different bubble stabilization.

Image digital analysis and texture profile of breads

The effect of emulsifier addition on technological characteristics is summarized in Table 2. Compared to the control, significantly smaller longitudinal area was produced by PGEF addition, which meant a reduction in the size of the loaves. The rest of the emulsifiers did not significantly modify this parameter. Previous studies reported that adding SSL and DATEM resulted in higher area and volume of breads, due to the increase in dough aeration and volume.[27,28] Probably the use of high hydrated doughs is responsible for the differences with previous studies. In addition, a negative correlation ($r = -0.8754$) was observed between the longitudinal area of the small-scale breads and the maximum height of the proofed dough (Hm). This correlation indicated that dough volume increased during fermentation with emulsifiers addition but likely they did not confer enough resistance to improve final volume. Likewise, no significant differences were found in the longitudinal perimeter, except with DATEM and PGEF that gave smaller values.

The analysis of the bread cross-section revealed significant differences in the number of gas cells (in cell cm^{-2}) and mean cells area (in mm^2) on account of emulsifiers addition (Table 2, Fig. 4). The more number of cells, the less mean cell area and vice versa. DMG-45, DMG-75, DATEM and lecithin showed greater cell number with smaller area than the control. In the case of distilled monoglyceride emulsifier with different particle size (DMG-45 and

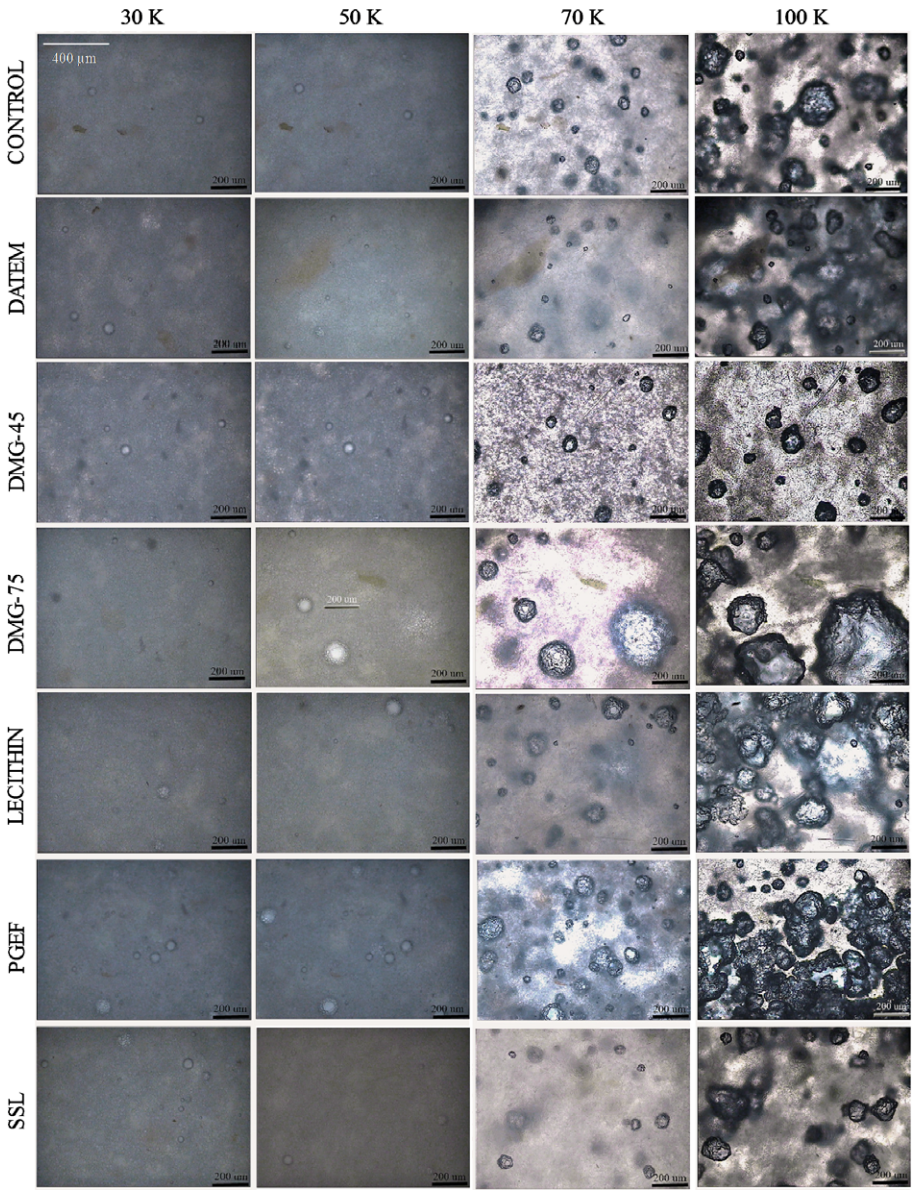

Figure 2. Captured images of gas bubbles during simulated microbaking at microscope.

DMG-75), no significant difference was observed in these parameters, showing that particle size did not affect the cell number and area. Emulsifiers did not induce a significant effect on the circularity compared to the control. However, significant differences between DMG-75 (0.60) and PGEF (0.74) were found. Perfect circularity is difficult to obtain in bread, due to the pressure differences in the gas bubbles and changes occurred during process.[24]

All samples showed significant differences in all texture parameters compared to control (Table 2). With the hydrated recipe very soft crumbs were obtained, and emulsifiers increased the crumb hardness although variation ranged from 79 to 100 g. The highest hardness was obtained with the PGEF, followed by SSL and DATEM. DMG-45, DMG-75 and lecithin were the emulsifiers that gave a smaller rise in the crumb hardness. Hardness showed a strong positive correlation ($r = 0.9373$) with the maximum height of dough (Hm) during fermentation, but that contrasts with results obtained when optimum hydration of wheat flour (500–600 mL kg^{-1}) was used.[12] Dough hydration affects the size of the diameter of the bubbles,[29] leading to higher bubbles, but since all recipes were prepared with the same hydration it should be expected no additional effect due to the liquid phase. Considering the high number of smaller cells mostly found in the breads containing emulsifiers, the hardness increase must respond to the thickness of the cell walls. Therefore, in this study the interaction of emulsifiers with proteins and starch leading to the cell walls had greater impact on texture than the bubbles feature. It has been previously reported that a higher degree of gluten polymerization during baking results in higher firmness of the baked products.[11] At the same time, emulsifiers, like SSL or DATEM interact with gluten, changing the solubilization of polymeric aggregates and that interaction was dependent on the type of emulsifier,[19] particularly SSL reduces the incorporation of gliadins into the gluten network having a direct effect on the subsequent polymerization during baking,[11] and in turn affecting crumb firmness. Therefore, at the level of hydration used in the present study, emulsifiers contributed to increase dough aeration and in consequence

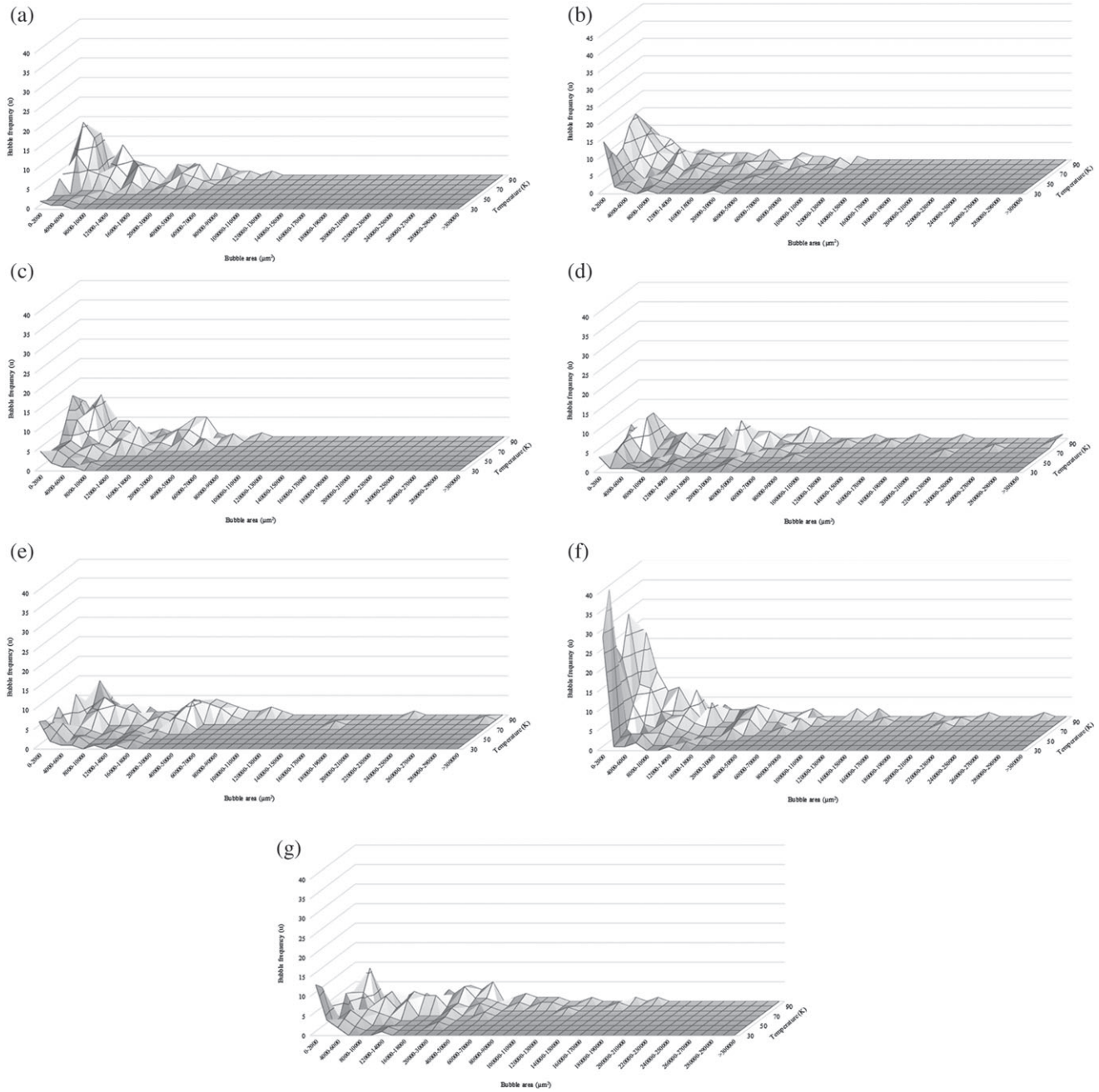

Figure 3. Bubble size distribution during baking for each emulsifier: Control (a), DATEM (b), DMG-45 (c), DMG-75 (d), lecithin (e), PGEF (f) and SSL (g).

the number of gas cells in the crumb, but simultaneously their interaction with gluten changed the proteins polymerization during baking affecting cell wall thickness and in turn crumb firmness.

Considering the other texture parameters, chewiness was significantly higher in the samples with emulsifiers, except with DMG-75, than in the control, and resilience decreased especially in samples with distilled monoglycerides (DMG-45 and DMG-75), which again differed from previously reported with optimum hydrated doughs,[12] confirming the important role of water on the dough aeration and crumb texture.

Statistical analysis

In order to understand the effect produced by the emulsifiers and the differences or correlations between them, a cluster analysis (Fig. 5) and an analysis of principal components (Fig. 6) were carried out. Cluster analysis showed the discrimination between breads containing emulsifiers and the control by combining the two observations that were closest to form the groups. Three well differentiated groups were drawn, with the control and DMG-75 being more separated from the rest of the samples. The other emulsifiers were closer in relation to the analyzed variables, evidencing more similar effects in the doughs and final product. According to their performance with dough and bread, the closest emulsifiers were DATEM and lecithin, followed by DMG-45, SSL and finally PGEF. In this study, the closeness observed between lecithin and DATEM was attributed to the production treatment of lecithin that included a hydrolysis stage, thus it behaves as a monoglyceride despite being from a diglyceride.

Table 2. Emulsifiers effect on loaves morphogeometrics characteristics, cell crumb distribution and texture profile of small-scale breads

Sample	Control	DATEM	DMG-45	DMG-75	Lecithin	PGEF	SSL
Longitudinal section							
Area (cm^2)	5.37 ± 0.48^{bc}	5.31 ± 0.24^{bc}	5.09 ± 0.19^{ab}	5.12 ± 0.32^{ab}	5.00 ± 0.24^{ab}	4.95 ± 0.20^{a}	5.50 ± 0.30^{c}
Perimeter (cm)	1.25 ± 0.04^{c}	1.17 ± 0.07^{ab}	1.23 ± 0.05^{bc}	1.25 ± 0.07^{bc}	1.23 ± 0.1^{bc}	1.11 ± 0.04^{a}	1.20 ± 0.08^{bc}
Cross section							
Number of cells cm^{-2}	10 ± 2^{a}	13 ± 2^{bc}	15 ± 1^{cd}	16 ± 2^{d}	15 ± 2^{cd}	9 ± 2^{a}	12 ± 3^{ab}
Mean cell area (mm^2)	3.31 ± 0.81^{b}	1.92 ± 0.89^{a}	1.57 ± 0.47^{a}	1.61 ± 0.40^{a}	1.88 ± 0.34^{a}	3.23 ± 1.70^{b}	2.48 ± 0.48^{ab}
Minimum cell area (mm^2)	0.15 ± 0.02^{c}	0.14 ± 0.04^{c}	0.10 ± 0.03^{ab}	0.06 ± 0.02^{a}	0.12 ± 0.03^{bc}	0.13 ± 0.02^{bc}	0.12 ± 0.03^{bc}
Maximum cell area (mm^2)	11.80 ± 3.61^{bc}	7.88 ± 1.79^{ab}	7.22 ± 1.94^{a}	9.12 ± 2.60^{abc}	10.13 ± 2.37^{cd}	17.73 ± 4.77^{d}	13.67 ± 3.65^{abc}
Circularity	0.68 ± 0.16^{ab}	0.68 ± 0.12^{ab}	0.62 ± 0.11^{ab}	0.60 ± 0.11^{a}	0.70 ± 0.06^{ab}	0.74 ± 0.07^{b}	0.72 ± 0.05^{ab}
Crumb texture							
Hardness (g)	55 ± 4^{a}	85 ± 6^{c}	79 ± 3^{b}	79 ± 3^{b}	80 ± 4^{bc}	100 ± 6^{e}	93 ± 5^{c}
Springiness	0.95 ± 0.01^{c}	0.93 ± 0.01^{c}	0.94 ± 0.02^{a}	0.86 ± 0.06^{ab}	0.94 ± 0.04^{c}	0.91 ± 0.02^{bc}	0.85 ± 0.08^{c}
Cohesiveness	0.83 ± 0.03^{c}	0.73 ± 0.02^{b}	0.72 ± 0.03^{a}	0.66 ± 0.05^{a}	0.73 ± 0.04^{b}	0.74 ± 0.03^{b}	0.64 ± 0.04^{b}
Chewiness (g)	35 ± 5^{a}	56 ± 6^{bcd}	53 ± 5^{bc}	47 ± 5^{ab}	56 ± 3^{bc}	58 ± 6^{d}	51 ± 7^{b}
Resilience	0.49 ± 0.03^{c}	0.38 ± 0.01^{b}	0.37 ± 0.04^{a}	0.29 ± 0.03^{a}	0.39 ± 0.04^{b}	0.40 ± 0.03^{b}	0.26 ± 0.01^{b}

Mean ± standard deviation. Different letters within the same parameter differ significantly ($P < 0.05$).

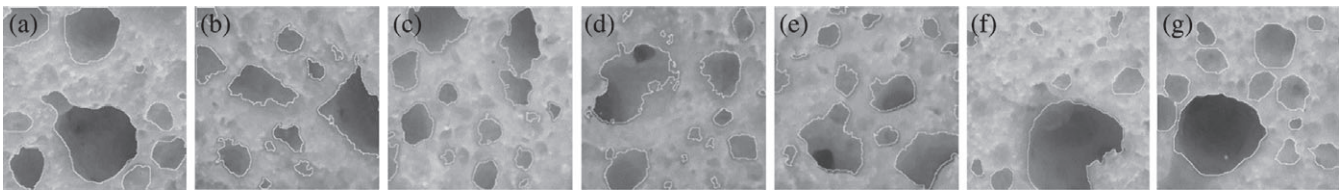

Figure 4. Captured images and bubbles count of small-scale breads. (a) Control, (b) DATEM, (c) DMG-45, (d) DMG-75, (e) lecithin, (f) PGEF, (g) SSL.

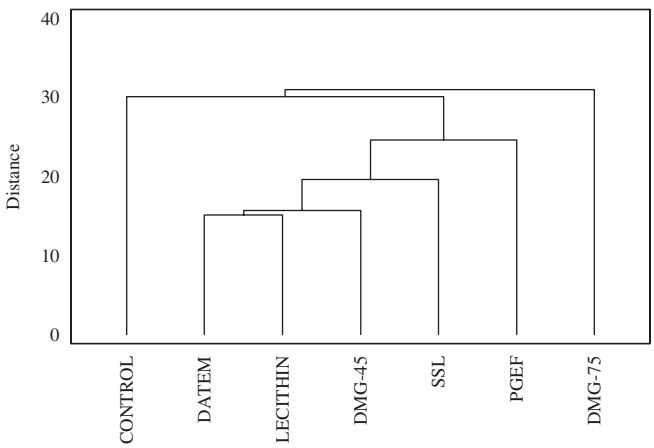

Figure 5. Cluster statistical analysis by using closest neighbor method.

PCA of the experimental data obtained containing emulsifiers resulted in two principal components explaining 48.3% and 22.0% of the data variation (Fig. 6). Thus, the model explained 70.3% of the total variation in data. The first principal component weight (PC1) was defined by the longitudinal 2D area, the longitudinal 2D perimeter and cell cm^{-2} in the positive axis, and on the negative axis were located resilience, cohesiveness, springiness, Tx and mean cell area. The second component (PC2) was defined by T1, bubbles cm^{-2}, longitudinal 2D area, H'm and the mean bubble area. DATEM, lecithin, SSL, and PGEF were found in the negative PC1 component where the majority of dough and bread responses were located. As shown in cluster analysis (Fig. 5), DMG-75 was the furthest emulsifier attending to its experimental responses, particularly longitudinal 2D area and perimeter, and H'm. Results obtained from DATEM and lecithin were explained due to responses to chewiness, cohesiveness, resilience, cell circularity and Tx. However, PGEF and SSL, adjacent in cluster analysis, were related with the mean cell area, hardness, T'1 and Hm. Eventually, DMG-45 position was related to T1 and the number of gas cells cm^{-2}. Overall, emulsifiers could be grouped into four categories attending to the responses obtained with dough and bread performance. In the first group, DATEM and lecithin due to their effect on crumb texture and dough permeability; second group would include SSL and PGEF that showed larger bubbles, with fewer and larger gas cells and higher crumb hardness; third group with an intermediate behavior respect to the previous ones DMG-45; and finally DMG-75, with a more distant behavior than the control, which led to large bubbles.

CONCLUSIONS

The role of emulsifiers on the progress of bubbles during proofing and baking was evaluated. Emulsifiers showed different functionality that was attributed to their diverse chemical structure and also physical characteristics (particle size). Furthermore, results shown that emulsifiers functionality was dependent on the dough hydration. All emulsifiers studied, increased the maximum dough volume during proofing, but showing different effect on dough permeability or ability to retain CO_2. Digital image analysis of the recorded baking under a microscope, allowed both the number of bubbles and size to be quantified and allowed the role of the emulsifiers on aeration to be recognized. Emulsifiers allowed

Figure 6. Score plot from a principal component analysis of the combination of components weight (■simulated microbaking, ◆texture properties, ● rheofermentometer variables and ▲ digital image analysis of breads) and principal components (× emulsifiers).

greater air incorporation into the dough observing higher number of bubbles, particularly with PGEF. Major changes in dough occurred at 70 °C when bubble size augmented and became more heterogeneous, and emulsifiers affected the size and number of bubbles, with DMG-75 producing the largest bubbles. In bread, emulsifiers tend to increase the number of gas cells with lower size, but that gave greater crumb firmness, which suggested different interactions between emulsifiers and gluten, affecting protein polymerization during baking. Despite the diverse chemical structure of the emulsifiers, experimental data following dough proofing and bread features allowed to discriminate among them.

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Modifying gluten-free bread's structure using different baking conditions: Impact on oral processing and texture perception

P. Puerta [1], R. Garzón [1], C.M. Rosell, S. Fiszman, L. Laguna [*], A. Tárrega

Institute of Agrochemistry and Food Technology (IATA-CSIC), Agustín Escardino, 7, Paterna, Valencia, Spain

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ABSTRACT

This study evaluated the impact of different gluten-free bread's inner structure on oral processing and texture perception. Four rice-based breads were prepared varying water hydration (H: 85 or 100 g of water/100 g of flour) and fermentation time (F: 30 or 75 min). Mechanical properties of breads, bolus properties (after three chews and at swallowing point), subject's chewing and swallowing activities, and perceived sensations using Temporal Dominance of Sensations were measured. Long fermentation breads (H100F75 and H85F75) had bigger air cells, less density, than short fermentation breads (H100F30 and H85F30). High hydration breads were harder, chewier, less cohesive, and resilient. Bolus from H100F75 and H85F75 breads was moister, softer, less dense, and generated smaller particles than the H100F30 and H85F30 bolus, which was more consistent and adhesive. Before swallowing, H85F30 and H85F75 required more saliva, H100F30 and H85F30 required more time, chews and swallows. At the beginning of the mastication, short fermentation breads were perceived compact, whereas long fermentation, aerated. Independently of the formulation, all were perceived sticky. Thus, by varying breadmaking conditions like fermentation time, the structure of gluten-free breads can be modified to improve its texture sensations, elicited during consumption.

1. Introduction

The rise in coeliac disease diagnosis and its social awareness has expanded the gluten-free products available. For the food industry this represents a technical challenge, as gluten is the main structure-forming protein in baked goods (Espinosa-Ramírez, Garzon, Serna-Saldivar, & Rosell, 2018). In breads, gluten forms a network that provides cohesiveness and helps CO_2 retention, produced during fermentation, creating an open crumb structure (Deora, Deswal, & Mishra, 2014). Gluten absence drastically affects bread characteristics, commonly resulting in low volume, a crumbly texture, pale colour, and poor flavour; thus, less quality than their gluten containing counterparts (Espinosa-Ramírez et al., 2018; Houben, Höchstötter, & Becker, 2012; Matos Segura & Rosell, 2011).

Commercial gluten-free-breads are characterised by an extensive list of ingredients, including acidifiers, emulsifiers, leavening agents, preservatives, and aromas or flavourings (Matos Segura & Rosell, 2011; Puerta et al., 2020). Additionally, because of consumer demand for a clean label breadmaking (Asioli et al., 2017), breadmaking conditions have been investigated as a novel approach to improve gluten-free bread characteristics, (Vallons, Ryan, & Arendt, 2011). For example, fermentation time has shown to increase volume and produce softer texture (Cao et al., 2020), a strategy, that although not tested in gluten-free breads could change and improve the gluten-free bread structure and texture. Another approach could be to change one of the main ingredients such as water. In a previous study, this altered the bread volume and the texture of gluten-free breads (Sahin, Wiertz, & Arendt, 2020).

However, for both approaches (changing conditions or reformulation), studies are mainly based on bread's mechanical properties (Cao et al., 2020; Morreale, Garzón, & Rosell, 2018; Sahin et al., 2020; Schober, Bean, Boyle, & Park, 2008; Ziobro, Witczak, Juszczak, & Korus, 2013) and/or final sensory properties (Armstrong, Luecke, & Bell, 2009; Morais, Cruz, Faria, & Bolini, 2014). But it is still pendent to know how this alter the in-mouth process and the sensations along the different stages of the mastication.

Food oral processing studies have shown how products behave in-mouth and can help us better understand and modulate sensations

* Corresponding author.
E-mail address: laura.laguna@iata.csic.es (L. Laguna).
[1] These authors have equally contributed to the work.

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perceived during consumption of products (Chen, 2014; Jourdren, Saint-Eve et al., 2016). Through food oral processing, the structure of foods is broken down by teeth and tongue comminution, dissolving components in saliva (Devezeaux de Lavergne, Van de Velde, & Stieger, 2017) until a swallowable bolus is formed. This transition of food to a swallowable bolus, through oral processing, can be characterised at several stages with rheology (hardness, cohesiveness, viscosity, tribology) (Jourdren, Panouillé, et al., 2016; Peyron et al., 2011) or particle size characterisation (Aleixandre, Benavent-Gil, & Rosell, 2019). Particle size characterisation also provides insight about the fragmentation pattern during mastication, by using sieving (Devezeaux de Lavergne, van de Velde, van Boekel, & Stieger, 2015; Van Der Bilt & Fontijn-Tekamp, 2004), or image analysis (Aleixandre et al., 2019; Rizo, Peña, Alarcon-Rojo, Fiszman, & Tárrega, 2018; Tournier, Grass, Zope, Salles, & Bertrand, 2012).

Additionally, to study the dynamics of perception during the full duration of the mastication process, different sensory dynamic techniques can be used, such as Time Intensity, Progressive Profiling, Temporal Check-All-That-Apply (TCATA) or Temporal Dominance of Sensations (TDS).

The TDS method allows a subject to select a dominant parameter from a list of attributes, providing the dynamics of sensations that dominate along eating time (Pineau et al., 2009). While in TCATA, each panellist can select and deselect the attributes that apply to the tested sample moment-to-moment (Castura, Antúnez, Giménez, & Ares, 2016). Both dynamic techniques have been used to further understand bolus formation and sensation in ham (Rizo et al., 2019), gels (Devezeaux de Lavergne, van de Velde, et al., 2015), and sausages (Devezeaux de Lavergne, Derks, Ketel, de Wijk, & Stieger, 2015). Furthermore, in breads, the TDS method has been used to link bolus formation and sensations along the mastication (Jourdren, Saint-Eve et al., 2016; Panouillé, Saint-Eve, Déléris, Le Bleis, & Souchon, 2014).

Previous bread studies have investigated structural breakdown linked to perceived sensations, showing that, by only varying bread structure, the perception of salt changes, as a denser bread crumb is related to a less salty perception (Panouillé et al., 2014; Tournier, Grass, Septier, Bertrand, & Salles, 2014). Moreover, the initial bread structure is critical for gluten-free products, conditioning the textural sensations, the breakdown pattern in oral activity, and the perceived sensations (Puerta et al., 2020). Gluten-free breads with a greater number of smaller air cells in the crumb were less hard and springier than breads with few and bigger air cells. Gluten-free breads (with smaller air cells) were perceived as softer and spongier than breads with few air cells, perceived as hard breads (Puerta et al., 2020). Further, bread is usually consumed combined with other foods such as sauces (for example, gravy), or spreads (such as butter, cheese spread), which can also alter the breakdown pattern and thus, the oral behaviour, bolus properties, and sensory perception (van Eck, Fogliano, Galindo-Cuspinera, Scholten, & Stieger, 2019). Recent studies have shown how the addition of a sauce like mayonnaise facilitates the bolus formation (decreases the number of chewing cycles before swallowing), changing the perceived sensations, in special at the beginning of the oral processing (van Eck, Hardeman, et al., 2019; van Eck, Franks, et al., 2020).

This study's hypothesis was that the modification in the structure of gluten-free breads achieved by varying breadmaking conditions can affect the texture of the bread and its breakdown pattern in mouth, eliciting the dynamics of critical sensations during consumption.

Therefore, this study aimed to study how structural changes in gluten-free breads, caused by different baking conditions (level of dough hydration and fermentation time), impact oral processing (bread breakdown and bolus formation) and sensory perception.

2. Materials and methods

2.1. Bread preparation

Gluten-free bread formulation was rice flour (Harinera La Meta, S.A., Lleida, Spain) and corn starch (EPSA Aditivos Alimentarios, Valencia, Spain) in a 70:30 (g/g) blend ratio. The remaining ingredients are expressed proportionally to the blend of flour and starch (g/100 g of flour-starch blend): compressed yeast: 3 (Lessaffre, Lille, France), salt: 1.5, seed oil: 2, hydroxypropylmethylcellulose (HPMC): 2 (Methocel K4M, Dow Chemical, Midland, MI, USA), and sugar: 1. Salt, sugar, and seed oil were purchased from a local market.

To obtain different crumb structures, preliminary tests were conducted, testing the impact of levels of hydration and fermentation times. Hydration is referred to as the amount of water added to the blend of rice flour and corn starch. Hydration levels were used because the recipe was expressed based on flour addition, as is currently used in breadmaking. Breads with two levels of hydration (H85 and H100) were obtained by adding different water amounts that corresponded to 85 and 100 g of water/100 g of the blend of flour and starch. Fermentation times were 30 or 75 min at 35 °C (F30 or F75). Combining both levels of hydration and fermentation times, four breads were obtained: H85F30, H85F75, H100F30, and H100F75.

Solids for obtaining 6 breads were blended in a kneader with a dough hook, then water was added and mixed for 8 min. Then 600 g of dough were moulded (20 × 10 × 10 cm, length, width, height) and fermented in a controlled cabinet (Salva, Guipúzcoa, Spain) at 35 °C for 30 or 75 min. After fermentation, dough was baked at 185 °C for 45 min in a convection oven (Eurofours, Gommegnies, France).

After cooling, breads H100F30, H100F75, H85F30, and H85F75 were placed in sealed plastic bags and stored at −20 °C.

2.2. Bread characterisation: grain crumb appearance, moisture content, crumb density, and texture analysis

Crumb grain appearance, moisture content, and crumb texture were evaluated after cooling for 1 h at room temperature.

2.2.1. Grain crumb appearance using imaging analysis

The bread crumb was studied using digital image analysis as previously reported (Espinosa-Ramírez et al., 2018). Bread slices were scanned using a flatbed scanner (HP Scanjet 4370, Hewlett–Packard, Palo Alto, CA, USA) in the RGB (Red-Green-Blue) standard format with 1200 dpi resolution.

Image analysis of bread slices was conducted using Fiji Image J software (Schindelin et al., 2012). For image segmentation and determination of crumb grain characteristics, image contrast was improved and then the predefined algorithm "Otsu" was applied. The crumb grain measurements included: cell density (cell/cm^2), mean cell area (mm^2), surface porosity (total cell area/total slice area in percentage), and mean cell shape using the circularity factor (0-perfect circle to 1-perfect rectangle). Two slices of bread obtained in two different batches (six breads per batch) were analysed for each sample.

2.2.2. Moisture content

The moisture content of gluten-free bread samples was determined according to the International Cereal Chemistry method (ICC 110/1, 1994). Moisture content was expressed as g of water/100 g of bread crumb. Four replicates were analysed for each sample.

2.2.3. Crumb density

The crumb density was obtained from the weight and volume of a cylinder of the central part of the crumb (diameter = 3.3 cm; height = 2 cm). Three replicates were analysed for each sample.

2.2.4. Instrumental texture analysis

Crumb slices of 10 × 10 × 2 cm (length x width x thickness) were used for texture evaluation after removing the crust. Slices were double compressed to 50% of their original height using a TA-XT plus Texture Analyser (Stable Microsystems, Godalming, UK) equipped with a 5 kg load cell and a 75 mm diameter aluminium cylindrical probe. Test speed was set to 2.0 mm/s with a trigger force of 0.049 N.

Eight replicates from two different batches (n = 4 × 2) were analysed. Parameters recorded were hardness (maximum force at the first compression), cohesiveness (calculated as the ratio between the areas under the second and the first peak), springiness (calculated as the ratio between the distances at which force is maximum during the second and the first cycle), chewiness (calculated from the multiplication of cohesiveness per hardness), and resilience (calculated by dividing the upstroke area of the first compression by the downstroke area of the first compression).

2.3. Bread bolus characterisation

2.3.1. Samples and participants

Bread samples for bolus characterisation and sensory evaluation comprised a cylinder of the central part of the crumb (diameter = 3.3 cm; height = 2 cm) of the different formulated gluten-free breads (H100F30, H100F75, H85F30, and H85F75), discarding the end of loaves and crust. In all studies, samples were presented monodically following a William's design in a different order across subjects, in sealable plastic cups labelled with three-digit codes.

Eleven subjects (six women and five men, aged 22–39 years old, average 30.5) with healthy complete dentition participated in the study. They gave informed consent and received compensation for their participation. Participants could rest between samples and were provided with still mineral water for rinsing their palates in all the experiments.

2.3.2. Bolus collection procedure

Subjects were asked to place the entire sample in the mouth, chew it as usual, and spit the bolus out into the plastic cup; either after three chewing cycles for determining particle size characterisation or after full mastication, for determining bolus moisture and mechanical properties.

2.3.2.1. Particle characterisation after three chewing cycles. Each bolus collected was spread out manually on a transparent glass surface (30 × 21 cm). Particles were manually separated and digitised in TIF format at 600 ppi on a scanner (Canon MP270 model K.10,339, Canon U.S.A. Inc. Lake Success, NY., USA) using a black background. Images were analysed using Nis-Elements® BR 3.2 software (Nikon, Tokyo, Japan). Sample images were binarised using a histogram-based segmentation process, according to the predefined intensity threshold value. Particle size distribution and number of particles were obtained for each bread and subject. The median particle area (a50), which is the particle area corresponding to 50% of total area occupied, was calculated. The interquartile ratio (a75/a25) between 75 and 25% of the cumulative area occupied by particles was calculated. A cumulative curve of total area occupied by particles of assorted sizes was obtained. Two boli per sample and subject were collected over two separate sessions.

2.3.2.2. Bolus characterisation at swallowing point

2.3.2.2.1. Bolus moisture. Bolus moisture was determined in triplicate. Four grams of bolus were weighed, mixed with 8 g of sand, and dried to a constant weight in an oven at 105 °C for >16 h. The difference in water content (g water/100 g bolus) between the initial bread and bolus at swallowing point was calculated as an indicator of saliva uptake. Three boli were collected over three separate sessions from each participant.

2.3.2.2.2. Bolus mechanical properties. Consistency and adhesiveness of bolus obtained at swallowing point were determined using a TA. XT plus Texture Analyser (Stable Micro System, Godalming, UK), fitted with a TTC Spreadability Rig containing a male cone (90° and 40 mm diameter) that matches a glass containing the female cone fixed in an HDP/90 platform. The bolus was placed into the female cone and carefully levelled. The force when penetrating the sample with the male cone at a constant rate of 2 mm/s until a depth of 28 mm was recorded.

From the force-displacement curves, consistency values corresponded to the maximum peak force during the downstroke (N.s). Adhesiveness value (N.s) corresponded to the area under the curve while rising the probe (negative area). For each subject, three boli of each sample were collected over three different sessions. Six boli were collected over separate sessions from each participant.

2.4. Chewing and swallowing activity

Each subject was instructed to eat the bread cylinder in their usual manner while indicating the start of the test, and each of their swallows. This information was recorded using Compusense Cloud software (Compusense Inc., Guelph, Canada). From the data, the number of swallows, first swallow time, and chewing time were obtained. Participants were also recorded on video to visualise the chewing cycles count.

Three replicates per sample were performed in three separate sessions.

2.5. Sensory evaluation

Texture sensations perceived during consumption of the four bread samples were assessed by 11 subjects using the TDS method. Two previous sessions were held to establish the list of texture attributes. In the first session, each subject generated a list of attributes by comparing the different bread samples. A second session comprised a discussion and sample tasting to achieve, by consensus, a final list of attributes and their definitions. Table 1 shows the list of nine attributes and definitions (Table 1). In addition, each panellist attended one session for familiarisation with the TDS.

In the evaluation sessions, participants introduced the whole sample into the mouth and initiated the test while they pressed the "start" button and started chewing. They were asked to select the dominant sensation at each moment among the list of nine attributes while eating. They could select each attribute as often as they considered and until they stopped perceiving any sensation in the mouth.

The order of the attributes in the list was varied among participants according to a balanced design. Each panellist evaluated the four samples in each session. Six replicates were performed over six separate sessions. The serving size and sample presentation were as described in Section 2.5. The assessments took place in a temperature-controlled room under white light and in standard sensory booths, designed in accordance with ISO 8589 (ISO, 2007). Data collection from TDS tests

Table 1
List of texture attributes (and definitions) used in the sensory evaluation of the gluten-free breads with the TDS method.

Attributes	Definition
Compact	The bread is hard to compress when chewing because it is dense
Aerated	The bread is easy to compress, light
Roughness	The bread scratches the internal surfaces of the mouth (palate, tongue, throat)
Crumbly	The bread disaggregates and breaks down in particles easily
Bolus hard to form	Difficulty to aggregate or agglutinate the bread particles in a swallowable bolus, it is not cohesive
Hard to swallow	Bread particles remain retained through the throat
Gritty	Presence of particles in the mouth
Sticky particles	Presence of bread particles adhered to oral mucosa that need to be removed with the tongue
Dry	Bread that resulted difficult to moisturize/hydrated

and analysis were conducted using Compusense Cloud (Compusense Inc., Guelph, Canada).

Experimental procedure of this work was approved by the Ethical Committee of CSIC (ethics references 018/2019).

2.6. Statistical analysis

For each instrumental parameter of breads, a one-way analysis of variance (ANOVA) was applied. Tukey's test was used to assess significant differences ($\alpha = 0.05$) among samples.

Values of bolus characterisation after three chewing cycles and at swallowing point, as well as chewing and swallowing activity parameters showed a non-normal distribution according to the Shapiro-Wilks test, and showed heteroscedasticity according to Levene's test. Therefore, they were analysed by the non-parametric Friedman's test. Nemenyi's test was used to assess significant differences ($\alpha = 0.05$) among samples.

TDS curves representing the dominance rate for each sensation at various times of the chewing period were obtained for each bread. TDS data were normalised according to individual evaluation time on a scale from 0 to 100% of consumption. Both chance and significance limits were included in the graphs. The chance level represented the dominance rate of an attribute that can be obtained by chance (chance level, $P_0 = 1/9$, as there were 9 attributes), and significance level representing the minimum value significantly higher than the chance level (significant level $= P_s = P_0 + 1.645 \sqrt{[P_0 (1 - P_0)/\text{number of replicates}]}$).

XLSTAT statistical software (Version 2010.5.01, Microsoft Excel®, Addinsoft, Paris, France) was used for the statistical analysis.

3. Results

3.1. Gluten-free bread characteristics: mechanical properties, moisture content, size, and crumb grain appearance

Values of instrumental texture profile parameters for the breads are shown in Table 2. Significant variation was found among samples in hardness, cohesiveness, chewiness, and resilience ($P < 0.05$) but no significant for springiness ($P > 0.05$). Hardness values showed that longer fermentation time (F75) provided softer crumbs than short fermentation time (F30). Only in short fermentation breads, hydration influenced hardness values and were higher for higher hydration samples (H100F30). Cohesiveness and resilience (capacity to retain original height) were similar in all breads for the different fermentation times.

Gluten-free bread crumb moisture content was significantly different among samples ($P < 0.05$) according to the initial hydration level (Table 2).

As shown in Fig. 1, breads presented distinct size and crumb structure. Cell density and mean cell area values significantly varied among samples ($P < 0.05$). Main differences were related to the fermentation time. Long fermentation resulted in breads with lower cell density and higher air cell size (H85F75 and H100F75) that corresponded to the slices of greater height (Fig. 1). Accordingly, crumb density decreased with fermentation time, especially for the low hydration breads (Table 2).

Table 2
Texture parameters (n = 8), crumb moisture content (n = 4), crumb density (n = 3) and internal crumb structure (n = 2) of the gluten-free breads.

	H85F30	H85F75	H100F30	H100F75	F-ratio	*P*-Value
Texture parameters						
Hardness (N)	40 ± 3 b	15 ± 2 a	48 ± 7 c	16 ± 4 a	116.6	<0.001
Springiness	0.85 ± 0.05 a	0.86 ± 0.09 a	0.88 ± 0.07 a	0.91 ± 0.05 a	1.0	0.4
Cohesiveness	0.73 ± 0.03 ab	0.76 ± 0.04 b	0.69 ± 0.03 a	0.73 ± 0.02 ab	4.9	0.01
Chewiness (N)	24 ± 4 b	10 ± 2 a	30 ± 5 c	11 ± 3 a	61.4	<0.001
Resilience	0.40 ± 0.02 ab	0.42 ± 0.03 b	0.37 ± 0.02 a	0.40 ± 0.02 ab	5.1	0.01
Crumb moisture (g water/100 g bread crumb)	48.8 ± 0 a	49.3 ± 0.2 a	52.3 ± 0.9 b	52.7 ± 0.2 b	110.8	<0.001
Crumb density	0.54 ± 0.02 d	0.29 ± 0.01 a	0.49 ± 0.01 c	0.41 ± 0.01 b	214.8	<0.001
Crumb structure						
Cell density (cell/cm^2)	30.0 ± 0 b	15.0 ± 1 a	26.0 ± 1 b	15.0 ± 1 a	108.9	<0.001
Surface porosity (%)	33.6 ± 0.3 a	37.9 ± 1.5 a	32.8 ± 2.5 a	36.9 ± 2.3 a	2.5	0.20
Mean cell area (mm^2)	1.17 ± 0.17 a	2.47 ± 0.11 b	1.25 ± 0.07 a	2.50 ± 0.47 b	16.3	0.01

Different letters within rows indicate significant differences among breads according to Tukey's test ($\alpha = 0.05$).
H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

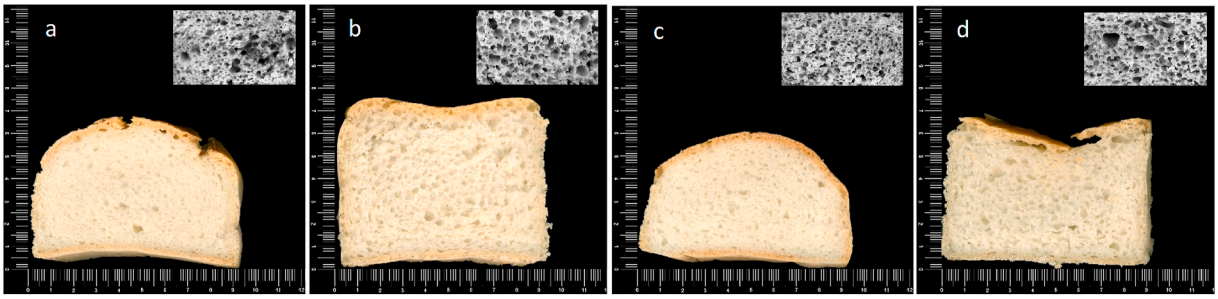

Fig. 1. Scanning of the sliced gluten-free breads: a) H85F30, b) H85F75, c) H100F30 and d) H100F75. Units in the scale are expressed in cm. H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

Table 3
Mean and standard deviation of the median particle area (a50) and interquartile ratio (a75/a25) between 75 and 25% of the cumulative area occupied by particles of bolus obtained after three chews of gluten-free breads (n = 2).

Bread	a50 (mm^2)	a75/a25
H85F30	53.1 ± 4.7 b	7.4 ± 26.7 b
H85F75	24.8 ± 0.6 a	3.4 ± 8.4 a
H100F30	45.8 ± 4.1 b	6.6 ± 19.1 b
H100F75	27.9 ± 0.9 a	3.4 ± 7.3 a
Friedmans's Q	43.8	39.66
p-Value	<0.001	<0.001

Different letters within columns indicate significant differences among breads according to Nemenyi's test (α = 0.05).
H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

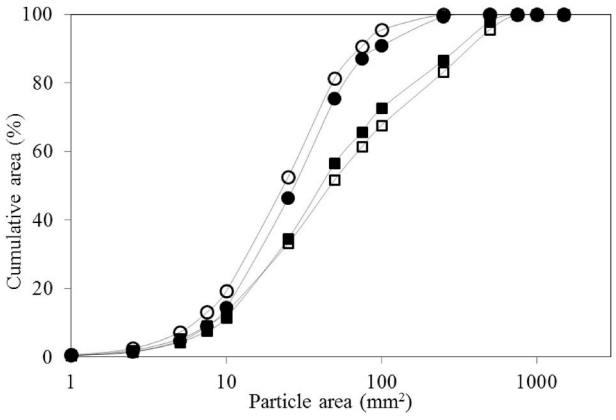

Fig. 2. Cumulative curve representing the total area occupied by particles of bolus obtained after three chews of gluten-free breads H85F30 (-□-), H85F75 (-○-), H100F30 (-■-) and H100F75 (-●-). H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

3.2. Bolus characterisation

3.2.1. Particle size distribution

The median particle area (a50) and interquartile ratio (a75/a25) after three chews are presented in Table 3. Significant differences ($P < 0.001$) were found among samples for both a50 and a75/a25 ratio parameters. Long fermentation breads (H85F75 and H100F75) generated more particles smaller than those obtained from short fermentation breads (H85F30 and H100F30), indicated by a50 values. Particles obtained from long fermentation breads presented lower a75/a25 ratio values, indicating that these breads fragmented into particles more homogeneous in size than short-fermented breads.

The cumulative curve of the total area occupied by particles (Fig. 2) showed that for long fermentation breads, the area is occupied mostly by smaller particles, while for short-fermented breads, particles with bigger size indicating a heterogeneous pattern of fragmentation were present.

3.2.2. Moisture content of bolus at swallowing point

Table 4 shows the moisture of bolus, at swallowing and saliva uptake, for the four gluten-free breads. Bolus moisture at the swallowing point slightly varied (64.3–66.4 g water/100 g bolus) among breads ($P < 0.001$); and significant differences were found between short-fermented bread (H85F30) and long-fermented breads (H85F75 and H100F75).

Saliva uptake significantly varied among bread samples ($P < 0.001$), being higher in low hydration breads (H85F30 and H85F75) than high hydration breads (H100F30 and H100F75). Particularly, the highest significant difference was found between H85F30 and H100F75 (15.5 and 13.2 g water/100 g bolus, respectively).

3.2.3. Mechanical properties of bolus at swallowing point

Consistency and adhesiveness values of bolus obtained at swallowing point were significantly different among bread samples ($P < 0.05$, Table 4). Long-fermented breads (H85F75 and H100F75) resulted in a less consistent bolus at the swallowing point than shorter fermented breads (H100F30 and H85F30). This effect was higher and only significant in low hydration breads (H85). Likewise, bolus adhesiveness varied with fermentation, long-fermented breads resulted in less adhesive boli than short-fermented breads, but this effect was only significant for low hydration breads.

Table 5
Mean and standard deviation values of the oral activity parameters (number of chewing cycles, number of swallows, time for first swallow and total chewing time) when consuming gluten-free breads, (n = 3).

Bread	Chewing cycles	Swallows	Time first swallow (s)	Total chewing time (s)
H85F30	53 ± 18 b	5 ± 2 c	32 ± 7 b	59 ± 13 b
H85F75	38 ± 14 a	4 ± 2 a	24 ± 8 a	44 ± 12 a
H100F30	52 ± 16 b	5 ± 2 bc	32 ± 9 b	58 ± 14 b
H100F75	42 ± 14 a	4 ± 2 ab	27 ± 8 a	47 ± 12 a
Friedmans's Q	68.03	29.41	29.41	53.44
p-Value	<0.001	<0.001	<0.001	<0.001

Different letters within columns indicate significant differences among breads according to Nemenyi's test (α = 0.05).
H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

Table 4
Mean and standard deviation of the mechanical properties and moisture content of bolus obtained from gluten-free breads at swallowing point and saliva uptake values deviation (n = 6 for mechanical properties and n = 3 for bolus moisture and saliva uptake values).

Bread	Bolus consistency (N)	Bolus adhesiveness (N.s)	Bolus moisture (g water/100 g bolus)	Saliva uptake (g water/100 g bolus)
H85F30	10 ± 6 c	1.6 ± 0.9 c	64.30 ± 0.04 a	15.50 ± 0.04 bc
H85F75	7 ± 5 a	1.0 ± 1.4 a	65.80 ± 0.04 b	16.50 ± 0.04 c
H100F30	9 ± 5 bc	1.3 ± 0.7 b	65.50 ± 0.05 ab	13.20 ± 0.04 a
H100F75	8 ± 5 ab	1.1 ± 0.6 ab	66.40 ± 0.03 b	13.70 ± 0.03 ab
Friedmans's Q	41.16	45.49	19.07	29.62
p-Value	<0.001	<0.001	<0.001	<0.001

Different letters within columns indicate significant differences among breads according to Nemenyi's test (α = 0.05).
H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

3.3. Chewing and swallowing activity

The number of chewing cycles, swallows, time to the first swallow, and total chewing time significantly varied among breads ($P < 0.005$, Table 5), because of the fermentation time. Increasing fermentation time resulted in breads (H100F75 and H85F75) that required fewer chews, swallows, time to the first swallow, and chewing time than breads with short fermentation. The effects of fermentation time were more noticeable for low hydration breads.

3.4. Sensory evaluation

The TDS curves in Fig. 3 show the fundamental differences among breads occurred at the beginning of the oral processing. Initial sensations described by consumers when eating short-fermented breads were compact and dry, whereas aerated was the first sensation described in long-fermented breads. Immediately after, crumbly sensation appeared as dominant for all breads, but frequency of selection was higher in long than in short fermentation breads. Bolus hard to form and grittiness were the sensations with maximum frequency in the middle of consumption for all breads. Grittiness seemed more frequent in long fermentation breads. At the end of mastication, having a sticky particles sensation was dominant in all samples, lasting longer in short fermentation breads.

Among breads with different hydration level, no differences in the TDS were found.

4. Discussion

In this study, breadmaking conditions (fermentation time and hydration level) of gluten-free breads were modified, affecting gluten-free breads' structure. Longer fermentation resulted in gluten-free breads with a more open structure (bigger air cells and less density) with crumbs presenting lower instrumental hardness values. This is explained by a greater gas expansion caused by long fermentation time, which increased the volume of bread (Hong & Kweon, 2020). Because of that expansion, the long-fermented gluten-free loaves were bigger than loaves using short fermentation. Hydration level also affected the structure, especially for short fermentation gluten-free breads, where low level of hydration increased the crumb density, as air cells were smaller. Morreale et al. (2018) reported strong and moderate correlations between hydration level and instrumental parameters; with strong positive link between water quantity and crumb hardness in gluten-free breads.

Here, structural changes affected the gluten-free bread mechanical properties, and instrumental texture measurements showed that longer fermentation produced gluten-free bread with lower instrumental hardness and less chewiness.

Structural changes of gluten-free breads also affected the in mouth behaviour. During eating, chewing movements first aim to break down food. This breakage pattern was different among the gluten-free breads of this study. After three chews, breads with long fermentation broke easily into more homogeneous and smaller particles; as a lack of gluten in bread results in crumblier structures, because there is no gluten to

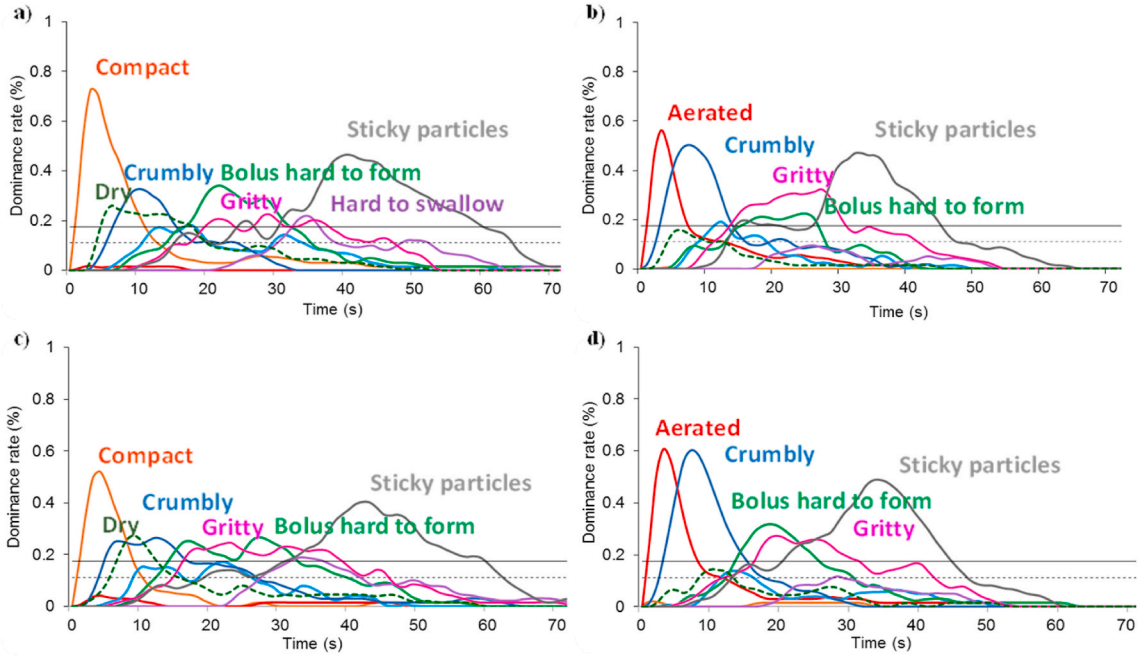

Fig. 3. Dominance curves of attributes compact (—), aerated (—), roughness (—), crumbly (—), bolus hard to form (—), hard to swallow (—), gritty (—), sticky particles (—) and dry (– –) obtained by TDS when consuming gluten-free breads: a) H85F30, b) H85F75, c) H100F30 and d) H100F75. Dotted black line indicates the chance level and solid black line, significance level. H85 and H100: addition of 85 or 100 g of water/100 g of the flour and starch blend, respectively. F30 and F75: fermentation time of 30 or 75 min, respectively.

provide a structural network (Espinosa-Ramírez et al., 2018). In our study, we showed that gluten absence and the processing can change the breakdown pattern, as longer fermentation time produced crumblier gluten-free breads. Furthermore, during eating, saliva is incorporated to foods until a safe and comfortable-to-swallow bolus is formed (Panouillé et al., 2014). At the end of the mastication, samples with lower initial hydration required more saliva incorporation, during mastication, to achieve the critical moisture level to swallow. Bolus consistency and adhesiveness were lower for long fermentation gluten-free bread bolus and higher for short fermentation and low hydration gluten-free bread bolus. Previous authors hypothesised that bolus' mechanical properties depend on the initial water content and absorbing capacity (Jourdren, Panouillé, et al., 2016). In this study, the initial hydration level and fermentation time influenced the structure, because bread boli with short fermentation were more consistent and adhesive. The plasticiser role of water contributed to this behaviour, as bread de-structuration has been related to hydration and crumb fragmentation (Le Bleis, Chaunier, Montigaud, & Della Valle, 2016).

Oral activity also varied among the gluten-free breads studied. Short fermentation breads required more time and chews to form a swallowable bolus, because breads were denser, harder, less breakable. This agrees with previous studies that showed dry and hard products required more chewing cycles and time to form a swallowable bolus (Tournier et al., 2012; Engelen, Fontijn-Tekamp, & Van Der Bilt, 2005).

Structural changes also affected the sensations dynamics, different among breads at different times. These differences stand out at the beginning of the mastication, where longer fermented breads were perceived as more aerated and less dry, improving the sensory characteristics of gluten-free breads. As the mastication of the gluten-free breads progressed, all provided similar sensations. Previous studies have shown that at the beginning of the mastication the initial structure is still somewhat intact (Gao, Tay, Koh, & Zhou, 2018), having a higher impact on the sensory perception. In this study, these differences seem to be diluted as the mastication progressed, in contrast from previous studies with different commercial gluten-free breads, where sensations differed along mastication (Puerta et al., 2020). Comparing sensations of commercial gluten-free breads with structures like regular bread, authors observed perceptual changes at the beginning (soft and spongy vs. dry and crumbly), at the middle (compact vs. sandy), and the end (pasty, sticky vs. sticky) of mastication (Puerta et al., 2020). The difference in perception between the commercial gluten-free breads and the breads of this study can be attributed to the diverse ingredients added in commercial gluten-free breads; for example, the flour; or starches blends; hydrocolloids, and fats. In this study, the recipe was kept constant and only breadmaking conditions were altered, but further investigation might be needed to improve the perceived texture sensations along the entire mastication process, not only at the beginning.

5. Conclusions

Changes in the structure of gluten-free breads were achieved by varying two conditions: water hydration and fermentation time. Both conditions influenced mechanical properties of food boli, but only fermentation time influenced oral behaviour and sensations perceived during mastication.

Oral processing was enlightened by a better understanding of the bread structure and has helped to comprehend the initial bolus formation, related with different sensations as compact or aerated. Furthermore, the dominant sensations were not discriminant, although the bolus had a different consistency before swallowing.

The implication of this study for the gluten-free bread industry is that baking conditions, such as fermentation time, can improve gluten-free bread's sensory characteristics regarding texture, creating more aerated sensations that are usually missed in gluten-free breads. However, further investigation, such as reformulation, might be needed to improve textural sensations along the entire mastication process.

CRediT authorship contribution statement

P. Puerta: Formal analysis, Investigation, Writing - original draft. **R. Garzón:** Formal analysis, Investigation, Writing - original draft. **C.M. Rosell:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **S. Fiszman:** Conceptualization, Writing - original draft. **L. Laguna:** Conceptualization, Resources, Writing - original draft. **A. Tárrega:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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