International Degree of Doctor in Food Science

Programa de Doctorado en Ciencias de la Alimentación

Starch-based matrices: viscoelastic behavior and their effect on starch digestibility by using different methodologies

Matrices con base almidón: comportamiento viscoelástico y su efecto en la digestibilidad del almidón utilizando diferentes metodologías.

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List of original publications

The present thesis is based on the following publications:

1. Santamaria, M., Garzon, R., & Rosell, C. M. (2023). Chapter: Gluten free bakery products. In *ICC Handbook of 21st Century Cereal Science and Technology*. G. Schleining, P. Shewry, H. Koksel, J. Taylor (Eds.), Elsevier, 1st Edition May 1, 2023.

2. Santamaria, M., Garzon, R., Moreira, R., & Rosell, C. M. (2021). Estimation of viscosity and hydrolysis kinetics of corn starch gels based on microstructural features using a simplified model. *Carbohydrate Polymers*, *273*, 118549.

3. Santamaria, M., Montes, L., Garzon, R., Moreira, R., & Rosell, C. M. (2022). Unraveling the impact of viscosity and starch type on the *in vitro* starch digestibility of different gels. *Food & Function*, *13*(14), 7582-7590.

4. Santamaria, M., Montes, L., Garzon, R., Moreira, R., & Rosell, C. M. (2022). Performance of starch gels on *in vitro* enzymatic hydrolysis assessed by rheological methodologies. *Starch - Stärke*, *75*(1-2), 2200189.

5. Santamaria, M., Garzon, R., & Rosell, C. M. (2023). Impact of starch-hydrocolloid interaction on pasting properties and enzymatic hydrolysis. Submitted.

“There is only one thing that makes a dream impossible to achieve: the fear of failure”

Paulo Coelho

Als meus pares, el meu germà Vicent i Joan

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RESUMEN GLOBAL

Introducción

El gluten está formado por un conjunto de proteínas conocidas como gliadinas y gluteninas presentes en los cereales. Se encuentra en las especies *Triticum, Triticeae* (cebada y centeno), trigos híbridos y alguna variedad de avena. Este compuesto tiene una funcionalidad tecnológica importante en los productos basados en cereales debido a sus propiedades viscoelásticas. Para simular su función tecnológica, en los productos de panadería sin gluten, se utilizan otros ingredientes y/o aditivos como: hidrocoloides, proteínas, enzimas o almidones modificados. Sin embargo, la ingestión de gluten en individuos genéticamente predispuestos induce a padecer la enfermedad celiaca. La prevalencia mundial de dicha patología es del 1 al 3%, y su tratamiento se basa en seguir una dieta sin gluten. La Comisión del Codex Alimentarius define un producto sin gluten aquel que no supera los 20 mg/kg. Asimismo, la enfermedad celiaca está relacionada con la diabetes mellitus tipo 1, la cual se basa en una deficiencia de insulina debida a la pérdida de células β-pancreáticas, lo que provoca que la glucosa no entre en las células, dando lugar a hiperglucemia. A su vez, se asocia con posibles enfermedades cardiovasculares.

Los productos sin gluten están formulados principalmente por mezclas de harinas y almidones. Desde un punto de vista nutricional, estos alimentos son deficientes en proteínas, fibra dietética, vitaminas y minerales, pero son ricos en grasas e hidratos de carbono. El elevado contenido de almidón en estos productos ha generado muchas investigaciones sobre la digestión del almidón debido a su asociación con el índice glucémico. El índice glucémico (IG) es una medida basada en la rapidez con que se incrementa el nivel de glucosa en la sangre tras la ingesta de un alimento. Según el IG, los alimentos se clasifican en bajo (≤55%), medio (55-69%), o alto (≥70%). Existen distintos factores que afectan a la respuesta postprandial, como el procesado del alimento, sus propiedades, los nutrientes que contienen, la viscosidad del quimo durante la digestión, los inhibidores enzimáticos o la composición del almidón. Todo ello ha impulsado a la industria alimentaria a desarrollar alimentos sin gluten saludables que aporten nutrientes de calidad, mejoren la saciedad o disminuyan la respuesta glucémica, llevando a cabo estrategias que reduzcan la velocidad de digestión del almidón.

El almidón es uno de los ingredientes más utilizados para la elaboración de productos con base de cereales, debido a su porte energético y sus amplias aplicaciones tecnológicas como espesante, gelificante o estabilizante. El almidón está formado por dos polisacáridos, amilosa y amilopectina, los cuales se unen mediante enlaces α-1,4, que se interconectan con enlaces α-1,6, dando lugar a estructuras ramificadas. La amilosa es el compuesto minoritario (25-28%) y muestra una estructura lineal, mientras que la amilopectina es el componente

mayoritario (72-75%) y presenta una estructura ramificada. A nivel mundial, la principal fuente de almidón es el maíz (82%), seguido del trigo (8%), la patata y la tapioca (5%). Sin embargo, otras fuentes de interés incluyen el arroz, guisante, garbanzo, boniato, sorgo, o cebada. Cabe decir que, las características del almidón pueden variar en función de su origen botánico, su estructura y/o el empaquetamiento entre los gránulos. La morfología de los gránulos de almidón puede ser ovalada, redonda, esférica, angular o elipsoidal y el tamaño granular oscila entre 1 y 100 µm. Según la cristalinidad del almidón, se clasifican en almidón de tipo A, presente en los cereales, almidón de tipo B en los tubérculos y almidón de tipo C, que es una mezcla de las formas de tipo A y B y se encuentra en leguminosas.

Sus aplicaciones funcionales se deben principalmente al fenómeno conocido como gelatinización. Este proceso ocurre cuando el almidón en presencia de agua se somete a una temperatura de calentamiento. Durante la gelatinización, se produce el hinchamiento de los gránulos de almidón y, simultáneamente, la lixiviación de la amilosa. Tras la gelatinización, las moléculas de almidón se someten al proceso de retrogradación, el cual consiste en la formación de una estructura de doble hélice resultante de la reorganización de las cadenas lineales de la amilosa. Estos fenómenos originan cambios en las características de la matriz. A partir de una pasta viscosa/líquida dan lugar a la formación de un gel. En el caso del almidón, los geles consisten en una red tridimensional que posee una cantidad variable de agua, obteniendo matrices con diferentes características. La viscosidad mide la resistencia del material frente al flujo y deformación, viéndose afectada por parámetros como la temperatura, velocidad de cizallamiento y/o presión. La pasta de almidón se define como no newtoniana, por lo que la viscosidad cambia con la temperatura y la tensión de cizallamiento aplicada. Como se ha comentado anteriormente, los procesos de gelatinización y retrogradación dan lugar a la viscosidad en las suspensiones de almidón. Por ello, se dispone de varias técnicas para determinar las propiedades viscosimétricas del almidón, por ejemplo, viscosímetros (de flujo capilar, rotacional o vibracional), el analizador rápido de viscosidad (RVA), el analizador de fuerza rápida (RFA) o el reómetro dinámico.

Dada la importancia de los geles de almidón en el procesado de los alimentos y al nivel de la respuesta postprandial, se han desarrollado distintas metodologías para determinar las características de estos geles: análisis de textura (dureza, elasticidad, cohesividad y masticabilidad), propiedades viscoelásticas, propiedades de hinchamiento, análisis de microestructura, análisis de estructura cristalina y digestibilidad *in vitro*, entre otras. Dichas características de los geles tienen un gran impacto en la calidad de los alimentos y sus propiedades o atributos sensoriales. Asimismo, la reología de los sistemas alimentarios tiene impacto sobre la digestión de los alimentos. La digestión se define como un proceso fisiológico esencial para los seres humanos, el cual se basa en la absorción de nutrientes de los alimentos ingeridos. A lo largo de la digestión oro-gastrointestinal, el almidón

sufre muchos cambios físicos y químicos a causa de la acción enzimática y por los cambios del pH en el medio. La primera fase es la oral, donde los alimentos se mezclan con la saliva. En ella se inicia la hidrólisis del almidón mediante la alfa-amilasa salivar a pH 6-7. Seguidamente, ocurre la fase gástrica durante una o dos horas, en la cual, los alimentos se rompen en fragmentos más pequeños. En esta etapa el pH cambia a 1,5-3,5, y como consecuencia del medio ácido la enzima alfa-amilasa salivar se inactiva, y se hidrolizan las proteínas. Los alimentos que se disgregan en forma de partículas grandes y con alta viscosidad ralentizan el vaciamiento gástrico. Tras esta etapa, se produce la fase intestinal. En ella el almidón es sometido a una hidrólisis enzimática catalizada por la actuación de la alfa-amilasa pancreática y la alfa-glucosidasa en el intestino delgado. En dicha fase se genera glucosa que se absorbe y pasa al torrente sanguíneo. Las fracciones resultantes de la digestión del almidón según su susceptibilidad a ser hidrolizadas se clasifican en: almidón rápidamente digerido (RDS), el cual se digiere durante los primeros 20 minutos y se relaciona con un aumento rápido de los niveles de glucosa en sangre; almidón lentamente digerido (SDS), el cual se hidroliza lentamente en el intestino delgado entre los 20-120 minutos tras la digestión; y el almidón no hidrolizado tras los 120 minutos de digestión, que se define como almidón resistente (RS). Esta fracción es fermentada por las bacterias intestinales a ácidos grasos de cadena corta, principalmente acetato, propionato y butirato, los cuales se han descrito como beneficiosos para la salud. A nivel nutricional el almidón de digestión lenta se ha relacionado con una menor respuesta glucémica postprandial, mientras que el almidón resistente es considerado como fibra dietética.

Dada la relación existente entre las propiedades del almidón y su susceptibilidad a sufrir hidrólisis enzimática, así como el impacto sobre los niveles de glucosa en sangre, la investigación sobre los cambios en el almidón tiene un gran interés por su relación con los procesos metabólicos que suceden a lo largo de la digestión humana. Se han propuesto diversas estrategias para modular la digestión del almidón y así reducir la respuesta glucémica. Entre ellas la modificación de las propiedades del almidón mediante tratamientos fisicoquímicos, o la adición de otros ingredientes funcionales. Sin embargo, la digestión del almidón no solo se ve influenciada por sus propiedades intrínsecas, sino que las propiedades físicas del medio también podrían afectar a la difusión de las enzimas y su accesibilidad hacia los gránulos de almidón. Por un lado, se han descrito algunas propiedades intrínsecas del almidón con un gran impacto en la digestión como el origen, tamaño granular o el contenido de amilosa. Por otro, en la digestión es importante diferenciar el estado en el que se encuentra el almidón, es decir, si está en forma nativa o gelatinizado. Los gránulos de almidón nativos son más resistentes a la actividad enzimática frente a los almidones gelatinizados. Por este motivo, se aplican diversos tratamientos como: enzimáticos, físicos o químicos para modificar las propiedades de los almidones nativos. Sin embargo, una de las prácticas más comunes ha sido la incorporación de ingredientes funciona-

les como polisacáridos, proteínas, lípidos o polifenoles. Además, se han descrito algunos mecanismos mediante los cuales las fibras solubles pueden reducir la hidrólisis del almidón: (i) creando una barrera en la superficie del gránulo para la acción enzimática y restringiendo la lixiviación de la amilosa, (ii) generando una red alrededor de los gránulos de almidón que limita el acceso de las enzimas o (iii) aumentando la viscosidad del alimento, lo que ralentiza la liberación de glucosa. A su vez, los complejos proteína-almidón o lípido-almidón pueden encapsular a los gránulos de almidón creando una barrera que impida la accesibilidad enzimática.

La información previamente expuesta pone de manifiesto la importancia de la digestibilidad del almidón y su impacto sobre el metabolismo. Es por ello por lo que se han descrito diferentes metodologías para evaluar la digestión de los alimentos. Aparte de las consideraciones éticas, los métodos *in vivo* con animales o humanos, presentan diversos inconvenientes como la dificultad de controlar la digestibilidad y biodisponibilidad de los nutrientes, el elevado coste, y tiempos largos de análisis. Por este motivo, se han desarrollado otras metodologías *in vitro*, aunque la variedad de condiciones experimentales que se describen en la literatura científica dificulta la realización de comparaciones y limita el alcance de las conclusiones. En 1992 se definió uno de los métodos más extendidos para evaluar la digestión de carbohidratos propuesto por Englyst *et al*. (1992). Posteriormente, a nivel internacional (COST INFOGEST) se propuso un método estandarizado de digestión oro-gastrointestinal *in vitro* (Minekus *et al*., 2014). Cabe resaltar que estos protocolos recomiendan cuantificar la hidrólisis del almidón solamente en la fase intestinal. No obstante, se han aplicado otros métodos indirectos basados en el registro del comportamiento reológico para estudiar la digestión.

Como se ha comentado anteriormente, los hidrocoloides aportan propiedades tecnológicas y nutricionales que justifican su amplia utilización en la industria alimentaria, por ejemplo, en productos como sopas, salsas, helados, mermeladas, postres gelificados, pasteles, etc. Los hidrocoloides son polímeros de cadena larga compuestos por polisacáridos y proteínas. Proporcionan una gran capacidad para unir moléculas de agua debido a la presencia de grupos hidroxilo, dando lugar a geles con mayor viscosidad. Esta propiedad hace que posean una gran diversidad de aplicaciones como gelificantes, espesantes, emulsionantes o estabilizantes. Existe una gran variedad de hidrocoloides con estructuras químicas muy diversas, por lo cual una de las clasificaciones que se utiliza es basada en su origen. Existen hidrocoloides de origen vegetal (celulosa, pectina, goma guar, goma garrofín), animal (gelatina, proteínas del suero, quitosano), algas (agar, carragenina), bacterias (goma xantana) o procedentes de modificaciones químicas de otros compuestos como la celulosa. Asimismo, están atrayendo mayor interés debido a los beneficios nutricionales que pueden aportar, por ejemplo, como prebióticos o aumentando en el tiempo de vaciado gástrico, así como favoreciendo la digestión de nutrientes. Es por ello por lo que se ha relacionado la viscosidad

originada por los hidrocoloides con un posible impacto en la respuesta glucémica. Específicamente, se ha postulado que el aumento de la viscosidad de los sistemas alimentarios originado por la adición de hidrocoloides afecta a la movilidad intestinal, disminuye la transferencia de masa y dificulta la actividad enzimática, con la consiguiente reducción de la liberación de glucosa en el intestino delgado. No obstante, hay una escasa información sobre el papel fundamental que tiene la viscosidad en matrices alimentarias con su velocidad de digestión.

Objetivos y metodología

El objetivo principal de la presente tesis doctoral fue estudiar el impacto de la viscosidad de geles de almidón sobre la digestión. Para ello se formularon diferentes matrices, simples o binarias, que permitieran entender el impacto de las propiedades intrínsecas de los geles de almidón, o bien del medio, sobre su susceptibilidad a la digestión utilizando diversas metodologías enzimáticas para evaluar su hidrólisis.

Para alcanzar el objetivo principal, se definieron los siguientes objetivos específicos:

1. Analizar las estrategias utilizadas para la obtención de productos alimentarios sin gluten saludables, y relación con en el índice glucémico de estos productos.

Se realizó una revisión sobre la situación actual de las diversas estrategias, tanto tecnológicas como nutricionales, que se han empleado para obtener productos sin gluten de calidad y saludables. La gran mayoría de estos productos están compuestos por almidón, lo cual se relaciona con altos índices glucémicos debido a una dieta rica en carbohidratos.

2. Identificar el impacto de la viscosidad y microestructura de los geles de almidón sobre su hidrólisis enzimática, mediante sistemas simples, elaborados únicamente por almidón.

Se prepararon geles de almidón de maíz utilizando diferentes concentraciones (1:4 a 1:16), con el objetivo de tener sistemas homogéneos con diferentes viscosidades, pero sin la adición de ningún otro compuesto que pudiera afectar en su hidrolisis enzimática. Por un lado, se analizaron las propiedades de los geles (viscosidad y microestructura), y por otro lado su digestibilidad simulada mediante una digestión oro-gastrointestinal y una hidrólisis enzimática *in vitro* con alfa-amilasa pancreática.

3. Validar la relación entre las propiedades viscoelásticas de geles de almidón con su hidrólisis enzimática *in vitro* mediante la utilización de almidones de distinto origen.

Se utilizaron geles de almidón de maíz, trigo y arroz. Se prepararon dos tipos de

muestras: (VV) geles con la misma concentración (1:4) con viscosidad variable y (CV) geles con diferentes concentraciones (1:4, 1:5,5 y 1:5,2) con el fin de obtener viscosidades constantes. Se analizó el comportamiento reológico de los geles, el cual se correlacionó con los parámetros obtenidos tras su hidrólisis enzimática.

4. Desarrollar métodos reológicos rápidos y continuos para evaluar el comportamiento del almidón durante la gelatinización y su digestión enzimática.

Se pusieron a punto dos metodologías reológicas, utilizando el analizador rápido de viscosidad (RVA) y el reómetro, con el fin de estudiar el comportamiento de la viscosidad de los geles durante ciclos de calentamiento y enfriamiento. Además, se registró la caída de viscosidad tras la adición de la alfa-amilasa debido a la hidrólisis del almidón, lo que se definió como "digestograma". Finalmente, estos datos se modelizaron y se correlacionaron con los datos obtenidos en la hidrólisis enzimática mediante la cuantificación de glucosa.

5. Explorar la relación entre la viscosidad de sistemas binarios (almidón-hidrocoloide) con su hidrólisis enzimática mediante el método reológico rápido desarrollado.

Se utilizaron sistemas binarios con diferentes almidones (maíz, trigo, arroz, patata, guisante y tapioca) e hidrocoloides (goma garrofín, goma xantana, goma guar, hidroxipropilmetilcelulosa y psyllium) a distintas concentraciones (0% - 0,5% - 2,5%). Se registró el comportamiento viscoelástico durante la gelatinización de los geles y la caída de viscosidad tras la adición de alfa-amilasa. Se correlacionaron los parámetros reológicos con la velocidad de hidrólisis del almidón (k).

Resultados y discusión

El mercado de los productos sin gluten está en continuo cambio debido al incremento de la demanda por parte de los consumidores. Este interés creciente por los productos libres de gluten se ha relacionado con el aumento en la incidencia de enfermedades relacionadas con la ingesta de gluten, y con creencias sobre la relación directa entre alimento sin gluten y dieta saludable. El gluten es una fracción proteica que desempeña un papel tecnológico fundamental en los productos derivados de cereales, especialmente, en los productos de panadería. Las formulaciones de estos productos sin gluten se basan principalmente en mezclas de harinas y/o almidones. El almidón permite retener el dióxido de carbono que se produce durante la fermentación de la masa, generando una estructura similar a la miga que tienen los productos con gluten. Además, se adicionan otros ingredientes como, hidrocoloides, proteínas, enzimas o almidones modificados, los cuales imitan las propiedades viscoelásticas del gluten debido a su capacidad para unir moléculas de agua y formar estructuras tridimensionales, dando lugar a panes con mejores propiedades tecnológicas (mayor contenido de humedad, textura o volumen), sensoriales y alargar la vida útil de los productos sin gluten.

Inicialmente, las investigaciones sobre el desarrollo de los alimentos sin gluten se centraron en aspectos tecnológicos, tratando de superar las barreras que suponía la sustitución o el reemplazo del gluten durante su procesamiento. Sin embargo, una vez superados dichos retos mediante la utilización de los ingredientes anteriormente mencionados, el interés se centró en la mejora de la calidad nutricional de los productos sin gluten. Estos alimentos incluyen un gran número de productos en base almidón con un elevado contenido en carbohidratos y grasas, y menor contenido en proteínas. Por ello, existe un gran interés en reducir la digestibilidad del almidón, puesto que una dieta alta en carbohidratos se ha asociado con un elevado índice glucémico. Para ello, se están desarrollando estrategias innovadoras en la elaboración de productos de panadería sin gluten que alteren la digestibilidad del almidón. Por ejemplo, la utilización de almidones de otros orígenes como son las legumbres; o la aplicación de tratamientos físicos como el fraccionamiento de las harinas; o la incorporación de masas madre, ya que se ha relacionado con la generación de ácidos orgánicos (láctico, acético y propiónico) durante la fermentación, asociándolo a panes con menor índice glucémico. Por último, otra estrategia ha sido modificar la viscosidad de las matrices alimentarias mediante la adición de fibras solubles para restringir la accesibilidad de las enzimas.

La relación entre la viscosidad de los sistemas alimentarios y la velocidad de hidrólisis se ha estudiado en matrices complejas mediante la adición de fibras alimentarias. Sin embargo, ningún estudio fundamental ha confirmado el papel que juega la viscosidad de los alimentos con base de almidón en su velocidad de digestión. En la investigación llevada a cabo en esta tesis, se definieron una variedad de matrices alimentarias, las cuales se caracterizaron y analizaron mediante diferentes modelos de digestión *in vitro*. Con ello, se determinaron las posibles correlaciones entre las propiedades viscoelásticas de dichas matrices y su impacto en la hidrólisis del almidón.

La primera matriz se basó en un modelo homogéneo para eliminar las posibles influencias del origen del almidón o la adición de alguna fibra soluble que modificara la viscosidad del medio. Por ello, se prepararon geles de almidón de maíz (almidón:agua) a diferentes concentraciones (1:4 a 1:16) en un analizador rápido de viscosidad (RVA). Como era esperable, el gel más concentrado mostró un pico de viscosidad mayor, ya que el contenido de almidón está vinculado con una mayor viscosidad aparente. Los geles de almidón presentaron una estructura en forma de panal. Entre las concentraciones 1:4 y 1:8, la microestructura fue más cerrada y el número de cavidades fue mayor. Estos sistemas homogéneos mostraron una relación positiva (r = 0,87) entre el número de cavidades y la viscosidad del gel. En cuanto a la cuantificación de las fracciones de almidón tras la digestión de los geles y su velocidad de digestión, los resultados indicaron que los geles más concentrados, es decir, con mayor viscosidad, aumentaron el contenido de almidón de digestión lenta (SDS) y disminuyeron la constante cinética (k).

El almidón de digestión lenta se relaciona con una menor respuesta glucémica. Finalmente, estos resultados indicaron que las propiedades de los geles como la viscosidad o su estructura pudo afectar a la actividad de la alfa-amilasa interactuando entre la enzima y el sustrato.

Estos resultados se basaron en un único tipo de almidón, el almidón de maíz. Para esclarecer la incógnita de si los resultados previamente obtenidos fueron debidos a la viscosidad o a la naturaleza del almidón, se prepararon geles de almidón de maíz, trigo y arroz, estableciendo dos sistemas distintos: controlando la relación de agua para obtener geles de viscosidad variable (VV) o geles de viscosidad constante (CV). En los geles VV, el almidón de maíz presentó una gelatinización más rápida y el almidón de trigo una fuerza máxima superior. Además, estos geles mostraron un comportamiento de geles tipo sólido ($G' > G''$), siendo el de trigo el que mayor modulo elástico (G') presentó durante su enfriamiento. Asimismo, el gel de maíz presentó un mayor contenido de almidón lentamente digerido y una menor velocidad de hidrólisis (k). En cambio, en los geles CV, el comportamiento reológico fue parecido pese a sus distintos orígenes y mostraron valores similares en el módulo viscoso (G''). Además, obtuvieron valores similares en el contenido de almidón lentamente digerido y en sus constantes cinéticas (k). Estos resultados volvieron a confirmar que la viscosidad de las matrices puede impedir la transferencia de masa afectando a la velocidad de digestión.

Por último, se analizaron matrices más complejas formadas por almidones de cereales, tubérculos y legumbres, junto con distintos hidrocoloides. Las suspensiones de hidrocoloides al 2% a 25 ºC mostraron diferencias significativas en sus viscosidades: psyllium > goma guar > goma xantana > goma garrofín > hidroxipropilmetilcelulosa. Los hidrocoloides mostraron un efecto sinérgico en la viscosidad del almidón de patata durante su calentamiento y enfriamiento. El almidón de patata presentó parámetros viscosimétricos más elevados que otros almidones, lo que se correlacionó con posibles enlaces covalentes entre los grupos fosfato presentes en dicho almidón. Por el contrario, los almidones de guisante y tapioca mostraron una menor viscosidad y por ende mayor velocidad de hidrólisis (k). Por otro lado, la incorporación de hidrocoloides modificó las propiedades de pasta de los geles. En general, se obtuvieron correlaciones negativas entre los parámetros viscosimétricos y la constante cinética. Finalmente, los sistemas binarios (almidón-hidrocoloide) presentaron mayores cambios en la velocidad de hidrólisis en el caso de almidón de patata junto con la goma xantana o el psyllium. Este resultado se asoció con la dificultad entre la interacción enzima-sustrato.

Para el estudio de estos sistemas con base de almidón se utilizaron diversas metodologías de hidrólisis enzimática *in vitro*. En primer lugar, los geles de maíz a diferentes concentraciones se analizaron mediante una digestión oro-gastrointestinal basado en la metodología INFOGEST. Sin embargo, este tipo de metodología no permitió el seguimiento de la hidrólisis del almidón en las distintas

etapas de la digestión oro-gástrica simulada. Por esta razón, los geles de almidón se hidrolizaron enzimáticamente con alfa-amilasa pancreática y su cinética se evaluó mediante la cuantificación de la liberación de glucosa durante 3 horas de ensayo.

Los métodos comentados anteriormente son metodologías que requieren tiempos prolongados, por ese motivo se desarrolló un método analítico rápido y continuo basado en medidas reológicas. Se utilizó el analizador rápido de viscosidad (RVA) y el reómetro. Además de registrar el comportamiento de los geles durante los ciclos de enfriamiento y calentamiento, la fase definida como "digestograma" permitió medir la hidrólisis enzimática del almidón registrando la viscosidad aparente (μ). La adición de la alfa-amilasa originó una caída de viscosidad generando un cambio de un gel sólido a un gel parcialmente fluido. Asimismo, se utilizó un modelo cinético de primer orden para modelizar los resultados y predecir la velocidad de hidrólisis (k) de la digestión de los geles de almidón, el cual se validó utilizando almidones de distinto origen. El almidón de maíz no presentó un buen ajuste, lo cual se atribuyó a la variación del pH observada en la suspensión, que pudo afectar a la actividad enzimática. Dada la importancia del pH en los ensayos enzimáticos, se modificó el método rápido para mantener el pH durante el análisis. Los resultados obtenidos en los métodos reológicos rápidos se correlacionaron con los análisis realizados mediante la cuantificación de glucosa. En este caso, los geles de arroz y trigo mostraron un buen ajuste entre las tres metodologías realizadas.

Conclusiones

La investigación realizada a través de los distintos capítulos permite concluir que la viscosidad de los sistemas compuestos principalmente por geles de almidón, influye significativamente en la velocidad de su digestión. Este efecto podría utilizarse como una estrategia para diseñar y formular productos sin gluten saludables que generen una menor respuesta glucémica, especialmente aquellos donde el almidón sea el ingrediente mayoritario.

En particular, se pueden destacar las siguientes conclusiones:

- Una vez optimizados los productos sin gluten a nivel tecnológico, es importante mejorar la calidad nutricional. Su elevado contenido en almidón se ha relacionado con una mayor respuesta glucémica. Se precisan estudios que relacionen los cambios estructurales del almidón con su impacto en la digestión, con el fin de reducir el índice glucémico tras el consumo de alimentos libres de gluten.

- La viscosidad en geles de almidón tiene un papel fundamental en su microestructura y digestión enzimática. Mediante la preparación de geles de almidón de maíz a distintas concentraciones, fue posible desarrollar un modelo simple que relacionara las propiedades macroestructurales y microestructurales con la

cinética de hidrólisis de los geles. El modelo mostró una relación lineal ($r = 0,98$) entre la estructura porosa (tamaños de las cavidades y grosor de las paredes) de los geles de almidón y su viscosidad. Estos resultados podrían aplicarse en el diseño de formulaciones alimentarias destinadas a la disminución de glucosa postprandial.

- La viscosidad desempeña un papel fundamental en las características en los geles de almidón y en la predicción de su comportamiento durante la digestión. Los geles de almidón elaborados a partir de distintos cereales (maíz, trigo, arroz) mostraron viscosidades significativamente diferentes cuando se elaboraron a partir de concentraciones constantes de almidón. Sin embargo, la fuerza resultante durante la gelatinización, las propiedades viscoelásticas y la tasa de hidrólisis de los geles de almidón se aproximaron cuando se modificaron las concentraciones de los geles, con el fin de obtener viscosidades similares. Por lo tanto, la viscosidad del gel podría ser un indicador rápido para estimar la hidrólisis cinética del almidón.

- Se desarrollaron pruebas rápidas y continuas para analizar el rendimiento de la gelatinización y digestión de diferentes geles de almidón. Los cambios de viscosidad fueron registrados mediante el RVA o reómetro, seguidos de una caída de viscosidad debido a su hidrólisis tras la adición de alfa-amilasa. Los gráficos, llamados digestogramas, se ajustaron mediante un modelo cinético de primer orden para predecir la digestión de los geles. Los geles elaborados con almidones de maíz, trigo y arroz confirmaron la validez de los métodos.

- Se ha mostrado la relación entre la viscosidad y la cinética de hidrólisis del almidón utilizando sistemas binarios formado por almidones e hidrocoloides. Los geles de cereales y patata mostraron una mayor viscosidad y una menor constante cinética, pero los geles de tapioca y guisante mostraron el comportamiento opuesto. En cuanto a los hidrocoloides, su impacto en la hidrólisis enzimática del almidón dependió en gran medida del tipo de almidón y del hidrocoloide, incluso de su concentración. La matriz de correlación confirmó las relaciones negativas entre la velocidad de hidrólisis (*k)* de los geles y su viscosidad a 37 ºC. Esta relación podría utilizarse como predictor de la susceptibilidad del almidón o del almidón-hidrocoloide a la hidrólisis enzimática mediante una prueba de viscosidad rápida.

ABSTRACT

Gluten free foods have been recognized as carbohydrate-rich, particularly high starch content, which has been related to high glucose postprandial levels. Numerous strategies have been applied to reduce starch enzymatic hydrolysis, but less attention has been paid to modulate the enzyme accessibility to starch. Some studies suggested the relationship between viscosity and starch digestibility, but without concluding results. The objective of this doctoral thesis was to determine the impact of the viscosity of the starch systems on their enzymatic hydrolysis rate (k), using different *in vitro* methodologies and starch-based systems. The different viscosity resulting from corn starch gels prepared at different concentrations indicated that higher viscosity led to more compact structure and reduced hydrolysis. Starch gels from corn, rice and wheat with constant viscosities displayed similar viscoelastic properties as well as hydrolysis rates, confirming the important role of viscosity on enzyme accessibility. Considering the importance of predict starch enzymatic hydrolysis, rapid and continuous rheological methods were developed based on the changes on the apparent viscosity after adding alpha-amylase. Furthermore, this methodology was tested with heterogeneous systems consisting of blends of different starches (corn, wheat, rice, potato, cassava, pea) and hydrocolloids (locust bean gum, guar gum, xanthan gum, hydroxypropylmethylcellulose, psyllium). A significant negative correlation (r = -0.55) between viscosity at 37 ºC and the kinetic hydrolysis rate (k) was obtained, particularly high in the system of xanthan gum with potato starch (r = -0.75). Therefore, viscosity of starch-based gels could be used as a predictor of their enzymatic hydrolysis, which could be assessed using rapid methods. Overall, the relationship between the viscosity of starch-based systems and their hydrolysis by alpha-amylase, revealed the importance of the system viscosity on the enzyme accessibility to starch. This result could be fundamental when designing starch-based foods that generated low postprandial glucose response.

RESUMEN

Se ha observado que los alimentos libres de gluten son ricos en hidratos de carbono, especialmente por su elevado contenido en almidón, lo cual se ha relacionado con altos niveles de glucosa postprandial. Se han desarrollado numerosas estrategias para reducir la hidrólisis enzimática del almidón, aunque no se ha profundizado en como modular la accesibilidad enzimática al almidón. Algunos estudios sugirieron una relación entre la viscosidad y la digestibilidad del almidón, pero sin resultados concluyentes. El objetivo de esta tesis doctoral fue determinar el impacto de la viscosidad de los sistemas de almidón en su tasa de hidrólisis enzimática (k), utilizando diferentes metodologías *in vitro* y sistemas con base de almidón. Los geles de almidón de maíz preparados a diferentes concentraciones mostraron diversas viscosidades. Los resultados presentaron que una mayor viscosidad daba lugar a geles con una estructura más compacta y con ello una menor hidrólisis. Los geles de almidón de maíz, arroz y trigo con viscosidades constantes mostraron propiedades viscoelásticas y tasas de hidrólisis similares, lo que confirma el importante papel que tiene la viscosidad en la accesibilidad de las enzimas. Dada la importancia de conocer y predecir la hidrólisis enzimática del almidón, se desarrollaron métodos reológicos rápidos y continuos basados en los cambios de la viscosidad aparente tras la adición de alfa-amilasa. Además, esta metodología se utilizó con sistemas heterogéneos basados en mezclas de diferentes almidones (maíz, trigo, arroz, patata, tapioca, guisante) e hidrocoloides (goma garrofín, goma guar, goma xantana, hidroxipropilmetilcelulosa, psyllium). Se obtuvo una correlación negativa significativa (r = -0,55) entre la viscosidad a 37 ºC y la cinética de hidrólisis (k), particularmente dicha correlación fue mayor en el sistema formado por almidón de patata con goma xantana (r = -0,75). En conclusión, la viscosidad de los geles con base de almidón podría utilizarse como predictor de su hidrólisis enzimática, estudiándose mediante métodos rápidos. En general, la relación entre la viscosidad de los sistemas con base de almidón y su hidrólisis debida a alfa-amilasa, reveló la importancia de la viscosidad de la matriz alimentaria en la accesibilidad de la enzima al almidón. Este resultado podría ser fundamental a la hora de diseñar alimentos con base de almidón que generaran una menor glucemia postprandial.

RESUM

S'ha observat que els aliments lliures de gluten són rics en hidrats de carboni, especialment pel seu elevat contingut en midó, la qual cosa s'ha relacionat amb alts nivells de glucosa postprandial. S'han desenvolupat nombroses estratègies per a reduir la hidròlisi enzimàtica del midó, encara que no s'ha aprofundit en com modular l'accessibilitat enzimàtica al midó. Alguns estudis van suggerir la relació entre la viscositat i la digestibilitat del midó, però sense resultats concloents. L'objectiu d'aquesta tesi doctoral fou determinar l'impacte de la viscositat dels sistemes de midó en la seua taxa d'hidròlisi enzimàtica (k), utilitzant diferents metodologies *in vitro* i sistemes a base de midó. Els gels de midó de dacsa preparats a diferents concentracions presentaren diverses viscositats, cosa que, mostrà que una major viscositat donava lloc a una estructura més compacta i amb això una hidròlisi menor. Els gels de midó de dacsa, arròs i blat amb viscositats constants ensenyaren similars propietats viscoelàstiques i taxes d'hidròlisis, la qual cosa confirma l'important paper que té la viscositat en l'accessibilitat dels enzims. Donada la importància de conèixer i predir la hidròlisi enzimàtica del midó, es van desenvolupar mètodes reològics ràpids i continus basats en els canvis de la viscositat aparent després de l'addició d'alfa-amilasa. A més, aquesta metodologia es va utilitzar amb sistemes heterogenis basats en mescles de diferents midons (dacsa, blat, arròs, creïlla, tapioca, pésol) i hidrocol·loides (goma garrofí, goma guar, goma xantana, hidroxipropilmetilcel·lulosa, psyllium). Es va obtindre una correlació negativa significativa (r = -0,55) entre la viscositat a 37 ºC i la cinètica d'hidròlisi (k), particularment aquesta correlació fou major en el sistema format per midó de creïlla amb goma xantana (r = -0,75). En conclusió, la viscositat dels gels a base de midó podria utilitzar-se com a predictor de la seua hidròlisi enzimàtica, a més d'estudiar-se mitjançant mètodes ràpids. En general, la relació entre la viscositat dels sistemes a base de midó i la seua hidròlisi per l'alfa-amilasa, revelà la importància de la viscositat de la matriu alimentària en l'accessibilitat de l'enzim al midó. Aquest resultat podria ser fonamental per al disseny d'aliments a base de midó que generaren una menor glucèmia postprandial.

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ABBREVIATIONS

AMG	Amyloglucosidase
ANOVA	Analysis of variance
AUC	Area under the curve
C∞	Equilibrium concentration of hydrolyzed starch
CD	Coeliac disease
DNS	3,5-dinitrosalicylic acid
DS	Digestible starch
DSC	Differential scanning calorimetry
DWB	Dry weight basis
*e*GI	Expected glycemic index
GF	Gluten free
GI	Glycemic index
GO	Glucose oxidase
GOPOD	Glucose oxidase-peroxidase
GPC	Gel permeation chromatography
HI	Hydrolysis index
HPMC	Hydroxypropylmethylcellulose
k	Kinetic constant / hydrolysis rate
LDS	Fisher's least significant differences test
MALS	Multi angle light scattering
MANOVA	Multivariate analysis of variance
Mw	Molecular weight
NPS	Non-starch polysaccharides
PCA	Principal component analysis
RDS	Rapidly digestible starch
RFA	Rapid force analyzer
RS	Resistant starch
RVA	Rapid visco analyzer
SCFA	Short-chain fatty acids
SDS	Slowly digestible starch
SEM	Scanning electron microscopy
T1DM	Type 1 diabetes mellitus
TS	Total starch

SAMPLE CODE

A	Agar
AG	Arabic gum
CA	Cassava
CMC	Carboxymethylcellulose
CO	Corn
CV	Constant viscosity
GG	Guar gum
KG	Konjac glucomannan
LBG	Locust bean gum
P	Psyllium
PE	Pea
PO	Potato
R	Rice
VV	Variable viscosity
W	Wheat
XG	Xanthan gum

INTRODUCTION

1. Gluten free foods and their nutritional and health implications

Gluten consists of three-dimensional proteins, named gliadins and glutenins, present in the *Triticum* species (*T. aestivum, T. dicoccum, T. durum, T. monococcum* and *T. spelta), Triticeae* tribe (barley and rye), wheat hybrids such as triticale, and probably oats (Rosell *et al*., 2014). From a technological point of view, in cereal-based products (bread, pasta, or noodles), gluten has an important functional role in defining their structure. In bakery products, gluten determines dough elasticity and extensibility, as well as appearance, volume, and crumb structure of breads (Gasparre & Rosell, 2022; Zoghi *et al*., 2021).

Coeliac disease (CD) is one of the most common food-induced pathologies. It is an autoimmune disorder generated by gluten digestion in genetically predisposed individuals. Nowadays, coeliac disease treatment is based on keeping a lifelong gluten free diet (Horstmann *et al*., 2017). The worldwide prevalence of CD is 1 to 3% (Gilissen *et al*., 2014). The Codex Alimentarius Commission defines gluten free (GF) as the food that does not exceed 20 ppm (parts per million) of gluten.

As mentioned before, GF diet is currently the unique treatment for coeliac disease. Several studies have suggested that this diet might have negative effects in the nutritional and metabolic status of coeliac patients (Valvano *et al*., 2020). CD and type 1 diabetes mellitus (T1DM) might be somewhat connected. The hypothesis involves that gluten consumption could be an environmental factor in T1DM, altering the function of the gut immune system and its association with the pancreatic immune system (Smyth *et al*., 2008). T1DM consist in an insulin deficiency due to pancreatic β-cell loss, which provokes that the glucose does not enter the cells, leading to hyperglycemia. The prevalence of CD within T1DM sufferers, goes from 3% up to 16% in children, and from 1.4% up to 6.8% in adults (Mahmud *et al*., 2015; Tokatly Latzer *et al*., 2018). Moreover, an association between T1DM and cardiovascular disease has been described and associated with higher risk for cardiovascular events, for example, retinopathy, neuropathy, obesity, and hypoglycemia (Atkinson *et al*., 2014).

In the absence of gluten, other ingredients are used to simulate gluten functionality when making bakery products, such as hydrocolloids, proteins, enzymes, or modified starches. In fact, a GF formulation for making those foods, mainly includes mixtures of GF flours and starches. As a result, GF bakery foods are

deficient in proteins, dietary fiber, vitamins, and minerals components, but they are rich in fats and carbohydrates, namely starch (Bello-Perez *et al*., 2020; Matos Segura & Rosell, 2011). The high starch content in those foods has motivated much research on starch digestibility for their association with a glycemic index (GI) or better referred as postprandial glucose responses (Bello-Perez *et al*., 2020; Jenkins *et al*., 1981; Matos Segura & Rosell, 2011). Glycemic index is defined as the increase in the area under the blood glucose response curve measured in individuals under standard conditions, determined two hours after that the intake of available carbohydrates (50 g). This parameter is expressed as a percentage from the same quantity of carbohydrates regarding reference food (glucose or white bread). There is a classification according to GI foods, low (≤55%), medium (55–69%), or high GI (≥70%) (Krupa-Kozak & Lange, 2019). The postprandial glucose responses are influenced by food process conditions, properties and structure of ingested food, nutrients, chyme viscosity, enzyme inhibitors, and starch composition (Priyadarshini *et al*., 2022). Considering that GF cereal-based foods give rapid postprandial responses, the search for strategies to reduce starch digestion rate is attracting much research (Punia Bangar *et al*., 2022). Frequently, physical treatments are applied to modify starch characteristics, and subsequently to alter starch fractions digestion (Yang *et al*., 2023). Furthermore, in starchy foods, the addition of sourdough has been related to the reduction of starch hydrolysis rate, either due to the generation of organic acids or the system viscosity that could slow down the digestibility (Giuberti & Gallo, 2018).

The relationship between different pathologies and a diet restricted to gluten free foods has driven the industry to develop healthy foods that provide more nutrients, improve satiety, or decrease the blood glucose response (Priyadarshini *et al*., 2022). Following sections will be explained these strategies in more detail.

2. Starch: composition, structure and functionality

One of the main components in cereals is starch, a polysaccharide composed by two polymers of glucose residues named amylose and amylopectin. Both polymers are linked by α-(1,4) bonds creating linear long segments, with α-(1,6) linkages making branch structures. Amylose is the minor component (25-28%) with mostly a linear structure, while amylopectin is more abundant (72-75%) and has a highly branched organization, composed of double helices kept by hydrogen bonds, and van der Waals forces (Bertoft, 2017; Korompokis *et al*., 2021; Wang *et al*., 2022). Starch granules are characterized by a semi-crystalline structure containing crystalline and amorphous lamellae. Amylopectin double helices are in crystalline lamellae, while amylose and amylopectin branch points are present in amorphous lamellae (Balet *et al*., 2019). Other minor constituents of starch

include protein, lipids, and minerals (phosphorous and phosphate), (Copeland *et al*., 2009).

Considering the global starch market, the main source of starch is corn (82%), followed from afar by wheat (8%), potato (5%), and cassava (5%) (Mohamed, 2021). Other commercial sources that are acquiring more interest are rice, pea, chickpea, sweet potato, sorghum, barley, waxy starches (with low or no amylose content), or Hylon (high amylose content) (Vamadevan & Bertoft, 2015). The individual starches have different intrinsic properties according to their original source, which will have a direct impact on their functional properties. The morphology of starch granules can be oval, round, spherical, angular, or ellipsoidal and the granular size are from 1 to 100 μm (Copeland *et al*., 2009). Bajaj *et al*. (2018) studied starch morphology, and the higher granule size was observed in potato starch (35.75 μm) with oval, irregular, or cuboidal shape, while rice starch displayed a smaller granule size (5.41 μm) with pentagonal and angular form. According to starch crystallinity, they are classification into A-type starch, which is present in cereals, B-type starch in the tubers and C-type starch is a mixture of A-type and B-type forms and exists in legumes and rhizomes (Buléon *et al*., 1998; Cui *et al*., 2022; Sinhmar *et al*., 2022; Tester *et al*., 2004; Zobel, 1988).

Starch is a functional ingredient in foods due to its applications as a thickener, stabilizer, gelling agent, and water retention agent. The functional applications are mainly due to starch gelatinization. This process occurs when starch is subjected to heating in the presence of water (**Figure 1**). Starch granules lose their semicrystalline structure (crystalline double-helices chains) and shift to an amorphous state (Ai & Jane, 2015). During the gelatinization process, swelling of starch granules happens, and simultaneously the amylose leaching (Cooke & Gidley, 1992; Garzon & Rosell, 2021; Lund & Lorenz, 1984; Schirmer *et al*., 2015). Those changes could be determined using different methodologies, such as polarized light microscopy coupled with a hot stage, thermomechanical analysis, and nuclear magnetic resonance spectroscopy. Although, the more commonly used is the differential scanning calorimetry (DSC), in which the enthalpy change (ΔH) indicates the energy consumption required for the separation of the crystalline double-helices within the starch granules (Ai & Jane, 2015).

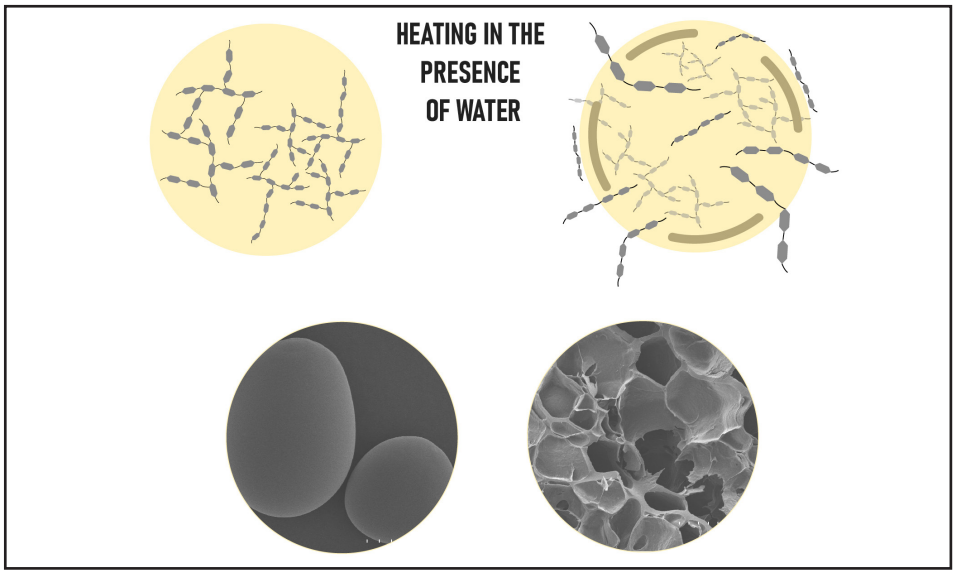

Figure 1: Starch granule structure and its gelatinization. Adapted from Cui *et al*., 2022.

After gelatinization, if the temperature decreases, starch molecules are subjected to the recrystallization or retrogradation process, which consists in the interaction of glucan molecules through hydrogen bond. In the recrystallization process, linear amylose chains can reassociate in a double helical structure at a short rate. Conversely, highly branched amylopectin molecules require longer time to recrystallize, and it consists of short chains combination (Copeland *et al*., 2009). For this reason, retrogradation predisposition of starches is associated with the amylose content in the starchy foods. These phenomena induce the conversion of starch slurries into viscous liquid/pastes and finally into gels (Cui *et al*., 2022; Sinhmar *et al*., 2022). Therefore, the process of transformation of starch granules into digestible food consists of several stages: glass transition, gelatinization, swelling, pasting, and retrogradation (Dona *et al*., 2010).

Starch-based gels are a three-dimensional network of starch containing a variable amount of water. Several methodologies have been used to determine the characteristics of starch-based hydrogels: texture analysis (hardness, springiness, cohesiveness, and chewiness), uniaxial tensile test, viscoelasticity test, swelling properties, structure analysis, crystal structure analysis, and *in vitro* digestibility (Cui *et al*., 2022; Singh *et al*., 2003; Wang & Copeland, 2013). Furthermore, the intrinsic properties of starch, as well as its source, will have an impact on the creation and characteristics of starch gels. Bajaj *et al*. (2018) analyzed starch gels from different sources using the techniques previously mentioned. Tuber starches presented higher viscosity associated with the presence of the esterified phosphate group and displayed extensive granule swelling. Aleixandre *et al*. (2021) reported

the honeycomb or sponge-like structure of starch gels. Pulses gels presented a more irregular structure than cassava or potato gels, which obtained a strong structure. Tuber and pulses gels had thicker wall cells than cereal gels. Conversely, their *in vitro* oro-gastrointestinal digestion revealed that rice gels had faster hydrolysis rate. Probably, the lower amylose content and weaker structure of the rice gels is responsible of that behavior. Similarly, in other gels like chickpea gel, the slower digestion rate was related to its high amylose content or amylose-lipid-protein complexes.

2.1 Viscosity in starch-based systems

Rheological properties of food matrices are fundamental to assess food quality, which is related to their sensory attributes (Steffe, 1996). The rheology or flow properties of the food systems has great influence on the food structure. Specifically, flow properties affect food processing (manufacturing and cooking), as well as the perception and its further performance during digestion (Fischer & Windhab, 2011). Viscosity measures the material resistance against flow and deformation. This property can be affected by different parameters like temperature, shear rate, and pressure. Starch paste displays a non-Newtonian feature, so the viscosity changes with the temperature and shear stress applied. Shear thinning, usually defined as thixotropy, is the non-Newtonian behavior of fluids whose viscosity decrease under a high shear rate in constant state flow (Tabilo-Munizaga & Barbosa-Cánovas, 2005). As mentioned above, during gelatinization the amorphous part of starch absorbs water and develops viscosity resulting in a starch paste. After cooling and storage, the starch paste can develop gels with viscoelastic properties. During cooking, amylopectin is responsible for swelling power and viscosity development. Nevertheless, amylose and other minor components (lipids or phosphate-monoester derivates) interlink with amylopectin and limit the starch granules swelling also affecting pasting performance (Ai & Jane, 2015).

Many techniques are used to determine starch pasting properties, like consistometer, viscometer (capillary flow, rotational or vibrational type), Rapid Visco Analyzer, Rapid Force Analyzer, or dynamic rheometer. Viscosity measurement by rotational viscosimeter is based on the force required to rotate an object immersed in a fluid. In vibrational viscometer, this parameter is related to the amplitude of the vibration changes.

The Rapid Visco Analyzer (RVA) mimics the cooking process of starch slurries, where starch-water suspension is subjected to heating and cooling cycles under a constant shear rate. The RVA records changes in viscosity as a function of temperature and time. In the heating stage, an increase in viscosity is observed.

During gelatinization, the breakdown of hydrogen bonds is produced, and amylose leaches out creating a paste, which consists of dissolved starch molecules, swollen granules, and granule fragments. At this stage, the peak viscosity is reached when a high number of swollen starch granules generates the paste. But after holding at high temperature (95 ºC) a decrease in viscosity is observed due to the melting of crystalline lamellar of starch and the access of water into the granule, with the final rupture of the granule. Finally, in the cooling stage viscosity increase due to starch retrogradation. Amylose and amylopectin chains recrystallization result in a more crystalline structure. The main parameters in RVA are peak viscosity (the highest viscosity during heating), trough viscosity (minimum viscosity after the peak viscosity), and final viscosity (viscosity at the end of the cooling stage). Further parameters are the following, breakdown that represents the viscosity stability during cooking at 95 ºC and it is the difference between peak viscosity and trough; and the setback calculated as final viscosity minus trough, which is related to amylose retrogradation (Balet *et al*., 2019; Mohamed, 2021).

On the other hand, Rapid Force Analyzer (RFA) is based on a rapid test for 90 seconds, employing constant temperature at 100 ºC and continuous stirring of the starch slurry. The equipment records the force changes during starch gelatinization. In this analysis, the force required to stir the starch suspension increases as the starch granules swell. The parameters defined are the initial force related to the slurry consistency, the alpha-slope measures the rate of starch swelling, the maximum force and the final force (Garzon & Rosell, 2021).

Dynamic rheometer measures rheological performance by exposing a viscoelastic liquid to oscillating deformation. The relevant parameters are storage modulus (G') defined as the amount of energy stored in the material, which represents the elastic part of the material, and the loss modulus (G'') described as the quantity of the energy dissipated, which represents the viscous part of the material. The relationship among these parameters is defined by tan δ (G''/G'), which indicates the physical behavior of the system. In starch suspensions, storage modulus (G') gradually increases when heating, which is related to the degree of granule swelling (Singh *et al*., 2003).

3. Starch digestion

Digestion is an essential physiological process, which involves the absorption of energy and nutrients from ingested food (Bornhorst & Singh, 2014). This process begins in the oral phase where food is mixed with saliva. Starch hydrolysis starts with salivary alpha-amylase action at pH 6-7. Then, at the stomach stage, food is broken into small fragments for 1 or 2 hours. The pH change to 1.5-3.5,

so the acidic environment inactivates the salivary alpha-amylase enzyme, and proteins are hydrolyzed. Food with large particle size and high viscosity, slow down gastric emptying. Finally, during 2 hours in the intestinal phase, starch undergoes enzyme hydrolysis in the small intestine by pancreatic alpha-amylase and alpha-glucosidase generating glucose, which goes into the bloodstream (Priyadarshini *et al*., 2022) (**Figure 2**). Furthermore, several factors that affect digestion have been identified: nutritional composition (proteins, lipids, or carbohydrates), food properties, or technological process. Along the oro-gastrointestinal digestion, starch undergoes many physical and chemical changes due to the enzymes' actions and also the stomach pH.

The digestion of starch has been attracting much attention due to its relationship with the postprandial glucose response (Bello-Perez *et al*., 2020). In fact, three different fractions of starch have been defined according to their susceptibility to be hydrolyzed (Englyst & Hudson, 1996). Rapidly digestible starch (RDS) is related to a fast increase in blood glucose level, and it is the amount of glucose released after 20 minutes of digestion. Slowly digestible starch (SDS) is slowly hydrolyzed in the small intestine, requiring between 20 and 120 minutes for its digestion. The starch not hydrolyzed after 120 mins of digestion is defined as resistant starch (RS). This fraction is fermented to short-chain fatty acids (SCFA) in the large intestine (Sajilata *et al*., 2006). Slowly digestible starch is related to moderate postprandial glycemic response, while resistant starch is considered dietary fiber (Wang *et al*., 2022).

Several strategies have been proposed to modulate the carbohydrate digestion, and particularly the starch hydrolysis to reduce the glycemic response. Among those, the reduction of the amount of carbohydrates available for digestion, the decrease of the food digestion rate, or the reduction of the glucose absorption rate. Regarding the reduction of the carbohydrates bioavailability, an important target has been the modification of starch through different approaches: (i) selecting appropriate starch properties, (ii) the modification of starches and (iii) the addition of other functional ingredients (Wee & Henry, 2020). Nevertheless, starch digestion is not only affected by its intrinsic properties, but also physical media properties can modulate enzyme diffusion to starch substrates (Bello-Perez *et al*., 2020; Yang *et al*., 2023).

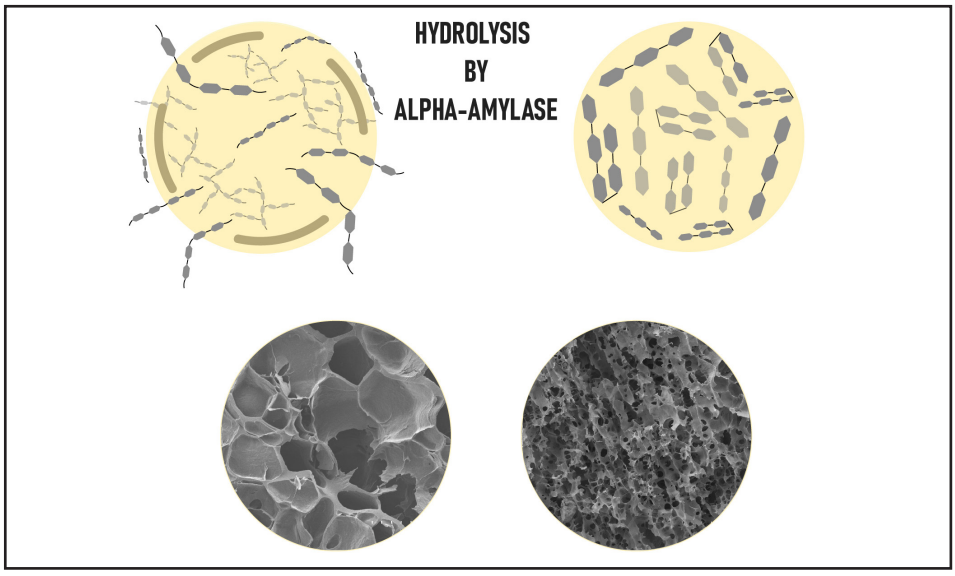

Figure 2: Starch gelatinized and its hydrolysis. Adapted from Korompokis *et al*., 2021.

Regarding starch properties, it has been described that its intrinsic properties (source, morphology, or amylose content) influence its digestibility (Copeland *et al*., 2009; Singh *et al*., 2010; Yang *et al*., 2023). Granule starch properties depend on the starch source. For this reason, some studies reported that cereal starches have easier digestibility due to the branch's bonds in the crystalline region (A-type), compared with B-type or C-type granules present in tuber or legume starches, respectively (Chi *et al*., 2021; Singh *et al*., 2010). Moreover, each type of starch granules presented variation in their morphology. It has been reported that the size and morphology of starch granules have an impact on their hydrolysis (Lindeboom *et al*., 2004). Small granules hydrolyze faster than large granules, which has been related to the minor granule specific surface area in large granules, which may decrease the extent of amylase binding. Furthermore, the amylose content or short-medium amylose chains affect starch hydrolysis (Gong *et al*., 2019). Higher amylose content reduces starch digestibility due to glucose chains being more packed through hydrogen bond linkages, hindering amylase activity. Conversely, the amylopectin chain has a larger surface area and does not affect enzyme accessibility.

The starch digestibility is greatly dependent on the starch form, that is in granular or gel state, because its susceptibility to enzyme digestion is different (Dona *et al*., 2010). Native starch granules are more resistant to enzyme hydrolysis than gelatinized starch. For this reason, several treatments explained below, are applied to modified starch surface granules, which facilitate the enzyme access to the glucose polymers.

Additionally, there are several, enzymatic, physical, and chemical processes that have been applied to modify native starches (Magallanes-Cruz *et al*., 2017). Physical treatments include hydrothermal methods like heat-moisture or annealing (Wee & Henry, 2020). Heat-moisture is done with low moisture content (< 35%) and high temperature (100 ºC - 130 ºC) and annealing is generated in presence of excess or intermediate water amount (> 35%) and at lower gelatinization temperature (Magallanes-Cruz *et al*., 2017). Those treatments provoke an increase in the RS fraction, without granule starch disruption with the subsequent impact on the glycemic response (Zavareze & Dias, 2011). Moreover, the extrusion process has been also described for changing the starch granule structure and the formation of complexes among starches, proteins, and lipids (Mohamed, 2021). Those complexes are more resistant to enzyme hydrolysis, and they have been associated with an increase in the SDS. Roman *et al*., (2019) related this SDS increase to the small fragments of amylopectin generated during the extrusion process. Those fragments showed faster retrogradation leading to molecules that were more difficult to hydrolyze. Instead, there are other physical non-thermal treatments, such as high hydrostatic pressure (10 – 1200 Mpa), that generates partial or complete starch gelatinization, and has been associated with slower starch digestibility, likely due to the formation of starch-lipid complex, higher RS content, or viscosity increase (Din *et al*., 2017; Mohamed, 2021).

Furthermore, the incorporation of functional ingredients (polysaccharides, proteins, lipids, or polyphenols) may also affect carbohydrate digestion (Mohamed, 2021; Wee & Henry, 2020; Yang *et al*., 2023). There is very extensive information about the impact of soluble polysaccharides on the starch hydrolysis (Singh *et al*., 2010). Soluble fibers can reduce starch digestion by three mechanisms: (i) creating a barrier in the granule surface for enzyme activity and restricting amylose leaching, (ii) generating a hydrated network around starch granules limiting enzyme access, or (iii) increasing the viscosity of food which slows glucose release. In addition, protein-starch or lipid-starch complexes can create a network surrounding the starch granules creating a barrier for enzymatic accessibility (Gularte & Rosell, 2011; Wee & Henry, 2020). Although there is not clear understanding of the polyphenols mechanism, numerous studies have reported the enzymatic inhibition induced by polyphenols rich extracts (Zhu, 2015). Polyphenols can inhibit digestive enzymes reducing the digestion rate (Sun *et al*., 2019; Takahama & Hirota, 2018). Aleixandre and Rosell (2022) determined that phenolic compounds can modulate postprandial glucose release in starch-based gels. Those authors highlighted the relationship between alpha-amylase inhibition and the degree of hydroxyl groups present in the structure of the phenolic compounds.

3.1 Digestion methods: *in vivo* or *in vitro* models

Different methodologies have been developed to record nutrients' digestion. *In vivo* methods with animals or human volunteers are the ones that can record the pathway changes along digestive system (Hur *et al*., 2011). Nevertheless, drawbacks of those methods, apart from ethically questionable, include the difficulty to control the digestibility and bioavailability of nutrients, as well as cost and being time-consuming (Havenaar & Minekus, 2019). For this reason, *in vitro* gastrointestinal studies or computational models have been established (Karthikeyan *et al*., 2021; Minekus *et al*., 2014). Nevertheless, it is difficult to compare results and obtain comparative conclusions because experimental conditions are rather different. For example, digestion steps (number and/or duration), the composition of the digestive fluids (enzymes activity, buffers, or food properties), mechanical stress, and fluid flows (Hur *et al*., 2011). The method reported by Englyst *et al*. (1992) has been one of the most extensively followed for carbohydrate digestion. Later, an international consensus within the COST INFOGEST network proposed a standardized oro-gastrointestinal digestion *in vitro* method (Brodkorb *et al*., 2019; Minekus *et al*., 2014). However, if the focus is the starch digestion, these protocols recommend quantifying the starch hydrolysis only in the intestinal phase.

Carbohydrate digestion generally is based on enzymatic hydrolysis of starch, and the record of the glucose release (Dupont *et al*., 2019). One of the common first-order kinetic models used for the time-course measurement of glucose release is the equation (1) proposed by Goñi *et al*. (1997).

$$C = C_{\infty} (1\text{-}e^{-kt}) \quad (1)$$

where C corresponds to the glucose concentration at any time (t), C_{∞} is the concentration of glucose at the end of the reaction and k is a first-order kinetic constant. Another related parameter is the hydrolysis index (HI), which is calculated as a percentage of the area under the hydrolysis curve (0-180 min) for the food product regarding the area for food standard (white bread or glucose). The HI can be used to calculate the expected glycemic index (*e*GI) by equation (2) proposed by (Granfeldt *et al*., 1992).

$$eGI = 8.198 + 0.862\ HI \quad (2)$$

Other indirect methods related to rheological behavior have been applied to study starch hydrolysis. Gee and Johnson (1985) reported a decline in ingesta viscosity during the simulated digestion process, using a rotary viscometer. Hódsági *et al*. (2012) presented significant correlations between hydrolysis and pasting parameters of corn and wheat starches, which can provide useful

information for estimating *in vitro* digestion. Other studies have employed the rheometer to record the rheological behavior during glucose release. Bordoloi *et al*. (2012) measure the viscosity performance of potatoes starch containing guar gum during intestinal digestion simulation using a dynamic rheometer observing a viscosity decrease due to the conversion of starch into glucose. Hardacre *et al*. (2016) studied the impact of shear rate (0.1, 1, 10 s^{-1}) on gelatinized corn and potato starches, reflecting a viscosity decline when dispersing enzymatic secretions at a shear rate of 0.1 s^{-1}. Besides, Rapid Visco Analyzer (RVA) was applied to record the change of apparent viscosity of starch gels by enzyme activity. Ferry *et al*. (2005) analyzed viscosity changes of wheat and waxy corn starch gels with several alpha-amylase concentrations. A similar study was carried out with amylase and amyloglucosidase in potato and waxy corn gels (Sorba & Sopade, 2013). Gamel *et al*. (2012) digested oat samples for two hours in the RVA canister in combination with digestive enzymes. However, there are no findings that combine the enzymatic hydrolysis results obtained by glucose release with results taken by rheological behavior.

For all that, the food viscosity property has a significant impact on starchy foods' digestibility and their digestion can be modulated by different *in vitro* models. The application of these methods can be used to design and formulate starch-based systems to reduce postprandial glucose levels, particularly for GF products with improved nutritional quality and health benefits.

4. Hydrocolloids: technological and nutritional attributes

Hydrocolloids are long-chain polymers composed of polysaccharides and proteins. Despite their diverse chemical composition, a common feature is their high content in hydroxyl groups in their structure that is responsible of their high ability to bind water molecules and consequently their solubility in water. There are many different types of hydrocolloids according to their origin. They could come from plants (cellulose, pectin, guar gum, locust bean gum), animals (gelatin, whey proteins, chitosan), seaweeds (agar, carrageenan), microbials (xanthan gum) or they can be obtained from chemical modifications of other compounds like cellulose. They are used in many foods like soups, sauces, ice-creams, jams, gelled desserts, and cakes, due to their technological properties as gelling, thickening, emulsifying or stabilizer agents (Appelqvist & Debet, 1997; Mahmood *et al*., 2017; Samant *et al*., 1993; Woomer & Adedeji, 2021). Specifically, hydrocolloids provide two basic properties to food systems: flow behavior (viscosity) and mechanical solid property (texture). They increase viscosity because of the water absorption and this property has a crucial role in foods processing and nutritional quality (Pirsa & Hafezi, 2023; Saha & Bhattacharya, 2010).

Hydrocolloids are getting even more attention due to their functionality in nutrition and health benefits for their prebiotic activity and effect on metabolic and chronic diseases. The role of hydrocolloids in human health has been explained by different mechanisms: physical effects, like gastric emptying time and transit absorption, which are related to gel structure formation in stomach digestion due to low pH; slow digestion of lipids; digestion of nutrients due to encapsulation or nutrient-hydrocolloid binding.

Similarly to previously mentioned information, the technological functionality of hydrocolloids, namely viscosity, has been related to glycemic response (Singh *et al*., 2010). Jenkins *et al*. (1978) observed a more effective postprandial glucose decrease in more viscous systems. Other studies reported the increase in the viscosity of food systems and the effect on intestinal mobility, besides the reduction in the glucose absorption in the small intestine (Gularte & Rosell, 2011; Krupa-Kozak & Lange, 2019; Liu *et al*., 2006).

The addition of hydrocolloids in cereal-based systems affect starch digestibility (**Table 1**). Furthermore, different hypotheses have been suggested to describe this correlation: starch-amylose-amylopectin interactions (Sasaki *et al*., 2015), the generation of a hydrated network around starch or higher viscosity of the digesta (Wee & Henry, 2020). Despite the extensive application of starch-hydrocolloid mixtures in the food procedure, and its association with a slower enzymatic activity due to digesta viscosity (Manzoor *et al*., 2020), there is a scarce information about the impact of starch systems viscosity on their hydrolysis rate.

Table 1: Studies about starch-hydrocolloid gels and their impact on starch hydrolysis.

Starch or flour (F)	Hydrocolloid	Percentage (w/w)	Results related to starch digestibility	Reference
Wheat (F)	Celite, AG, GG, LBG	1, 2.5, 5	GG and LBG decreased starch degradation, whereas AG and celite increased it	(Brennan *et al*., 2008)
Rice	A, XG, KG	30	XG and KG had more impact on starch hydrolysis No significant correlation was observed between the hydrolysis extent and the rheological properties	(Sasaki & Kohyama, 2011)
Corn Potato	Pectin, GG, XG, CMC, HPMC	1, 2, 3, 4	Hydrocolloids affect hydrolysis rate, especially GG reduced kinetic constant in potato gels	(Gularte & Rosell, 2011)
Waxy corn	GG, LBG, fenugreek gum, flaxseed gum, XG, and soy-soluble polysaccharide	4	XG lower glucose diffusion rate	(Fabek *et al*., 2014)
Potato	XG, GG, pectin, KG	5, 10, 15	XG reduced glucose levels earlier than GG and pectin, it was related to amylose-XG complexes	(Sasaki *et al*., 2015)
Rice (F)	XG, AG, GG, LBG	0.3, 0.5, 0.7	AG and XG presented minor glucose released and it was correlated with higher viscosity digesta due to less water content in gels	(Jung *et al*., 2017)
High amylose rice	XG, CMC, GG	0.4, 0.5	XG retarded starch hydrolysis, correlated with hydrocolloid-amylose complexes	(Oh *et al*., 2018)
Wheat	XG, GG, A, Mesona chinensis powder, LBG	0.3, 1, 2, 5	Starch digestion was reduced, correlated with the gel harness which limits enzyme accessibility	(Yuris *et al*., 2019)
Corn	GG, XG, AG	1, 0.8, 0.6, 0.4	GG decreased starch hydrolysis	(Zhou *et al*., 2020)

Hydrocolloids abbreviations: Agar, (A), Arabic gum (AG), Guar gum (GG), Locust bean gum (LBG), xanthan gum (XG), konjac glucomannan (KG), soluble cellulose derivatives: carboxymethyl cellulose (CMC) and hydroxypropylmethylcellulose (HPMC)

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Graphical introduction

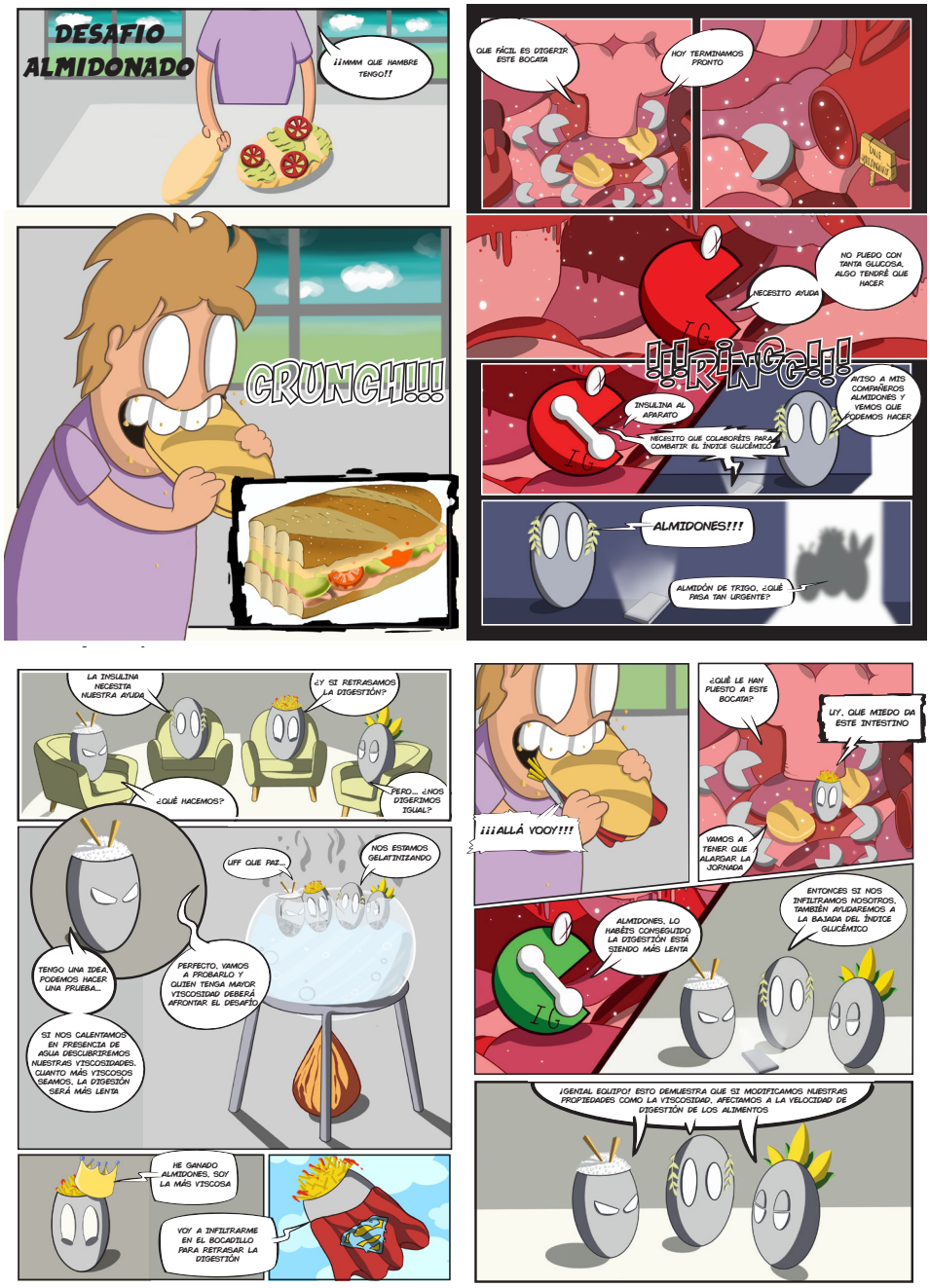


Figure 3: Comic presentated in the "2º Concurso de divulgación científica y cómics DESGRANANDO CÓMICS" contest.

OBJECTIVES

Considering the importance of starch hydrolysis in foods' digestibility, and particularly in gluten free systems that are mainly composed of starch, it is needed a better understanding of the factors affecting the glucose release. In general, approaches have been focused on the intrinsic properties of the starch and their impact on the starch digestibility. Nevertheless, there is scarce information about the role of viscosity in the starch digestion. Therefore, the main objective of the present research was to understand the role of the viscosity resulting from simple or binary food systems, containing starch, in the enzymatic hydrolysis of starch by applying *in vitro* methodologies.

To reach the main objective, the following specific objectives were defined:

1. Understanding gluten free foods gaps within the scientific context to obtain novel and healthy products.

2. Unraveling the impact of viscosity and gel microstructure on the enzymatic hydrolysis of starch gels, using homogeneous gels prepared only with starch.

3. Study potential relationship between the characteristics of starch gels obtained from different cereals and their *in vitro* hydrolysis.

4. Develop rapid methods to evaluate starch performance during gelatinization and their susceptibility to undergo enzymatic digestion.

5. Analyze the relationship between the viscosity of binary systems, containing blends of starch and hydrocolloid, and their enzymatic hydrolysis.

Working plan

The doctoral thesis has been organized into five different chapters that corresponds to specific scientific publications. **Figure 4** displays a summary of the different chapters above the results section.

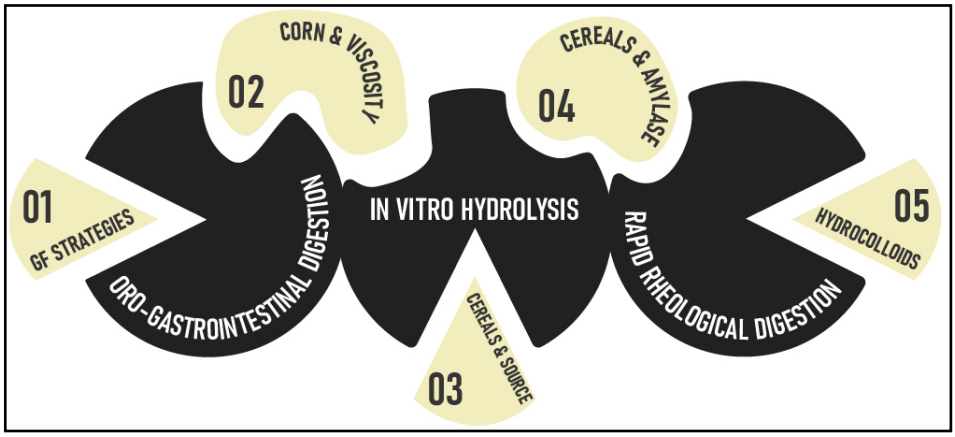


Figure 4: Overview of the chapters carried out.

Chapter 1 Corresponds to a handbook chapter that presents the current situation of gluten free cereal-based products. A revision of the strategies that have been used to obtain gluten free foods and their nutritional characteristics is presented to drive the design of healthy foods in the absence of gluten.

Chapter 2 Corn starch gels with different starch concentrations were prepared to study their digestibility applying both the oro-gastrointestinal digestion, and a direct *in vitro* enzymatic hydrolysis (**Figure 5**).

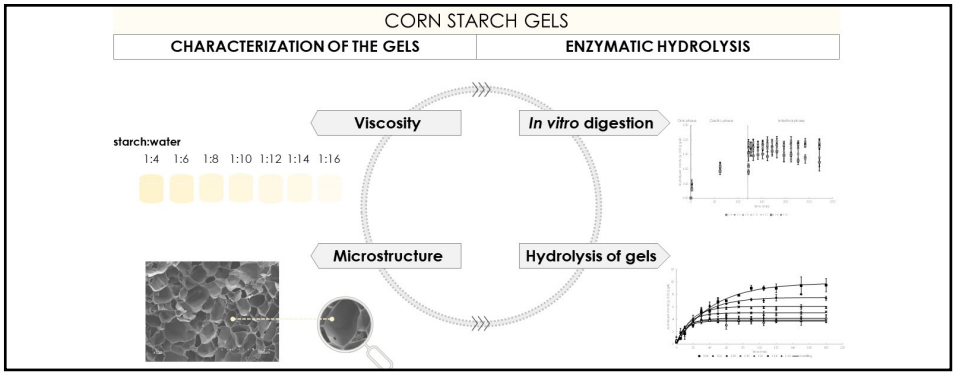


Figure 5: Graphical abstract of chapter two.

Chapter 3 Starch gels from corn, wheat, and rice with variable viscosity (VV) or constant viscosity (CV) were rheologically characterized and their properties correlated with the *in vitro* hydrolysis parameters (**Figure 6**).

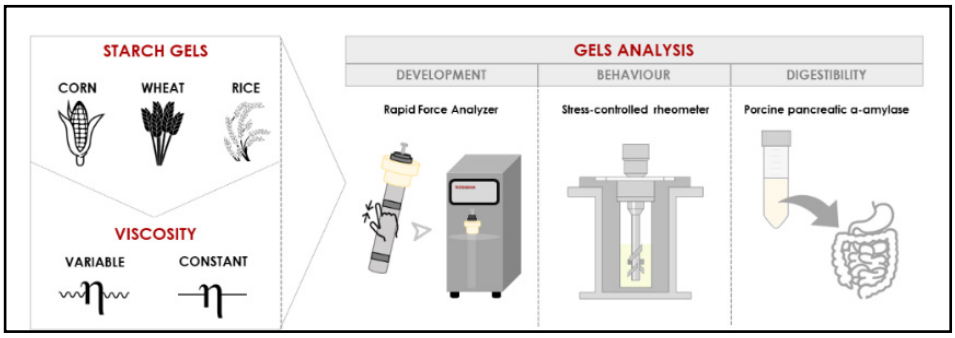


Figure 6: Graphical abstract of chapter three.

Chapter 4 Different rheological methods, including a rheometer and the Rapid Visco Analyzer, were developed to record the starch hydrolysis catalyzed by α-amylase activity. Moreover, correlations within them and the *in vitro* enzymatic hydrolysis of starch gels were performed (**Figure 7**).

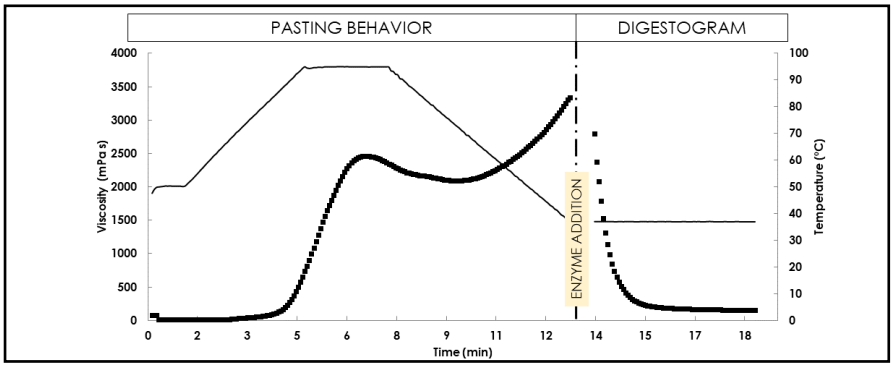


Figure 7: Graphical abstract of chapter four.

Chapter 5 Binary systems of different starches (corn, wheat, rice, potato, pea, and cassava) and hydrocolloids (locust bean gum, xanthan gum, guar gum, hydroxypropylmethylcellulose K4M and psyllium) added at several concentrations (0% - 0.5% - 2.5%) were used to investigate their impact in the rate of starch hydrolysis (**Figure 8**).

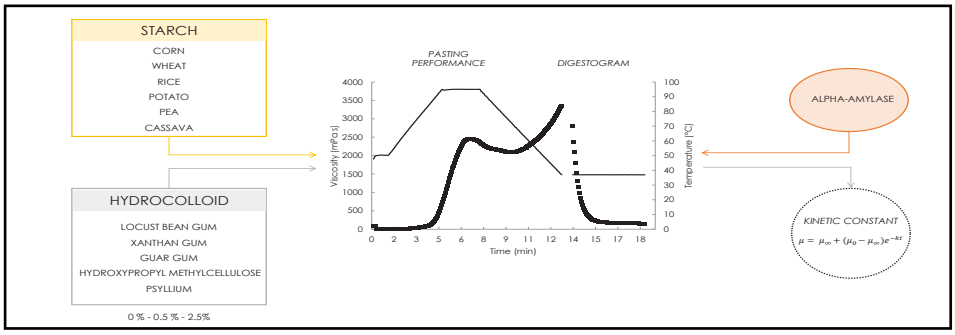
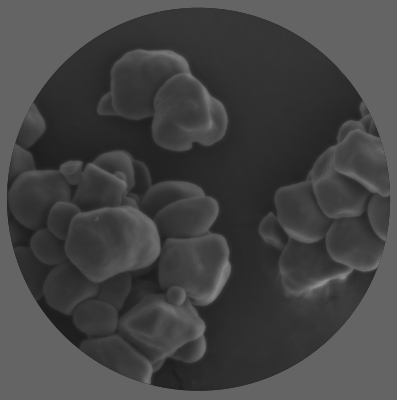
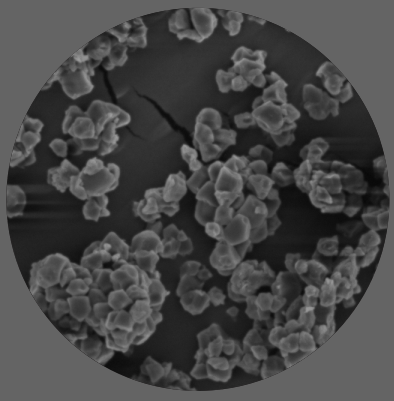
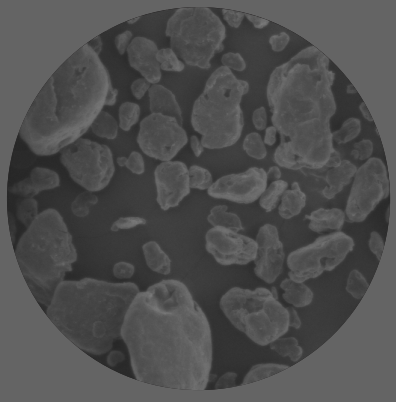


Figure 8: Graphical abstract of chapter five.





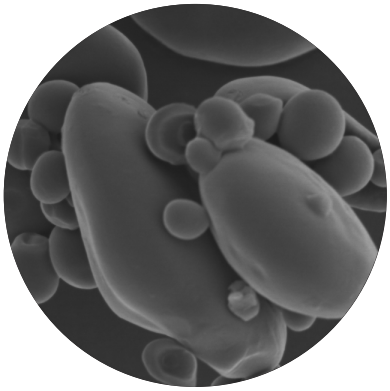


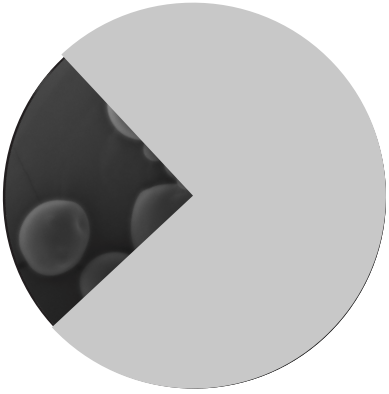
CHAPTER ONE

Gluten free bakery products

Maria Santamaria[1], Raquel Garzon[1], and Cristina M. Rosell[1,2]*

In *ICC Handbook of 21st Century Cereal Science and Technology*.
Elsevier, 1st Edition May 1, 2023





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ABSTRACT

Baked foods are mainly produced from gluten-containing cereals and the absence of gluten dramatically affects bakery products. The development of gluten free bakery products requires a global approach regarding recipes and breadmaking process. Considerable research has focused on finding gluten replacers to mimic gluten functionality. Blends of flours or starches, hydrocolloids, proteins, enzymes are frequently-used alternatives; but dough hydration and the optimization of proofing and baking are also important to improve the quality of gluten free products. In addition, special attention must be paid to increasing their nutritional quality, by increasing proteins and fibre, with a simultaneous reduction of the glycemic index. The use of sourdough and some physical treatments of gluten free flours should be additionally explored in the context of gluten free. Overall, there is still some way to go to obtain novel, healthy, and technologically accepted gluten free products to reach consumers' expectations.

1.1 Introduction

In the last decades, gluten free (GF) products have moved from a niche market to a mainstream business. Foresight still indicates further growth, with an increase from 5.6 billion $US in 2020 to 8.3 billion $US in 2025 in the global market for GF products. The motives for this change include increased numbers of individuals adhering to a GF diet, including people with gluten-related disorders (allergic, autoimmune, or immune-mediated) and those who associated GF with a healthier diet, regardless of lack of scientific evidence.

The Codex Alimentarius Commission stated that GF foods must not exceed 20 ppm (parts per million) of gluten. Gluten refers mainly to the storage proteins present in the genus Triticum, Triticeae tribe (barley and rye), wheat hybrids such as triticale, and possibly oats. From the technological point of view, gluten is a three-dimensional protein network, that underpins the structure of many bakery products, particularly fermented bread, where dough extensibility and elasticity are required for the aerated appearance, volume, and crumb structure. Gluten plays a major role in breadmaking process (**Figure 1.1**), gluten providing cohesion of the components after mixing and retaining the air nuclei incorporated during kneading. During fermentation, those nuclei are progressively filled with carbon dioxide (CO_2), with a simultaneous increase in their size, which continues until the initial stages of baking which fixes the crumb structure of bread. The expansion taking place during fermentation and baking is enabled by the viscoelastic properties of gluten. In fact, the absence of gluten significantly modifies dough rheology, being less cohesive and elastic, but stickier and with increased pastiness. Consequently, GF doughs are difficult to handle and frequently resemble batters. Similarly, gas retention is deficient during GF production, which leads to an unstable and irregular crumb structure. Overall, the post-baking quality of GF breads is deficient because they tend to present an unattractive appearance with a pale crust, low loaf volume, dry and crumbly texture, poor mouthfeel and short shelf life, besides having an un-balanced nutritional content compared with their gluten-containing counterparts.

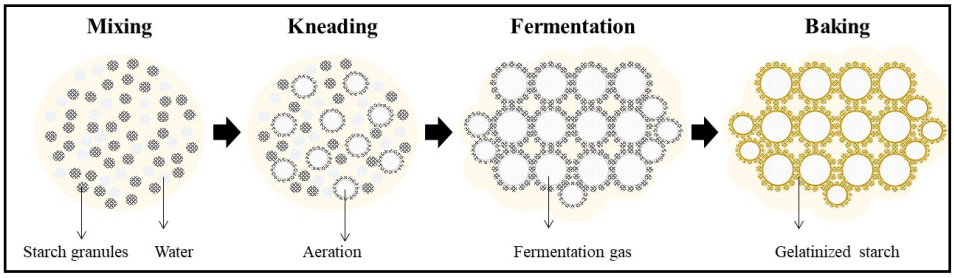

Figure 1.1: Breadmaking process in bakery products.

1.2 Challenges to obtain gluten free products

Initially, GF breads were conceived as bread-type foods for coeliac patients, in which complex GF blends were used, applying similar breadmaking processes to conventional breads. However, the removal of gluten from breadmaking processes could not be understood as a simple change of recipe, replacing gluten containing flours by GF flours. The development of GF bakery products is a technological challenge that requires a complete change in the breadmaking process, starting with the recipe and continuing with the processing. The removal of gluten from other bakery products such cakes or cookies has a less severe effect on processing because gluten plays a secondary role, due to the presence of high fat and/or sugar contents in these products. Starch is the main functional component in these systems and the secondary role of the gluten is related to the formation of a bi-continuous system composed of fat and non-fat phases (sugar and flour/starch).

1.2.1 Structuring agents to mimic gluten functionality

The starting point when making GF breads is to define a recipe, containing any structuring agent that could resemble gluten functionality. The basic ingredients are blends of GF flours and starches from different origins, but mainly from GF cereals (**Table 1.1**). Starch can provide a crumb-like structure after gelatinization and retrogradation during cooking and cooling, respectively. However, although starch has some gluten-like properties, such as the ability to hold carbon dioxide when gelatinized, it does not provide the level of stretch and recoil (viscoelasticity) that gluten provides, which very often results in big holes in the crumb structure (**Figure 1.2**). Because of this, a common practice is the addition of ingredients and additives as gluten replacers in GF formulations, to enhance the structure, acceptability, and shelf-life of GF products (**Figure 1.3**). This results in very complex systems, compared to gluten-containing systems, in which gas cells are stabilized by starch granules, proteins and strengthening agents.

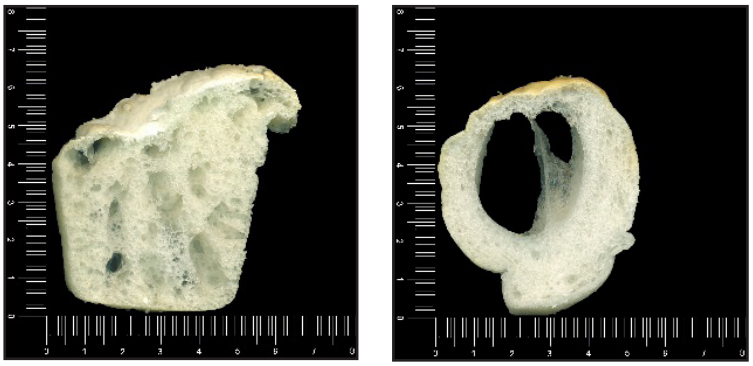

Figure 1.2: Deficient technological quality of starch-based GF bread.

The most common ingredients and additives in the GF industry include hydrocolloids, proteins, enzymes, and modified starches. Hydrocolloids are long-chain polymers formed by polysaccharides and proteins and are obtained from plants, seaweed, or microbial sources. Their function in food is to act as gelling and thickening agents, based on their high ability to bind water molecules. The incorporation of hydrocolloids has a significant impact on the dough/batter and in consequence on the final products. Hydrocolloids readily absorb water leading to three dimensional structures, that have similar viscoelastic properties to those of gluten, leading to cohesive doughs with improved gas-retention. GF breads containing hydrocolloids have higher moisture content, softer texture, higher specific volume, improved structure and sensory properties and longer shelf-life.

Functional proteins have been also proposed for improving the physical properties of GF formulations. Proteins from animal and vegetable sources have been used with animal proteins being associated with increased specific volume. In particular, dairy and egg proteins perform will in GF processing, acting as emulsifiers, increasing water holding capacity and gas stabilization. Enzymes are also proteins but have the capacity to catalyze biochemical reactions with very high substrate specificity. Starch degrading enzymes, non-starch degrading enzymes, lipases, proteases, transglutaminase, glucose oxidase (GO) and phytases are useful enzymes for bakery applications in gluten-containing foods. However, their performance cannot be directly extrapolated to GF breads, because recipes are completely different from conventional bread recipes and different functionalities are required to improve the dough/batter rheology. In fact, starch hydrolyzing enzymes, that are very useful in gluten bakery, must be carefully used in GF breadmaking, to avoid excessive hydrolysis of the starch polymers. Initially, enzymes with strengthening action were applied to create an internal network within the GF constituents. With this aim, transglutaminase, glucose oxidase or laccase have been used to create new covalent bonds between protein chains,

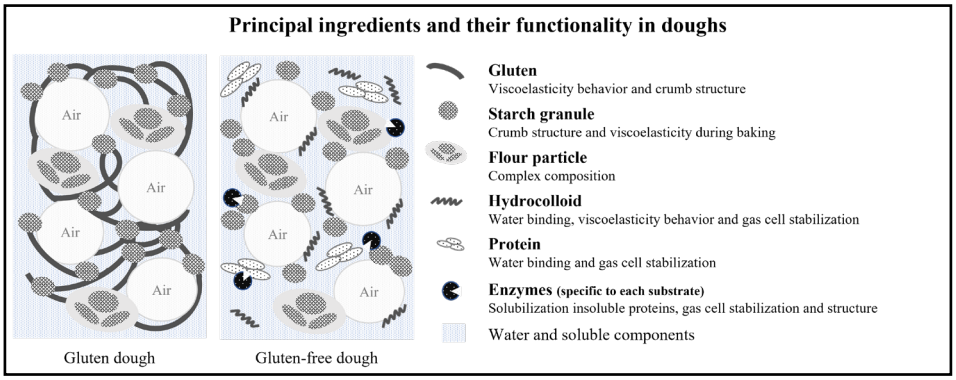

Figure 1.3: Analogy of breadmaking constituents between gluten or gluten free system.

building an inner structure that confers strength to dough, and in consequence, improving bread quality. However, the opposite action, protein breakdown, has also been demonstrated to be an effective way for improving GF bread quality. In fact, proteases with different specificities have been used to hydrolyze proteins, reducing their hydrophobicity, and in turn, stabilizing the GF matrix. However, the impact of crosslinking enzymes and proteases in GF systems is greatly dependent on the type of flour and the properties of the proteins, apart from the type of enzymes. Consequently, although enzymes are useful processing aids in clean label GF systems, their effectiveness must be tested in each specific system and their levels optimize.

1.2.2 Gluten free breadmaking processing: batter/doughs properties

Dough rheology measures the performance of gluten-containing doughs and the resulting impact on texture and crumb structure of the fresh breads. However, the rheology methods commonly used for gluten-containing doughs are not useful in GF systems and the complexity of the flours/starches blends makes it more difficult to predict their performance in GF breadmaking. Frequently, the dough/batter consistency has been defined as a quality indicator of GF systems, and it has been correlated with texture parameters such as the crumb hardness of baked rice GF bread. Farinograph, Mixograph, Mixolab and Doughlab have been used to determine the consistency of GF dough, while the rapid viscosity analyzer (RVA) is recommended to determine the consistency of batter. The complexity and diversity of GF recipes has prevented the identification of general indicators to predict breadmaking quality. However, the importance of hydration of flours/starches hydration, and consequently the resulting GF dough consistency, on the final bread quality, particularly bread volume is generally accepted. Dough hydration significantly affects the performance of the dough during fermentation (gas retention) and its further expansion during baking, which are related to the elastic behavior and viscosity properties, respectively. All those properties are also modified by the different ingredients. Flour-based doughs present higher viscoelastic moduli than starch doughs; this could be due to their diverse particle size, protein adhesion or granule-granule interactions, which strengthen the flour particles. Additionally, the gelatinization and retrogradation properties of starch affect their functional properties, having great impact on making GF foods. Starches from different sources vary in their transition temperatures and enthalpies of gelatinization, which play an important role in the sequential physical changes that GF dough undergoes during baking.

1.2.3 Technological quality and consumer acceptance

Recipes and processes are responsible for the end-product quality, including, moisture content, specific volume, color, crumb texture and structure, and sensory perception. Moisture content and its redistribution within the entire bread structure are critical, because this type of product generally dries very quickly. As mentioned before, the formulations of GF products are frequently based on starches and hydrocolloids, which require additional amounts of water. In general, increased hydration results in higher specific volume and softer crumbs. However, after baking moisture migration occurs within the crumb and to the crust, which is related to bread staling. Due to the high moisture content of these breads, the crusts are not usually crispy but rapidly become elastic and chewy.

Specific volume is widely used to assessing bread quality and is also used for GF bread quality, although the values are usually lower. The addition of hydrocolloids or enzymes can increase the specific volume of GF breads (**Table 1.1**). Furthermore, although flour-based bread has lower specific volume than starch-based bread, high consistency and viscoelastic moduli can be achieved with flour-based dough. This performance could be associated with the larger particles size, the presence of a protein layer and increased water absorption capacity of the flours compared to starches. Starch-based products have whiter colour due to their low protein content and because their high water content hinders browning reactions. Crumb structure is another quality indicator. The crumb structure is assessed by measuring the average size of pores or gas cells, and their number, with the porosity being related to texture parameters, including the hardness, cohesiveness, and elasticity of bread slices. The crumb texture of GF breads is crucial because these types of products tend to be harder with low cohesiveness compared to gluten-containing breads. All the above quality characteristics of the GF bakery products influence consumer acceptance (**Figure 1.2**). Analyses of consumer opinions and perceptions of commercial GF products show that texture and taste are the most relevant characteristic for the participants followed by appearance, freshness, aroma, volume, nutritional composition, or the ingredients list.

1.3 Advances in processes and recipes to produce gluten free goods

The world of GF bakery products has faced great changes from the initial technological challenge to obtain acceptable products, to the focus on nutritionally balanced foods that could also provide additional benefits to the health of consumers. There are numerous GF bakery products with a wide variety of formulations. Recipes differ depending on the type of product, the diversity of ingredients and additives, their concentrations, and the percentage of water in the

preparation. This diversity makes it very difficult to study the product properties in terms of processing, final quality, and nutrition; and specify quality parameters during the process. **Table 1.1** summarises the impacts of different ingredients on the production and quality of GF products. New thermal and non-thermal technologies have also been employed in the production of GF bakery products while high pressures or cold plasma have been applied to modify starch digestibility and viscosity or gelatinization temperature, respectively. A recent focus in GF foods is to enrich products by adding ingredients such as pseudocereals, pulses, fibre, fortified flours, etc. GF bakery products are low in essential nutrients and most are based on starchy foods with high fat and low protein contents and are often deficient in dietary fibre and vitamins and/or minerals. Combinations of flours from different origins, or even the inclusion of protein and fibers supplements in appropriate amounts result in better nutritional balance of bakery products.

An additional trend is the reduction of starch digestibility because the products have high starch contents and hence high glycemic index (GI). There is considerable interest in decreasing the GI in GF products because coeliac disease has been also associated with high incidence of type I diabetes mellitus. For this reason, starch digestibility is an important parameter in the nutritional quality of GF products. Starch fractions are classified as rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS), with RDS resulting in a faster increase in blood glucose than SDS. Innovative strategies are therefore being used to reduce the rate of starch digestion in GF foods. These include selecting the type of starches, applying physical treatments, incorporating sourdoughs and modulating the viscosity of the system. The incorporation of high amylose starches (amylose contents > 25% dry weight) increases the amount of resistant starch and decreases the RDS. Similar effect can be achieved by the addition of legume flours because they increase the content of soluble dietary fibre and increase the viscosity of pastes. The control of particle size distribution of the flours offers an additional alternative for controlling the starch hydrolysis. Breads made with flour with particle sizes >150 μm are digested more slowly and in consequence lead to lower GI. Moreover, sourdough is a good option to retard starch digestion. This is because the lactic acid bacteria present in the sourdoughs release organic acids (lactic, acetic, and propionic acids) during fermentation, which slow starch hydrolysis. Recent studies are focused on modifying the viscosity of the system, by incorporating ingredients such as inulin or other soluble fibers that limit enzyme activity, resulting in a decrease in GI. Similarly, ingredients from other sources, such as pumpkin, have been used to obtain more compact and stable gels that can hinder the α-amylase activity. These ingredients could act as a physical barrier and/or increase the viscosity of the medium.

Table 1.1: Impact of the most common ingredients used in GF bakery products on each parameter during the process (breadmaking, technological quality consumer acceptance, and nutrition).

Ingredients	Breadmaking			Technological quality and consumer acceptance					Nutrition
	Consistency	Gas retention	Pasting properties	Moisture loss	Specific volume	Structure	Texture	Acceptance	Digestion
Hydrocolloids Xanthan gum Agar-agar Carrageen Pectin β-glucan Gum arabic Locust bean gum Guar gum Hydroxypropylmethyl cellulose Carboxymethyl cellulose Methyl cellulose	↑	↑	↑	↓	↑	↑	↑↓ Hardness Cohesiveness Elasticity	↑	↓GI
Proteins Zein Pea Lupin Soy Albumin Collagen Dairy Egg	=	= ↑			↑↓=	↑	↑↓		↑ Amino acids ↓GI
Enzymes Tranglutaminase Glucose oxidase Protease Laccase Amylase Cyclodextrinase	↑	↑	↑	↓	↑	↑	↓		
↑ increase/improve ↓ decrease/less = no effect									

1.4 Future trends

GF bakery products are still a technological challenge because no other natural protein have similar viscoelastic properties to gluten. Up to now, the role of gluten has been replaced using blends of flours and starches, emulsifiers, hydrocolloids and fats. Future research should focus on understanding the behavior of GF doughs during processing, including mixing, kneading, fermentation, and baking. This should identify the technological conditions and processes required to give high quality products. Once the technological challenges have been overcome, work is required to improve the nutritional quality of GF products. Significant improvements have been achieved with some physical treatments of flours, the inclusion of pseudocereals or legumes, and other less conventional sources of flours such as acorn and chestnut. The use of sourdough systems also represents an alternative that requires further exploration for improving the technological and nutritional quality of the GF products, with a simultaneous reduction of the use of additives. Similarly, the importance of food digestibility has prompted the study of structural changes in starch and their relationship to reduced rates of starch digestion and hence lower glycaemic index, with benefits for the health of consumers. Finally, it is crucial that the sensory expectations are met, particularly regarding the appearance, aroma, texture, and especially taste of GF products.

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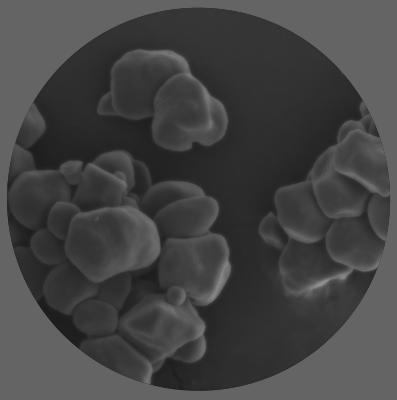

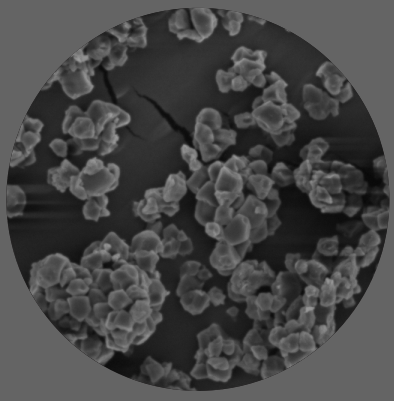

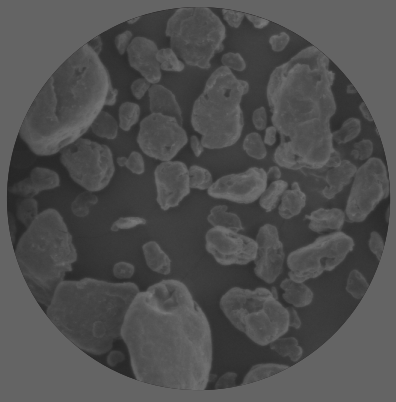

CHAPTER **TWO**

Estimation of viscosity and hydrolysis kinetics of corn starch gels based on microstructural features using a simplified model

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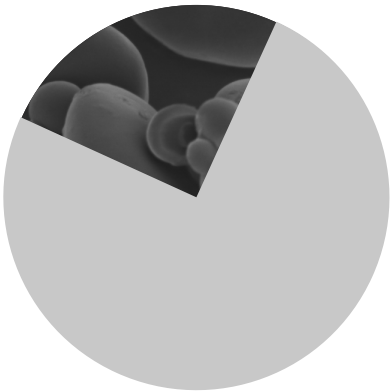

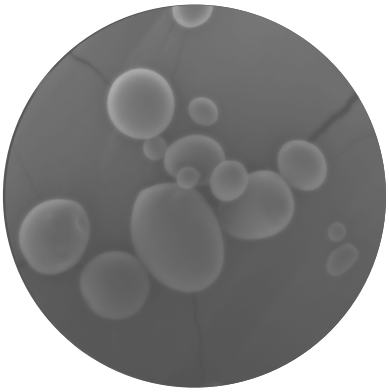

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ABSTRACT

Viscosity is an important rheological property, which may have impact on the glycemic response of starchy foods. However, the relationship between starch gels viscosity on its hydrolysis has not been elucidated. The aim of this work was to assess the effect of gels viscosity on the microstructure, and the kinetics of enzymatic hydrolysis of starch. Corn starch gels were prepared from starch:water ratios varying from 1:4 to 1:16. A structural model was proposed that correlated (R-square = 0.98) the porous structure (cavity sizes, thickness walls) of gels and its viscosity. Kinetics constants of hydrolysis decreased with increasing starch content and consequently with gel viscosity. Relationships of viscosity with the microstructural features of gels suggested that enzyme diffusion into the gel was hindered, with the subsequent impact on the hydrolysis kinetics. Therefore, starch digestibility could be governed by starch gels viscosity, which also affected their microstructure.

2.1 Introduction

The understanding of starch hydrolysis is attracting much research owing its relationship with the metabolic processes occurring along human digestion, particularly the postprandial blood glucose levels (Hardacre *et al*., 2016). Previous to the glucose absorption in small intestine, starch is hydrolyzed by salivary and pancreatic α-amylase in the mouth and small intestine, respectively, generating short oligomers, such as maltose or maltotriose (Dona *et al*., 2010). According to the rate of hydrolysis, starch is commonly categorized into three fractions (Englyst & Hudson, 1996): rapidly digestible starch (RDS) associated with a fast increase in blood glucose level, slowly digestible starch (SDS) slowly hydrolyzed in the small intestine, and resistant starch (RS), which is not digested by the enzymes in the superior gastrointestinal tract, but microorganisms can ferment it to short chain fatty acids (SCFA) in the large intestine (Dura *et al*., 2017; Zhou *et al*., 2020).

Despite the interest in starch digestion, there is uncertainty about the factors that could affect the hydrolysis of starch catalyzed by α-amylase. The starch concentration, its botanical origin, or the starch status as native or gelatinized form are important properties that may influence the hydrolysis. Previous studies suggested that cereal flours are digested more rapidly than tubers and legume flours, due to their difference in starch microstructure and chemical composition (Gularte & Rosell, 2011; Liu *et al*., 2006). Furthermore, Dhital *et al*. (2017) described that mechanisms limiting enzymatic activity are related to binding or blocking the access of α-amylase. Those authors differentiated when enzymatic hydrolysis is in aqueous solution as occurs in the gelatinized starch or in slurry as the case of granular starch. In both cases the amylase hydrolysis might be limited by, first the barriers that prevent the binding of the enzyme to starch and secondly, the structural features of starch that impede amylase access to the substrate. Consequently, physical characterization of the starch granule as size, pores in the granular surface or the supramolecular structure are properties that can impact the adsorption and binding of the α-amylase. Besides starch structure, viscosity of the system has been incorporated as one important element in the starch digestion (Hardacre *et al*., 2016). However, studies investigating viscosity have been focused on the impact of soluble and insoluble dietary fiber, but not on the role of gels viscosity produced as a result of starch gelatinization. The addition of hydrocolloids (usually labelled as non-starch polysaccharides, NPS) modifies the gelatinization/gelation process of the starch (Brennan,*et al*., 2008; Sasaki & Kohyama, 2011). A study carried out with corn and potato starches and different hydrocolloids (pectin, guar gum, xanthan gum and soluble cellulose derivatives CMC and HPMC) confirmed that hydrocolloids affected the hydroly-

sis rate to different extent, depending on the hydrocolloid and type of starch (Gularte & Rosell, 2011). Authors observed an increase in initial rate of starch amylolysis in the presence of hydrocolloids, with the exception of guar gum that decreased the kinetic constant in potato gels (Gularte & Rosell, 2011). Yuris *et al*. (2019) studied the digestibility of wheat starch gels in the presence of several polysaccharides (xanthan, guar, agar) and explained the reduction in the starch digestibility by the increase in gel hardness that limits the enzyme accessibility to starch. Similarly, guar and xanthan gums added to high-amylose corn starch affected starch viscosity and retarded starch hydrolysis leading to lower estimated glycemic response (Chung *et al*., 2007; Zhang *et al*., 2020). The different studies discussed the relationship between the extent of starch hydrolysis and the system viscosity, but divergences on the role of viscosity accelerating or slowing down the starch hydrolysis have been encountered, which might be attributed to a possible viscosity threshold required for that enzymatic inhibition. Additionally, some studies analyzed the relation between insoluble fiber like cellulose and the α-amylase activity. Nsor-atindana *et al*. (2020) reported that amylase can bind cellulose and act as a reversible and non-specific inhibitor, and the inhibition becomes more apparent as the particle size of the polymer decreases (Dhital *et al*., 2015; Nsor-atindana *et al*., 2020).

Therefore, although it has been found out that the viscosity of exogenous sources of hydrocolloids impacts the rate of digestive hydrolysis of starch to our best knowledge there are no studies regarding the viscosity effect of starch gels on their hydrolysis by digestive enzymes. Based on this, we initially hypothesized that starch gels viscosity could affect their digestion, and furthermore, that their structural features also might influence the enzymes accessibility to the starch. The aim of this study was to unravel the impact of viscosity and gel microstructure on the enzymatic hydrolysis of starch gels, using homogeneous gels prepared only with starch, in order to avoid possible artifacts derived from the interaction between heterologous polymers as it occurs in the presence of different hydrocolloids. Corn starch gels were prepared with different starch concentrations leading to gels with different properties and microstructure. To simulate starch digestion, the oro-gastrointestinal digestion (Minekus *et al*., 2014) and a direct *in vitro* enzymatic hydrolysis (Benavent-Gil & Rosell, 2017) were applied to the different gels.

2.2 Materials and methods

2.2.1 Materials

Corn starch EPSA (Valencia, Spain) of 95% purity (20.25% amylose content) and 13.22% moisture content was used. The enzymes used were type VI-B α-amylase from porcine pancreas (EC 3.2.1.1), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232.468.9), bile salts and 3,5-dinitrosalicylic acid (DNS) were acquired from Sigma Aldrich (Sigma Chemical, St. Louis, USA). Amyloglucosidase (EC 3.2.1.3) was provided by Novozymes (Bagsvaerd, Denmark). Glucose oxidase/peroxidase (GOPOD) kit (Megazyme International Ireland Ldt., Bray, Ireland) was used. Solutions and standards were prepared by using deionized water. All reagents were of analytical grade.

2.2.2 Preparation of gels and pasting properties

The preparation of starch gels and the pasting performance of each samples was determined by Rapid Visco Analyzer (RVA 4500; Perten Instruments, Hägersten, Sweden). Corn starch gels were prepared at different concentrations with deionized water (w:w, 1:4; 1:6; 1:8; 1:10; 1:12; 1:14; 1:16). Slurries were subjected to heating and cooling cycles consisting of: 50 ºC for one min, heating from 50 to 95 ºC in 3 min 42 s, holding at 95 ºC for 2 min 30 s, then cooling down to 50 ºC in 3 min 48 s and holding at 50 ºC for 2 min. The pasting parameters evaluated included the peak viscosity (maximum viscosity during heating), breakdown (viscosity difference between peak viscosity and trough), and the pasting rate calculated as the slope of the apparent viscosity during heating until 95 ºC. The apparent viscosity of the formed gels was measured at 37 ºC with a vibrational viscometer VL7-100B-d15 (Hydramotion Ltd, Malton, UK). This apparatus measures viscosity at high shear rate where the strong shear-thinning behavior of samples is less relevant. Moisture of gels was determined in two steps using an infrared balance (KERN, Balingen, Germany). Three different batches for each gel were prepared.

2.2.3 Total starch

The amount of total starch of the gels was quantified using a commercial assay kit (Megazyme International Ireland Ldt., Bray, Ireland). Two replicates were measured for each sample.

2.2.4 Scanning Electron Microscopy (SEM)

Fresh gels were immersed in liquid nitrogen and then freeze-dried. The microstructure of the different freeze-dried gels was observed using scanning electron microscopy (S-4800, Hitachi, Ibaraki, Japan). Samples were examined at an accelerating voltage of 10 kV and 100x magnification. Micrographs (1.3x0.98 mm) were captured. The microstructure analysis was carried out using the ImageJ analysis program (ImageJ, National Institutes of Health, Bethesda, Maryland, USA) and NIS-Elements software (Nikon Instruments Inc., Tokyo, Japan). An auto local thresholding was applied using ImageJ software and measured the wall thickness, and then the measurement of gel cavities or holes was carried out with Nis-Elements software. Parameters assessed were number of cavities/mm^2, mean cavity area (μm^2), porosity (%) calculated as ratio of total area of cavities and total image area, and wall thickness (μm) as previously described by Garzon and Rosell (2021). Three images were used to calculate the average of previous parameters.

2.2.5 *In vitro* oro-gastrointestinal digestion

The oro-gastroinstestinal digestion was carried out following the standardized static digestion method described by Minekus *et al*. (2014) and adapted by Aleixandre *et al*. (2019). Minor modifications included the use of five grams of gel prepared in the Rapid Visco Analyzer (RVA) and 27 U/mL of α-amylase solution. Aliquots were withdrawn along digestion. Specifically, at the end of oral and gastric digestion and during the three hours of intestinal digestion. Aliquots were immediately heated to 100 ºC for 5 min to stop enzyme hydrolysis. Hydrolysis was quantified with 3,5-dinitrosalicylic acid (DNS) spectrophotometrically using an SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany) at 540 nm, using maltose as standard. Resistant starch was determined at the end of the digestion.

2.2.6 Hydrolysis kinetics and expected glycemic index

Hydrolysis kinetics of starch gels were determined following the method described by Benavent-Gil and Rosell (2017) with minor modifications. One gram of gel was suspended into 4 mL of 0.1 M sodium maleate buffer (pH 6.9) with porcine pancreatic α-amylase (0.9 U/mL) and incubated in a shaker incubator SKI 4 (ARGO Lab, Carpi, Italy) at 37ºC under constant stirring at 200 rpm during 3 h. Aliquots (100 μL) were taken during incubation and mixed with 100 μL ethanol (96%) to stop the enzymatic hydrolysis. Then, it was centrifuged for 5 min (10,000 x*g*, 4°C). The pellet was suspended in 100 μL of ethanol (50%) and centrifuged as described before. Supernatants were pooled together and kept

at 4°C. Supernatant (100 µL) was diluted with 885 µl of 0.1 M sodium acetate buffer (pH 4.5) and incubated with 15 µL amyloglucosidase (214.5 U/mL) at 50°C for 30 min in a shaking incubator, before quantifying glucose content.

The remnant starch after 24 h hydrolysis was solubilized with 2 mL of cold 1.7 M NaOH. The mixture was homogenized with Polytron Ultra-Turrax T18 (IKA-Werke GmbH and Co. KG, Staufen, Germany) for 5 min at 14,000 rpm in an ice bath. The homogenate was diluted with 8 mL 0.6 M sodium acetate pH 3.8 containing calcium chloride (5 mM) and incubated with 100 µL AMG (143 U/mL) at 50 ºC for 30 min in a shaking water bath. Afterwards, the glucose content was measured using a glucose oxidase–peroxidase (GOPOD). The absorbance was measured at 510 nm. Starch was calculated as glucose (mg) × 0.9.

The hydrolysis results allowed to calculate the amount of starch fractions. Rapidly digestible starch (RDS) was the starch fraction hydrolyzed within 20 min of incubation, slowly digestible starch (SDS) was the fraction hydrolyzed within 20 and 120 min, total digestible starch (DS) the amount of hydrolyzed starch after 24 h of incubation and resistant starch (RS) was the starch fraction that remained unhydrolyzed after 24 h of incubation (Calle *et al*., 2020). The *in vitro* digestion kinetics were calculated fitting experimental data to a first-order equation (Eq.1) (Goñi *et al*., 1997):

$$C = C_{\infty} (1\text{-}e^{-kt}) \quad (1)$$

where was the percentage of starch hydrolyzed at time, was the equilibrium concentration or maximum hydrolysis of starch gels, k was the kinetic constant and t was the time chosen. In addition, the time required to reach 50% of (t_{50}) was calculated. The hydrolysis index (HI) was obtained by dividing the area under hydrolysis curve (0-180 min) of the sample by the area of the sample more concentrated (1:4) over the same period. The expected glycemic index (*e*GI) was calculated with the proposed Eq. (2) (Granfeldt *et al*., 1992).

$$eGI = 8.198 + 0.862\ HI \quad (2)$$

2.2.7 Statistical analyses

Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as mean ± standard deviation, using Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA). Fisher's least significant differences test (LSD) was used to estimate significant differences among experimental mean values. Differences of $P < 0.05$ were considered significant. Furthermore, Pearson correlation analysis was used to identify possible relationships among experimental parameters.

2.3 Results and discussion

2.3.1 Formation process of gel

The pasting properties were recorded to identify the impact of starch concentration on the gel performance. Rapid Visco Analyzer (RVA) registered the apparent viscosity during heating and cooling cycle; the logarithmic scale for the apparent viscosity was used for comparison purposes (**Figure 2.1**). The pasting behavior in RVA cycle was different among samples. At high starch content the maximum peak viscosity was reached earlier with higher slope (pasting rate) during heating, indicating faster increase of apparent viscosity. Peak viscosity is considered the equilibrium point between swelling and rupture of starch granules (Balet *et al*., 2019). Therefore, at low starch content the granules can swell more freely, without the contact of other swollen granules. In consequence the rupture was delayed and reached at higher temperatures. As a result, the peak temperature decreased from 95 to 84 ºC with increasing starch content. Eerlingen *et al*. (1997) reported similar performance when different concentrations of potato starch were subjected to different hydrothermal treatments. At low concentrations, the starch particles are completely swollen, but the space is rather limited at a higher starch concentration and swollen granules can only fill up the available space referred as close packing concentration. At the lowest concentration, a shoulder was visible before reaching the maximum peak viscosity, likely evidencing differences in swelling rate of starch granules associated to their particle size distribution. It has been reported that the average size of individual corn starch granules ranged within 1-7 µm for small and 15-20 µm for large granules (Singh *et al*., 2003). Mishra and Rai (2006) observed that corn starch exhibited polyhedral granules with size ranging from 3.6 to 14.3 µm. Differences in the granular size led to diverse surface area that could interact with water, and in consequence modifying the swelling rate. Nevertheless, the viscosity shoulder was only visible in the more diluted system, probably at higher concentration the predominant granules size population masked the swelling of the less abundant one.

Regarding the maximum apparent viscosity, as expected, the most concentrated starch gel (starch:water, 1:4) showed the highest peak of apparent viscosity (21,727 mPa s), observing a progressive decrease of that viscosity when increasing the starch dilution up to 1:16. Similar trend was observed in the final viscosity. This result was expected based on the amount of starch added in each slurry, because the apparent viscosity was directly related to the amount of starch.

The viscosity decay observed along holding at 95 ºC (breakdown), associated with the disintegration degree of starch granules, exhibited also differences among samples. Major differences were observed within the most concentrated gels up

to 1:8, at higher dilution changes in apparent viscosity were less visible, even during cooling. Standard methods for recording apparent viscosity of starches are usually carried out with starch:water slurries of 1:8, obtaining pasting profiles similar to the present study (Calle *et al*., 2021; Mishra & Rai, 2006). Nevertheless, no previous study showed the apparent viscosity of gels with different starch concentration and how it impacts on the starch digestibility.

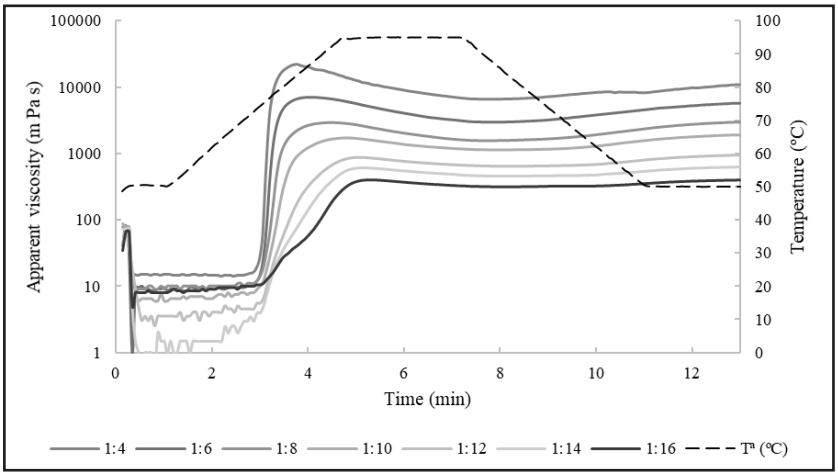

Figure 2.1: RVA pasting profiles of corn starch gels prepared with different starch concentrations. Values in the legend are referred to the ratio starch:water (w:w). Discontinuous line shows the temperature applied during the heating-cooling cycle.

2.3.2 Characterization of the gels

Considering the potential impact of gels characteristics on their hydrolysis performance, a thorough analysis of the gels was carried out. Viscosity at 37 ºC and the content of total starch in tested gels are presented in **Table 2.1**. The total starch content decreased as the dilution increased. The wide range of gels concentrations, from 4.5% to 18.6%, could cover the concentration existing in very diverse starch foods, from soups to salad dressings (4-15%). As expected, starch concentration had a significant impact on the gels' viscosity (R-square = 0.97). Sample with the highest content of total starch (18.6%) also showed the highest viscosity (768 mPa s). Conversely, the viscosity of the more diluted gel was 48 mPa s. A significant power law correlation was observed between the starch content and the resulting gels viscosities, which was related to the change on flow resistance when modifying the amount of solid per volume unit (Moreira *et al*., 2012).

The structural impact of starch concentration on the resulting gels was evaluated by analyzing the SEM micrographs (**Figure 2.2**). The gels morphology considerably varied with the starch content. Gel microstructure resembled a

network with small cavities. As the starch dilution increased, an enhancement in the size of cavities was observed with samples 1:4 and 1:6 having more closed structures (**Figure 2.2a and 2.2b)**. The disintegration of granules during heating, as indicated the breakdown observed for those gels in the RVA, might be responsible for that tight structure. The results of the image analysis (**Table 2.1**) confirmed significant differences ($P < 0.05$) in the microstructure of the gels, except for porosity. The number of cavities or holes in the gels showed a steady decrease as the starch dilution increased up to 1:8. Further dilutions did not induce significant differences in the number of cavities/mm^2. Simultaneously, the mean area of the cavities progressively increased with the starch dilution in the gels, again until sample 1:8, with no additional changes at higher dilution values. There was a significant positive relationship between number of cavities with viscosity (R-square = 0.87) and total starch (R-square = 0.82). Conversely, negative significant relationships were obtained between the mean area of the cavities with viscosity (R-square = -0.84) and total starch (R-square = -0.84). When the median area of the cavities was used for comparing gels, the same trend was observed, except for the gel with the highest dilution (1:16) that exhibited significantly larger cavities.

Possible relationships among starch content, gels microstructure and their viscosity were analyzed. There was a positive logarithmic relationship (R-square = 0.98) between the thickness of the cavities' walls and the starch content of the gels, and exponential with the gels' viscosity (R-square=0.94). It was expected that the apparent viscosity of the gels depends mainly on the solid content, but viscosity values (**Table 2.1**) suggested that the 3-D network of the gel and its spatial distribution also must be considered. The gel structures shown in **Figure 2.2** were modelled as follows: pores (with an equivalent radius, r_{eq}) given by the median cavity area (A) and walls whose thickness (e) can be considered as two semi-thicknesses by the contribution of each neighboring pore covering. The area occupied by starch walls (A_{TP}) in relation to porous area can be evaluated by:

$$\frac{A_{TP}}{A} = \frac{A_e + A_s - A}{A} = \frac{A_e + A_s}{A} - 1 = \frac{(\pi+\sqrt{3}-\pi/2)\,(r_{eq}+e/2)^2}{A} - 1 \qquad (3)$$

where A_e is the area of the circle with radius given by the sum of r_{eq} and e; A_s is the area between three tangent circles with area A_e.

Table 2.1: Characterization of corn gels: total starch, viscosity at 37 ºC and microstructure parameters.

Sample	Total starch (g/100 g gel)	Viscosity (mPa s)	No. Cavities/mm²	Mean cavity area (µm²)	Median cavity area (µm²)	Porosity (%)	Wall thickness (µm)	W_{eq}[a]
1:4	18.6 ± 0.1^{a}	768 ± 23^{a}	226 ± 9^{a}	2,591 ± 119^{b}	1,027 ± 134^{d}	59.9 ± 3.7^{ab}	9.1 ± 1.1^{a}	24.9 ± 1.8^{a}
1:6	11.2 ± 0.2^{b}	422 ± 27^{b}	175 ± 60^{ab}	3,221 ± 1432^{b}	1,613 ± 946^{cd}	58.0 ± 4.7^{ab}	7.3 ± 0.2^{b}	14.8± 2.1^{b}
1:8	8.7 ± 0.2^{c}	112 ± 30^{c}	100 ± 16^{bc}	6,259 ± 685^{a}	3,321 ± 130^{bc}	61.6 ± 3.8^{ab}	5.8 ± 0.3^{cd}	6.5 ± 0.7^{c}
1:10	7.2 ± 0.1^{d}	111 ± 15^{c}	88 ± 22^{c}	7,709 ± 2155^{a}	5,493 ± 2371^{ab}	66.3 ± 2.2^{a}	4.8 ± 0.0^{de}	3.4 ± 1.3^{d}
1:12	5.7 ± 0.1^{e}	74 ± 1^{d}	93 ± 14^{c}	7,650 ± 246^{a}	5,209 ± 520^{b}	69.8 ± 8.5^{a}	3.5 ± 0.3^{ef}	2.7 ± 0.6^{de}
1:14	5.4 ± 0.1^{e}	62 ± 7de	122 ± 4^{bc}	7,050 ± 1750^{a}	4,691 ± 117^{b}	65.5 ± 7.7^{ab}	2.7 ± 0.2^{fg}	2.4 ± 0.4^{de}
1:16	4.5 ± 0.0^{f}	48 ± 4^{e}	93 ± 33^{c}	8,806 ± 930^{a}	7,668 ± 871^{a}	65.8 ± 3.6^{ab}	1.8 ± 0.2^{g}	1.0 ± 0.3^{e}
P-value	**0.0001**	**0.0001**	**0.0050**	**0.0012**	**0.0009**	**0.2623**	**0.0001**	**0.0001**

Means within the same column followed by different letters indicate significant differences $P < 0.05$.
[a] W_{eq} was obtained from Eq. (4): $W_{eq}=A_{TP(1:16)}/A_{TP} \bullet e/e_{1:16}$

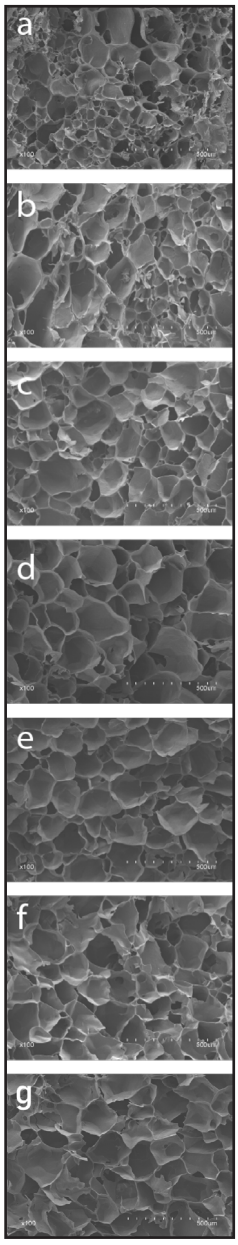

Figure 2.2: Scanning electron micrograph of corn starch gels. Magnification 100x. The starch:water ratio is: 1:4 (a); 1:6 (b); 1:8 (c); 1:10 (d); 1:12 (e); 1:14 (f); 1:16 (g).

Spatial distribution of the starch and the thickness of the wall depended on the starch gel content. As r_{eq} was in all cases longer than *e*, the highest A_{TP} (Eq. 3) was obtained with the highest cavity area (in this case 1:16). A_{TP} is employed to evaluate the number of cavities equivalent to contain the same amount of starch than in other gels. Nevertheless, these cavities have thicker walls and the number of equivalent walls, W_{eq}, regarded to the reference wall (thinnest wall, $e_{1:16}$) must be evaluated by means of:

$$W_{eq} = \frac{A_{TP(1:16)}}{A_{TP}} \frac{e}{e_{1:16}} \quad (4)$$

Eq. (4) allows the determination of the number of the walls with the same thickness (1.8 μm) per unit of starch gel. Introducing the corresponding data collected in **Table 2.1** and by evaluation of Eq. (3), the number of walls increased with increasing starch content from 1 (1:16) up to 24.9 (1:4). A linear relationship (*R*-square = 0.98) between number of equivalent walls (W_{eq}) and viscosity (μ, mPa s) was found, Eq. (5), achieving a structural model that involves the porous characteristics of starchy gels and a physical property such as viscosity.

$$\mu = 30.46\ Weq - 14.97 \quad (5)$$

2.3.3 *In vitro* digestion and hydrolysis of gels

The method INFOGEST was used to simulate the digestion of corn starch gels in the oro-gastrointestinal tract (**Figure 2.3**). Experimental results are displayed as g of hydrolyzed starch per 100 g of gel, since the *in vitro* method is directly based on the amount of food ingested, in this case gels. Starch hydrolysis during oral and gastric phase presented very low hydrolysis considering the percentage of starch hydrolyzed. This was already reported by Iqbal *et al*., (2021) because of a short residence time during oral phase and the inhibition of α-amylase at low pH in the gastric phase. In the intestinal phase, there was only an initial increase in the amount of hydrolyzed starch, but no further changes were observed along the intestinal digestion time. The oro-gastrointestinal digestion did

not show a trend with the different starch gels, although the most concentrated gel (1:4) exhibited the lowest level of starch hydrolysis (1.5 g of hydrolyzed starch/100 g gel). Some authors indicated that samples with high starch content underwent slow hydrolysis, which has been related with the viscosity impeding the diffusion of enzymes, and in consequence, the enzymes accessibility and their binding to their substrate (Sanromán *et al*., 1996; Wu *et al*., 2017).

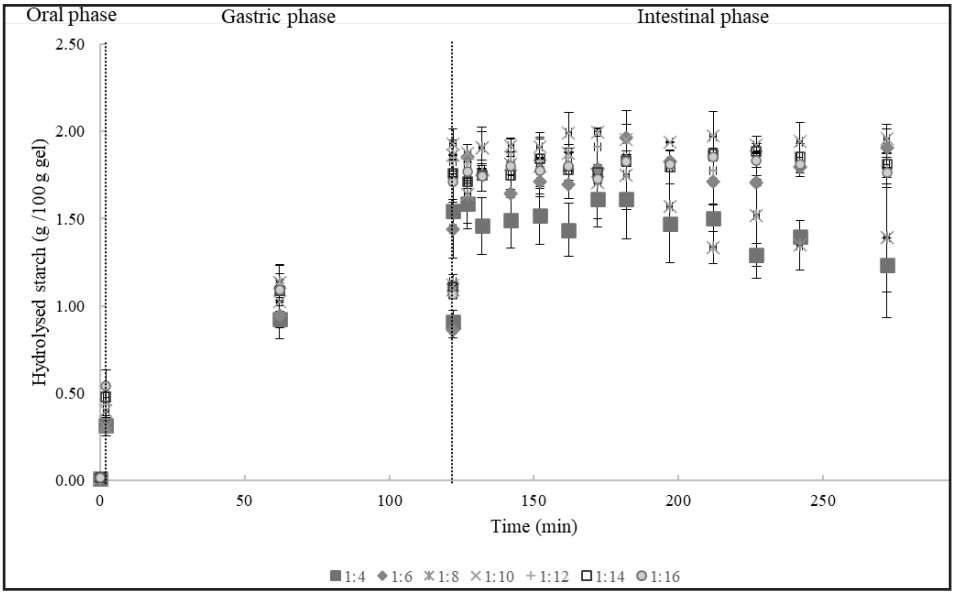

Figure 2.3: *In vitro* oro-gastrointestinal digestion of gels prepared with different starch concentration. Legend is indicating the ratio starch:water used to prepare the gels.

Overall, the application of the oro-gastrointestinal *in vitro* digestion to starch gels did not allow us to identify the possible impact of gels viscosity and microstructure on the enzymatic hydrolysis, since the progressive dilution of the samples in each digestion phase masked differences associated to intrinsic characteristics of the gels. For this reason, the starch hydrolysis was directly carried out with porcine pancreatic α-amylase following methodology previously reported (Benavent-Gil & Rosell, 2017).

According to the rate and extent of *in vitro* digestion of starch, rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) were quantified, obtaining significant differences ($P < 0.05$) among the gels (**Table 2.2**). RDS, starch digested in the first 20 min, is the fraction that causes rapid increase in blood glucose after digestion of carbohydrates (Dona *et al*., 2010). In this study, RDS did not present a linear correlation with the starch concentration.

Sample 1:8 showed the highest amount of RDS. According to Dhital *et al*. (2017), the hydrolytic activity of the amylase could be reduced when the enzyme access to the starch is limited. In the present system, a decrease of the RDS might be expected when increasing gel viscosity, and thus the starch concentration of the gel. Nevertheless, that decrease was only observed at higher starch concentrations until 1:8, which suggests that a viscosity threshold was required in order to affect the enzyme accessibility. Conversely, SDS, related to low postprandial glycemic peak, showed steady decrease with the starch concentration, and the more diluted samples led to lower SDS. Chung *et al*. (2007) found that the incorporation of hydrocolloids increased the SDS, but without any clear trend on RDS content. Namely, samples with higher content of starch (1:4; 1:6) showed greater differences. Predictably, as the starch content in the gels was reduced, DS and RS decreased. Differences in DS were narrowed from sample 1:8 to 1:16, probably related to their viscosity differences at 37 ºC (**Table 2.1**). Concerning RS, the amount of this fraction was directly related to the total starch amount of the gels.

Table 2.2: Parameters of in vitro corn starch gels digestibility: rapidly digestible starch (RDS), slowly digestible starch (SDS), digestible starch (DS), resistant starch (RS).

Sample	RDS (g/100 g)	SDS (g/100 g)	DS (g/100 g)	RS (g/100 g)
1:4	3.51 ± 0.49^{bcd}	5.68 ± 1.16^{a}	9.99 ± 0.55^{a}	3.63 ± 0.24^{a}
1:6	3.77 ± 0.04^{ab}	3.64 ± 0.04^{b}	7.73 ± 0.17^{b}	2.41 ± 0.17^{b}
1:8	4.05 ± 0.22^{a}	1.95 ± 0.36^{c}	5.58 ± 0.69^{c}	1.59 ± 0.24^{c}
1:10	3.46 ± 0.18^{bcd}	1.57 ± 0.02^{c}	5.24 ± 0.67^{cd}	1.32 ± 0.13^{cd}
1:12	3.07 ± 0.07^{d}	1.43 ± 0.20^{cd}	4.17 ± 0.49^{de}	0.98 ± 0.06^{de}
1:14	3.14 ± 0.08^{cd}	0.86 ± 0.10^{cd}	4.23 ± 0.50^{de}	0.85 ± 0.15^{e}
1:16	3.59 ± 0.06^{abc}	0.27 ± 0.05^{d}	3.96 ± 0.14^{e}	0.70 ± 0.12^{e}
P-value	**0.0110**	**0.0001**	**0.0001**	**0.0001**

Values within the same column followed by different letters indicate significant differences $P < 0.05$.

For the more concentrated samples greater difference in viscosity was observed and the same trend was seen in the *in vitro* digestion parameters. Again, significant relationships were encountered with viscosity and the hydrolysis fractions SDS (R-square = 0.95) and RS (R-square = 0.96); and also the area of the cavities with SDS (R-square = -0.87) and RS (R-square = -0.84). The fraction of RDS content in relation to the initial starch content of the gel, *RDS(%),* decreased from 79.8% (1:16) up to 18.9% (1:4) with increasing starch content. It is worthy to mention that *RDS%* could be satisfactorily related with the structural parameter, W_{eq}, Eq. (4), by means of:

$$RDS\% = 74.45 - 16.73 \log(W_{eq}) \quad (6)$$

This relationship (*R*-square = 0.95) indicates that the presence of a high number of equivalent walls of starch results in a decrease of the initial amount of starch that is accessible by enzymes.

Starch hydrolysis of gels prepared with different concentration of corn starch is presented in **Figure 2.4**. Results have been plotted as both the amount of hydrolyzed starch per 100 grams of gels vs time or the amount of hydrolyzed starch per 100 grams of starch vs time. Those two different graphs for expressing results were chosen to understand the role of starch concentration in the gels. Hydrolysis plots confirmed the different behavior of the gels depending on the starch concentration. **Figure 2.4A** showed the initial starch hydrolysis with minor differences in the rate of hydrolysis but the maximum hydrolysis reached was dependent on the gels dilution. A progressive reduction in the maximum hydrolyzed starch was observed when increasing gels dilution. Samples with higher dilution (1:12; 1:14; 1:16) had a rapid initial hydrolysis but reached a plateau after hydrolyzing low amount of starch (ca. 4%) (**Figure 2.4A**). Regarding the starch content of the gels, when hydrolysis was followed recording the amount of hydrolyzed starch per starch amount on the gels (g starch/100 g of starch) (**Figure 2.4B**) the pattern was completely different. There was a slower hydrolysis in the more concentrated gels and faster hydrolysis in the diluted ones, which also reached higher hydrolysis extension (up to 86%), compared to the 53% hydrolysis observed in the gel 1:4. Other studies (Sasaki & Kohyama, 2011), reported the impact of viscosity, provided by the addition of different gums, on the decrease of the starch hydrolysis. Likewise, Ma *et al*. (2019) reported that the incorporation of pectin increased the viscosity in the gut lumen and showed slower rate of starch hydrolysis. This could be attributed to the formation of a pectin layer around starch granules that limited the access of enzymes. Conversely, in the present study, a homogenous system comprising only starch has been used and results confirm the real impact of viscosity on the starch hydrolysis.

The starch hydrolysis in all the gels showed a very good fitting (*R*-square = 0.96) to a first order kinetics model. The kinetics parameters derived from hydrolysis of gels including kinetics constant (k), equilibrium concentration of hydrolyzed starch (C_∞), area under the hydrolysis curve after 180 min (AUC 180), hydrolysis index (HI) and estimated glycemic index (*e*GI) are summarized in **Table 2.3**. These parameters were significantly ($P < 0.05$) different depending on the gel concentration. The kinetics constant (k) increased with the starch dilution and the time to reach 50% of the hydrolysis (t_{50}) showed a progressive decrease with the dilution. Therefore, more concentrated gels exhibited slower hydrolysis over the digestion time. At constant enzyme concentration and temperature of reaction, an increase of enzymatic reaction rate would be expected when increasing the substrate concentration. However, in the present gels, there is an

increase of reaction rate when diluting the starch and therefore, when decreasing the amount of starch in the gels, suggesting that the formation of enzyme-substrate complexes depended on the own structural gel features. High starch content hinders the enzyme diffusion into the gel and macroscopically this resistance associated to the mass transport can be related to gel viscosity (previously related to microstructural gel features with the proposed model). In fact, the hydrolysis kinetics constant depended inversely on the gel viscosity (**Figure 2.5**). Two different trends could be determined, associated with high (> 100 mPa s) and low (< 100 mPa s) viscosities corresponding to high (> 7 g starch/100 g gel) and low (< 7 g starch/100 g gel) amount of starch in the gels. At low viscosity range, the kinetics constant value drops linearly (*R*-square = 0.98) with gel viscosity. This regression allows the empirical prediction of enzymatic kinetics constant value (k_1 = 0.22 min^{-1}) at very low starch amount present in the gel (very low substrate concentration and gel viscosity assumed equal to water viscosity at 37 ºC, 0.692 mPa s) (Lide, 2005). This kinetics constant value could be interpreted like the kinetics constant in absence of mass transfer resistances within gel. In fact, the kinetics constant values collected in **Table 2.3** must be considered like a global kinetics coefficient where enzymatic reaction constant value (k_1, min^{-1}) and mass transfer coefficient (k_m, min^{-1}) are involved and the simplified relationship, after several assumptions for a model of resistances in series, is given by the Eq. (7) (Levenspiel, 1998):

$$1/k = 1/k_1 + 1/k_m \quad (7)$$

Eq. (7) allows the estimation of k_m of enzyme into the gels with different starch content and the corresponding values are shown in **Table 2.3**. The mass transfer coefficients value strictly depends on the characteristics of compound diffusing, turbulence conditions on the surface and properties of the fluid. In our case, in a simplified way, it was found a power relationship between k_m and viscosity (*R*-square = 0.996) and Eq. (7) can be written after substitution:

$$1/k = 1/0.22 + 0.196\ \eta^{0.8} \quad (8)$$

A very high correlation (*R*-square > 0.94) was obtained between experimental kinetics constant data and estimated values employing Eq. (8). The goodness of the first order model with the kinetics constant evaluated by Eq. (8) can be observed in the **Figure 2.4A** and **Figure 2.4B**. These results confirmed that the viscosity of starch gels must be considered to evaluate the hydrolysis rates. Previous hydrolysis studies dealing with changes in viscosity have been carried out with diverse hydrocolloids, and the slowdown of the enzymatic activity has been explained based on the hydrocolloid coating of the starch surface that block the enzyme accessibility to the substrate (Chung *et al*., 2007; Gularte & Rosell, 2011). However, the present research confirmed the role of the apparent viscosity of the gels on the enzymatic hydrolysis.

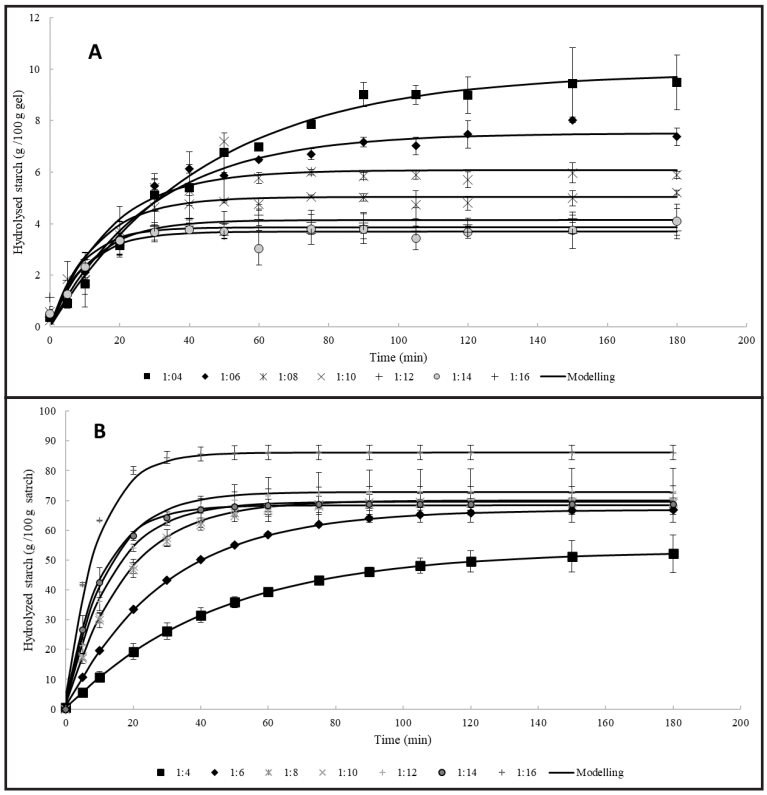

Figure 2.4: Enzymatic starch hydrolysis of different corn starch gels prepared with different starch concentration. Legend is indicating the ratio starch:water used to prepare the gels. Hydrolysis plots are expressed as: g/100 g gel (A) and g/100 g starch (B). Solid lines correspond to first-order model with kinetics constant evaluated by Eq. (8).

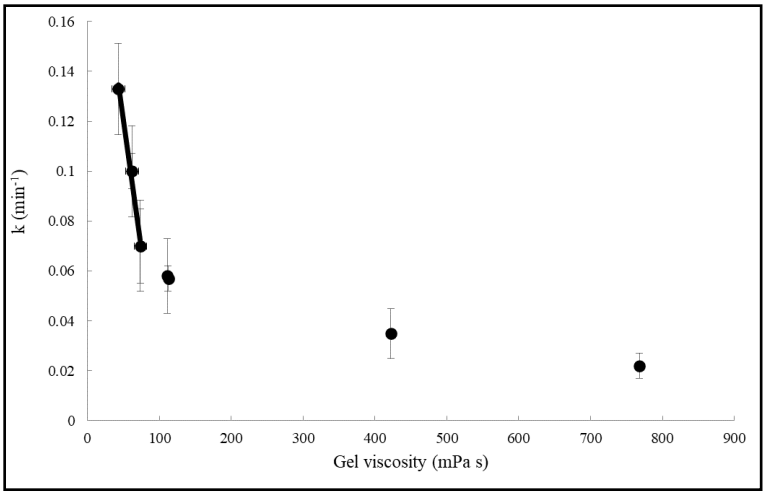

Figure 2.5: Relationship of the kinetics constant of first order model with gel viscosity.

Table 2.3: Kinetic parameters resulting from the enzymatic hydrolysis of corn gels with different starch concentrations**.** Kinetic parameters include: kinetic constant (*k*), time required to reach 50% of C_∞ (t_{50}); equilibrium concentration (C_∞), area under the hydrolysis curve after 180 min (AUC), hydrolysis index (HI) and estimated glycemic index (*e*GI) for corn gels with different concentration. Expressed per 100 grams of gels (**Figure 2.4A**).

Sample	k (min^{-1})	t_{50} (min)	C_∞ [a]	AUC	HI	*e*GI [b]	k_m [c] (min^{-1})
1:4	0.02 ± 0.01^{e}	35 ± 7^{a}	10.10 ± 1.53^{a}	1,335.00 ± 49.50^{a}	100.00 ± 2.99^{a}	94.40 ± 2.58^{b}	0.02 ± 0.01^{e}
1:6	0.03 ± 0.00^{de}	20 ± 0^{b}	7.52 ± 0.08^{b}	1,136.00 ± 12.73^{b}	85.09 ± 0.77^{b}	81.55 ± 0.66^{c}	0.04 ± 0.01^{de}
1:8	0.06 ± 0.01^{cd}	10 ± 0^{c}	6.01 ± 014^{c}	971.75 ± 8.27^{c}	72.79 ± 0.50^{c}	70.94 ± 0.43^{d}	0.07 ± 0.02^{cd}
1:10	0.06 ± 0.00^{cd}	10 ± 0^{c}	5.03 ± 0.20^{cd}	818.05 ± 34.29^{d}	61.28 ± 2.07^{d}	61.02 ± 1.79^{e}	0.08 ± 0.02^{cd}
1:12	0.07 ± 0.02^{bc}	10 ± 0^{c}	4.14 ± 0.44^{d}	683.65 ± 52.68^{e}	51.21 ± 3.18^{e}	52.34 ± 2.74^{f}	0.10 ± 0.02^{c}
1:14	0.10 ± 0.03^{ab}	8 ± 4^{c}	3.72 ± 0.33^{d}	628.00 ± 42.00^{e}	47.04 ± 2.54^{e}	48.75 ± 2.19^{f}	0.18 ± 0.03^{b}
1:16	0.13 ± 0.01^{a}	5 ± 0^{c}	3.86 ± 0.11^{d}	663.45 ± 17.04^{e}	49.70 ± 1.03^{e}	51.04 ± 0.89^{f}	0.34 ± 0.04^{a}
P-value	**0.0004**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**

Values followed by different letters within a column denote significant differences ($P < 0.05$). [a] C_∞ and k were determined by the equation, $C = C_\infty (1 - e^{-kt})$. [b] *e*GI was quantified following the equation proposed by Granfeldt *et al.* (1992). [c] obtained from Eq. (7):

In addition, the maximum hydrolysis (C_∞) reached with the different gels (**Figure 2.4A, Table 2.3**) showed a significant decrease when increasing gels dilution. A similar trend was observed for the total area under the hydrolysis curve (AUC), which is related to the glucose released over a hydrolysis period of 180 minutes (Goñi *et al*., 1997). To estimate the glycemic index (*e*GI), the hydrolysis index (HI) of each gel was calculated taking the sample 1:4 as a reference (HI = 100%). The *e*GI showed a steady decrease until 51% in the most diluted sample. Glycemic index is used to describe how the food starch is hydrolyzed in the digestive system and absorbed into the bloodstream (Dona *et al*., 2010). Some authors reported that the high viscosity induced by hydrocolloids might form a physical barrier for the α-amylase access, which would explain the decrease in glucose released and its absorption in the intestine (Dartois *et al*., 2010; Gularte & Rosell, 2011). Here, the same behavior was observed regarding the reduction in the hydrolysis rate, but now it is related to the increase of viscosity by the increase of starch content in the gels.

2.4 Conclusions

This study investigated for the first time the role of the viscosity of starch gels on the digestion of starch. Corn starch gels of varying starch concentration resulted in a range of different viscosities and microstructures. A structural model is proposed that connects by a linear relationship (*R*-square = 0.98) the porous structure (cavity sizes and thickness walls) of starch gels and their viscosity. The viscosity showed a linear relationship with the number of starch walls per area and its thickness (equivalent walls). The kinetics constant values of the starch hydrolysis decreased when increasing gel viscosity. Hydrolysis constants, considering mass transfer resistance within the gel, were successfully correlated with gel viscosity by means of a simple model, confirming the initial formulated hypothesis. Overall, the proposed simplified model links macrostructural properties (viscosity) and microstructural features (median cavity area and wall thickness) to analyze hydrolysis kinetics. It could also be extended to other physical and chemical processes where starch gels are involved and validated with other gels formed with starches from other sources. From the technological point of view, these findings could be applied in the design of food formulations aiming at postprandial glucose management.

Author Contributions: Credit roles: MS: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft; RG: Methodology; Supervision; Data curation; RM: Formal analysis; Writing - review & editing; Funding acquisition; CMR: Conceptualization; Funding acquisition; Investigation; Supervision; Writing - review & editing.

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Conflicts of Interest: The authors declare no conflict of interest.

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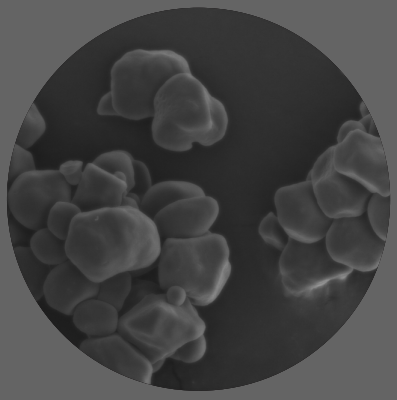

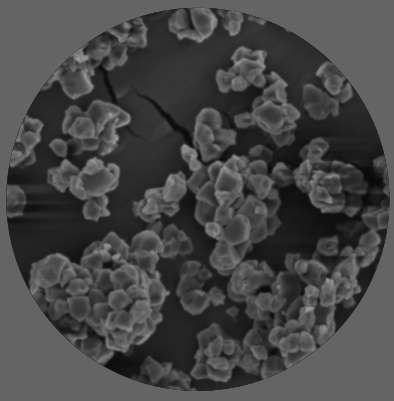

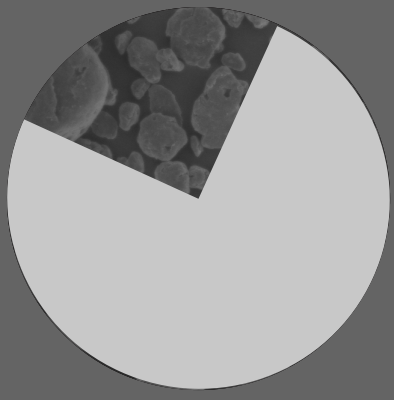

CHAPTER THREE

Unraveling the impact of viscosity and starch type on the *in vitro* starch digestibility of different gels

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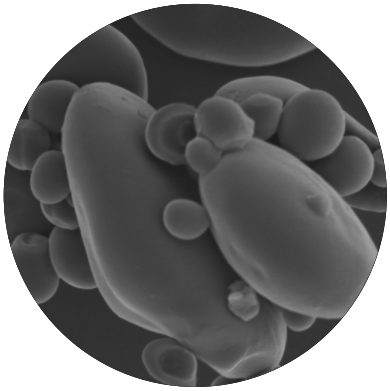

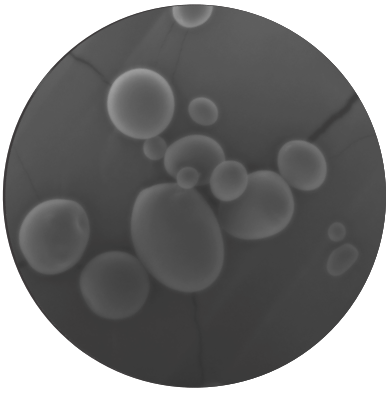

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ABSTRACT

Starch is one of the most important carbohydrate that is present in many foods. Gelatinization is an important property of starch, associated with physical changes that promotes an increase in viscosity. The objective of this research was to understand how viscosity of starch gels affects their hydrolysis and if that effect was dependent on the type of starch. Different gels (corn, wheat, and rice) with variable or constant viscosity were analyzed using diverse methodologies to determine changes in the pasting behavior. Rapid force analyzer, vibration viscometer and rheometer parameters discriminated the gels due to starch source and concentration. At fixed starch concentration, corn gel displayed the highest viscosity, slowing the enzymatic starch hydrolysis. Higher viscosity in those gels prepared with fixed starch concentration significantly enhanced the slowly digestible starch (SDS) and reduced kinetic constant (*k*). Nevertheless, gels with constant viscosity (550 mPa s) showed comparable hydrolysis kinetics, obtaining alike SDS, total hydrolyzed starch and *k*. The correlation matrix confirmed the relationship between the *k* and the gels viscosity (r = -0.82), gelatinization rate (α-slope) (r = -0.87), breakdown (r = -0.84) and elastic modulus (G' 37 ºC) (r = -0.73). Therefore, those parameters could be used as predictors of the hydrolysis performance of starch gels as well as in reverse engineering for the design of healthy foods.

3.1 Introduction

Starch is a polysaccharide extensively used as functional ingredient in many foods, due to its applications as thickener, stabilizer, gelling agent, and water retention agent (Ai & Jane, 2015). Because of that, besides intrinsic properties like amylose content, granule size, length of amylopectin branches and crystallinity, pasting properties or viscosity performance (peak viscosity, final viscosity, breakdown and setback viscosity) of the slurries during heating and cooling are always reported as key properties for starch characterization (Bajaj *et al*., 2018).

Consumers' health concerns have prompted to evaluate the food-related properties that could contribute to the human well-being and prevent diseases. In that scenario starch hydrolysis plays a fundamental role pertaining to postprandial glucose levels and in consequence the glycemic index of the foods (Singh *et al*., 2010). Starch digestion by the action of enzymes in the small intestine and the subsequent rate of absorption of the released glucose has been used to categorize the starch into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) (Englyst *et al*., 1992). Those facts have pointed out the importance of the starch hydrolysis kinetics, thus besides the intrinsic features of starch previously mentioned, digestive performance of the different starches is usually included in studies of starches characterization (Kaur *et al*., 2018). Different strategies have been developed to modulate the carbohydrate digestion, which include the reduction of available carbohydrate, reduce the rate of digestion or the delay of glucose absorption rate (Wee & Henry, 2020). In response to that, starches with low digestibility have been developed, like those rich in resistant starch either present in the native starch or obtained after chemical modification or processing (Bello-Perez *et al*., 2020).

Nevertheless, the digestion of starch is not only affected by starch features, but also physical properties of the media can modulate the rate of enzymes diffusion to starch substrates (Bello-Perez *et al*., 2020). Literature studies confirmed the role of bulk viscosity on the gastric emptying and on the reduction of glycemic index, opening the opportunity to modulate digestion with compounds that affect viscosity. This has been explored with diverse starches and hydrocolloids, which might restrict enzyme accessibility to starch by interacting with the surface of starch granules or creating a hydrated network surrounding that encapsulate the granule, or increasing the bulk viscosity (Gularte & Rosell, 2011; Qadir *et al*., 2021). In fact, results with different polysaccharides (guar gum, chitosan) indicated a

negative correlation between the peak viscosity (11,814-14,535 mPa s) and the SDS fraction of potato starches, suggesting that the effect might be more related to physical properties than chemical interactions (Sasaki, 2020). Nevertheless, very limited studies have correlated the viscosity of the starch gels with the digestion parameters. For instance, higher peak viscosity (480-5,076 mPa s) and viscosity breakdown, defined as the difference among the peak viscosity and lowest viscosity during holding stage at 95 ºC, (24-3,540 mPa s) of potato starches were correlated with lower hydrolysis rates of native starches but that correlation was not observed with the gelatinized starches (Noda *et al*., 2008). Bajaj *et al*. (2018) reported a reverse relationship between gel hardness and gelatinization temperatures with RS amount, but no relationship with peak viscosity in the range of viscosities 2,183 to 8,387 mPa s. Velásquez-Barreto *et al*. (2021) have recently reported the positive relationship of SDS, obtained in *in vitro* digestibility studies, with the Rapid Visco Analyzer (RVA) peak viscosity of gels (290-370 mPa s) and the viscosity upon cooling till 60 ºC (92-180 mPa s) of the starch gels isolated from un-conventional Peruvian tubers. Furthermore, other researchers used rheometric techniques to relate starch rheological behavior with their hydrolysis (Sandhu & Siroha, 2017). Yield stress (σ_o) or the minimum force required to initiate flow of starch paste was positively correlated with the peak viscosity (4,647-8,303 mPa s) in pearl millet starches, and negatively correlated with RS amount (Sandhu & Siroha, 2017). Overall, although previous research characterized the rheological properties of the different starch gels and their hydrolysis, results do not allow to identify the potential role of viscosity to explain encountered divergences.

Recently, authors studied the impact of viscosity of corn starch gels, obtained varying starch concentration, on the *in vitro* hydrolysis, observing that the hydrolysis kinetics constant depended inversely on the gel viscosity due to enzyme diffusion limitation (Santamaria *et al*., 2021). Specifically, positive significant relationship was defined between gel viscosity and the starch fraction SDS (*R*-square = 0.95) and RS (*R*-square = 0.96). In the case of RDS, results suggested that a viscosity threshold is required to affect the enzyme accessibility. Nevertheless, that impact of viscosity was only tested with corn starch gels, thus it remains to be investigated what happens with other cereal starches.

The possible correlation between starch gels characteristics and starch digestion might contribute to reverse engineering in the design of starch-based systems. In this way, foods could be design based on the knowledge

of food final food characteristics targeted. For this reason, the present study aims to validate the relationship of gel characteristics on the *in vitro* hydrolysis of starch gels obtained from different cereals. Starch gels from corn, wheat, and rice with variable viscosity (VV) or constant viscosity (CV) were rheological characterized, and their properties were correlated with the i*n vitro* hydrolysis parameters.

3.2 Materials and methods

3.2.1 Materials

Commercial food grade starches, having similar amylose content, from corn (20.15% amylose content and 12.43% moisture content) and wheat (23.98% amylose content and 12.72% moisture content) were supplied by EPSA (Valencia, Spain) and rice starch (20.71% amylose content and 10.30% moisture content) was purchased from Sigma Aldrich (Sigma Chemical, St. Louis, USA). The enzymes used were type VI-B α-amylase from porcine pancreas (EC 3.2.1.1) out of Sigma Aldrich (Sigma Chemical, St. Louis, USA) and amyloglucosidase (EC 3.2.1.3) from Novozymes (Bagsvaerd, Denmark). D-Glucose Assay Kit (GOPOD) was provided from Megazyme (Megazyme International Ireland Ldt., Bray, Ireland). Other chemicals were of analytical grade.

3.2.2 Preparation of starch gels with constant amount of starch (variable viscosity) or constant viscosity

Two sets of gels were prepared: first one using a fixed amount of starch, those gels were referred as variable viscosity (VV), and second one varying the amount of starch to obtain constant viscosity (CV). For gels under VV notation, 5 g starch (based on 14% moisture content) were suspended in 20 g water. Starches (corn, wheat, and rice) were manually dispersed in deionized water and the slurries were heated in a boiling water bath for 20 minutes, applying manual stirring every five minutes. Resulting gels were cooled down till 37 ºC for further analysis.

The viscosity of the rice gel, prepared as previously described, was measured at 37 ºC using a vibration viscometer VL7-100B-d15 (Hydramotion Ltd, Malton, United Kingdom). Although this viscometer measured at high shears, when reaching the Newtonian plateau, the complexity associated to shear-thinning materials is removed. Preliminary assays were conducted with corn and wheat starches to identify the amount of starch required to obtain similar viscosity to the one obtained with the rice gel. Afterwards, the second set of gels was prepared with starch: water, setting up the ratio for rice, corn, and wheat at 1:4, 1:5.5 and 1:5.2, respectively, to obtain gels with similar viscosity, referred as constant viscosity (CV).

The amount of total starch (TS) in the gels was quantified using a commercial assay kit (K.TSTA) (Megazyme International Ireland Ldt., Bray, Ireland) following the determination of total starch content of samples containing resistant starch (RTS-NaOH Procedure -Recommended).

3.2.3 Rapid Force Analyzer

The force changes during starch gelatinization were studied in the rapid force analyzer (RFA, Amylab® Chopin Technologies, Villeneuve-la-Garenne, Cedex, France), as previously described by Garzon and Rosell (2021). Briefly, starch slurry was placed into the precision test tubes of the device and manually shaken 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. The rapid test consisted of heating the sample at 100 ºC for 90 s subjected to continuous shearing. Plots recorded the force, expressed in Newtons, of the slurry/gel under continuous heating/shearing. The parameters defined include onset time indicating the start of gelatinization, initial (F0) and maximum force (F1), α-slope among F0 and F1, final force at 90 s (F2) and the force difference between F1 and F2 related to starch breakdown.

3.2.4 Gels viscoelastic behavior

The viscoelastic characterization was made on a stress-controlled rheometer (MCR 301; Anton Paar, Graz, Austria) using a starch pasting cell (ST24-2D/2V/2V-30, gap 2.460 mm, bob radius 12 mm) with a solvent trap kit to minimize water evaporation during tests. Different starches (corn, wheat, and rice) were dispersed in water (total weight 20 g) with constant and variable gel viscosity and poured into the rheometer cuvette at 95 ºC. First, a pre-shear of 100 s^{-1} was made for 1 min to homogenize the sample at 95 ºC. Secondly, a time sweep was carried out at 30 Pa, 1 Hz and 95 ºC for 19 min (previous assays were performed to ensure that frequency sweeps were carried inside the linear viscoelastic region of tested gels). Then, a cooling profile was made from 95 ºC to 37 ºC at 3 ºC/min with a constant stress of 30 Pa and a constant frequency of 1 Hz. The frequency sweep was carried out from 0.1 to 10 Hz at 1% strain and 37 ºC. Afterwards, a time sweep was carried out at 30 Pa, 1 Hz and at 37 °C for 30 min to observe the maturation of the gel. A second frequency sweep was made under the same conditions of the first one.

3.2.5 *In vitro* digestibility

Digestibility of starch gels was determined following the method described by Santamaria *et al*. (2021), with a few modifications. Fresh gel (200 mg) was

mixed with 4 mL of 0.1 M sodium maleate buffer (pH 6.9) containing porcine pancreatic α-amylase (0.9 U/mL) by using an Ultra Turrax T18 basic homogenizer (IKA-Werke GmbH and Co. KG, Staufen, Germany). The slurry was incubated in a shaker incubator SKI 4 (ARGO Lab, Carpi, Italy) at 37 ºC during 3 h under constant stirring (200 rpm). Aliquots were taken to quantify glucose release. The remnant starch after 24 h hydrolysis was solubilized with 2 mL of 1.7 M NaOH, using an Ultra-Turrax T18 homogenizer (IKA-Werke GmbH and Co. KG, Staufen, Germany) for 5 min 14,000 rpm in an ice bath and hydrolyzed with amyloglucosidase (143 U/mL) at 50 ºC for 30 min in a shaking water bath for its complete hydrolysis. Glucose determination was performed using a glucose oxidase-peroxidase (GOPOD) kit. The absorbance was measured by SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany) at 510 nm. Starch was calculated as glucose (mg) × 0.9.

From hydrolysis results, rapidly digestible starch (RDS) or the percentage of total starch hydrolyzed within 20 min of incubation, slowly digestible starch (SDS) or the starch fraction hydrolyzed within 20 and 120 min, digestible starch or total starch hydrolyzed after 24 h (DS), and resistant starch (RS) that remained after 24 h of incubation were calculated.

The *in vitro* hydrolysis data were fitted to a first-order equation (Eq.1) to describe the kinetics parameters of starch hydrolysis, as reported Goñi *et al*., (1997).

$$C = C_{\infty} (1\text{-}e^{-kt}) \quad (1)$$

where C was the concentration at t time, C_{∞} was the equilibrium concentration or maximum hydrolysis extent, k was the kinetic constant and t was the time chosen. Moreover, area under the hydrolysis curve in 180 min (AUC) was calculated and the hydrolysis percentage was the relation between C_{∞} and total starch content of each gel. All hydrolysis parameters were calculated in relation to 100 g of gel.

3.2.6 Statistical analysis

All experiments were carried out in triplicate and experimental data were statistically analyzed by Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA). Data was subjected to multivariate analysis of variance (MANOVA) and values were expressed as a mean ± standard deviation. Fisher's least significant differences test (LSD) was used to estimate significant differences among experimental mean values with a significance level ($p \leq 0.05$). Furthermore, Pearson correlation analysis was used to identify possible relationship between rheological and hydrolysis parameters.

3.3 Results and discussion

Two different types of gels were prepared using corn, wheat or rice, starches to identify the role of viscosity on the pasting, viscoelastic properties, and digestibility performance. First set of gels were prepared containing the same amount of starch and thus variable viscosity (VV). The initial amount of starch selected for those gels was based on a previous study (Santamaria *et al*., 2021), where that concentration (1:4 starch:water) for corn starch gels was the most limiting one regarding the relationship among closed gel structure, higher viscosity, and the slowest and more limited starch hydrolysis. In contrast, the second set was prepared varying the amount of starch for obtaining gels with the same viscosity (CV). The amount of total starch in samples with variable gel viscosity was 17.20 ± 0.20 g/100 g. On the other hand, gels having constant viscosity contained 12.63 ± 0. 08 g/100 g, 12.60 ± 0.18 g/100 g and 16.93 ± 0.15 g/100 g starch, for corn, wheat, and rice gels, respectively.

Viscosity of the gels prepared at VV was significantly ($p < 0.05$) influenced by starch source (**Table 3.1**). Corn gel presented the highest viscosity (1170 mPa s) at 37 ºC, followed by wheat gel (834 mPa s), and finally rice gel (525 mPa s). The viscosity of the rice starch was selected as the target to obtain CV gels.

3.3.1 Starches performance during gelatinization and viscoelastic properties of gels

After setting up the conditions to obtain the two types of gels, their textural performance during gelatinization was recorded using a rapid force analyzer (RFA) (Garzon & Rosell, 2021). It consists of a rapid (90 s) thermal method under continuous shearing. The force required to stir the slurries during gelatinization was different for each starch gel (**Figure 3.1**). Very low force was detected at the beginning of the test, till heating was high enough to promote the onset of starch swelling with a simultaneous increase of the stirring force. Pasting performance of gels was dependent on the source of starch and, obviously, on the amount of starch. However, the observed changes in the plots did not only reveal the starch dilution, but also changes in the force pattern of the gels. Parameters defined to analyze gels performance in the RFA are showed in **Table 3.1**.

Table 3.1: Rheological parameters of starch gels prepared at constant amount of starch giving variable gel viscosity (VV) or different amount of starch required to reach constant gel viscosity (CV). Gel development was recorded with a Rapid Force Analyzer and rheometric behaviour in the stages of cooling and mechanical spectra were evaluated with a rheometer. Gel made with rice starch was selected for defining the target viscosity at 37 ºC, because of that the same gel was used for VV and CV.

		Variable gel viscosity (VV)			Constant gel viscosity (CV)		*p*-Value	
		Corn VV 1:4	**Wheat VV 1:4**	**Rice VV Rice CV 1:4**	**Corn CV 1:5.5**	**Wheat CV 1:5.2**	Source	Viscosity
η adjustment	Vibration viscosimeter							
	η (mPa s)	1170 ± 293^{a}	834 ± 81^{b}	525 ± 15^{c}	542 ± 88^{c}	553 ± 55^{c}	**0.0297**	**0.0044**
Gel development	RFA parameters							
	Onset (s)	36 ± 1^{a}	28 ± 0^{b}	34 ± 2^{a}	34 ± 1^{a}	28 ± 3^{b}	**0.0005**	0.7310
	F0 (*N*)	2.10 ± 0.28	1.98 ± 0.49	1.90 ± 0.76	1.72 ± 0.12	1.51 ±0.62	0.8749	0.3515
	α-slope	1.23 ± 0.00^{a}	0.99 ± 0.01^{b}	0.57 ± 0.02^{c}	0.52 ± 0.04^{c}	0.39 ± 0.02^{d}	0.1314	**0.0043**
	F1 (*N*)	11.39 ± 0.30^{b}	15.29 ± 0.55^{a}	9.93 ± 0.86^{b}	6.11 ± 0.26^{d}	8.08 ± 0.68^{c}	0.1626	**0.0060**
	F2 (*N*)	6.74 ± 0.25^{c}	11.99 ± 1.14^{a}	8.78 ± 1.03^{b}	4.54 ± 0.02^{d}	7.92 ± 0.62^{bc}	**0.0030**	**0.0189**
	Breakdown (*N*)	4.65 ± 0.05^{a}	3.19 ± 0.44^{b}	1.16 ± 0.17^{c}	1.57 ± 0.28^{c}	0.15 ± 0.06^{d}	**0.0394**	**0.0046**
Gel behavior	Rheometric parameters							
	Cooling profile (initial and end values, at 1 Hz)							
	G´ 95 °C (Pa)	301 ± 2^{c}	575 ± 7^{a}	340 ± 8^{b}	171 ± 6^{d}	293 ± 16^{c}	**0.0134**	**0.0102**
	G″ 95 °C (Pa)	108 ± 39^{b}	233 ± 42^{a}	81 ± 21^{b}	73 ± 19^{b}	79 ± 0^{b}	0.1073	**0.0488**
	tan δ 95 °C	0.359 ± 0.125^{ab}	0.405 ± 0.069^{ab}	0.237 ± 0.057^{b}	0.428 ± 0.095^{a}	0.269 ± 0.016^{ab}	0.0824	0.6637
	G´ 37 °C (Pa)	3025 ± 49^{b}	3580 ± 141^{a}	872 ± 4^{e}	1380 ± 85^{d}	1580 ± 99^{c}	**0.0049**	**0.0045**
	G″ 37 °C (Pa)	155 ± 31^{b}	344 ± 4^{a}	99 ± 12^{c}	92 ± 9^{c}	173 ± 5^{b}	**0.0022**	**0.0175**
	tan δ 37 °C	0.051 ± 0.011^{b}	0.096 ± 0.003^{a}	0.113 ± 0.013^{a}	0.067 ± 0.011^{b}	0.109 ± 0.004^{a}	**0.0001**	0.1211
	Mechanical spectra							
	Slope linear *G′* (0.1-10 Hz)	0.020 ± 0.001	0.022 ± 0.002	0.026 ± 0.008	0.019 ± 0.003	0.023 ± 0.002	0.6419	0.1769
	Slope linear *G″* (0.1-10 Hz)	0.213 ± 0.035	0.195 ± 0.074	0.235 ± 0.042	0.247 ± 0.019	0.246 ± 0.002	0.1919	0.9474
	G′ (0.1 Hz)	4620 ± 71^{b}	5775 ± 7^{a}	1075 ± 35^{e}	2675 ± 148^{d}	3955 ± 92^{c}	**0.0000**	**0.0042**
	G″ (0.1 Hz)	154 ± 61^{ab}	255 ± 87^{a}	97 ± 24^{b}	68 ± 6^{b}	109 ± 14^{b}	0.1148	**0.0387**
	tan δ (0.1 Hz)	0.033 ± 0.013^{b}	0.044 ± 0.015^{b}	0.090 ± 0.020^{a}	0.025 ± 0.001^{b}	0.028 ± 0.004^{b}	**0.0003**	0.3128

Values followed by different letters within the same row denote significant differences $p < 0.05$. Parameters: η (viscosity), onset (starch gelatinization initial time), F0 (initial force), α-slope (between F0 and F1), F1 (maximum force), F2 (final force), breakdown (difference between F1 and F2), G´ (storage modulus) G" (loss modulus), tan δ (damping factor).

When adapted viscosity (CV), to have constant gel viscosity, differences within RFA plots were reduced, particularly during gelatinization. Regarding specific parameters, starch source significantly ($p < 0.05$) affected onset of gelatinization, force at 90 s (F2) and breakdown, whereas the gel viscosity (CV or VV gels) factor affected significantly ($p < 0.05$) α-slope, maximum (F1) and final force (F2), and breakdown. Wheat gels showed the lowest onset indicating that gelatinization began at lower temperatures (Garzon & Rosell, 2021). In VV gels made with the same amount of starch, corn gel showed higher α-slope, indicating faster gelatinization, and wheat gel displayed the highest maximum force (F1). Garzon and Rosell (2021) observed the same trend and correlated higher force with more porous gels, revealing thicker walls and big holes. Corn gel presented higher breakdown, indicating lower resistance to physical rupture during starch granule swelling. Similar result was reported using the RVA when comparing corn and rice starches and it was related to higher swelling of granules (Gupta *et al*., 2009). When adapting gels to obtain CV, corn and wheat gels showed lower forces with respect to rice gel, along gelatinization. Starches showed significant differences on F1, but onset, α-slope and breakdown of rice and corn starches were similar, confirming the proximity of the physical behavior of the starch gels when adapting viscosity.

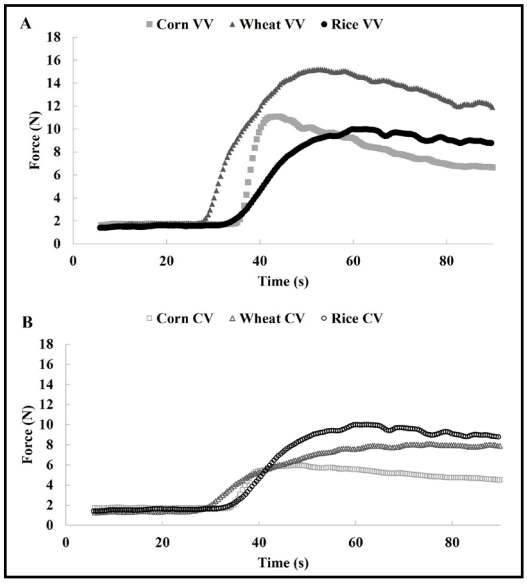

Figure 3.1: Plots of gel force during gelatinization of different starches using a rapid force analyser. (A) Gels were prepared at constant amount of starch giving variable viscosity (VV, closed symbols), or (B) different amount of starch required to reach constant viscosity (CV, open symbols). Corn: ■, wheat: ▲, rice: ●.

All starch gels, after fully developing a stable network structure, showed solid like behavior ($G' > G''$) (**Table 3.1**). During the cooling profile from 95 to 37 ºC both moduli increased, but greater differences were observed on G' than G''. In VV gels, $\Delta G'$ and $\Delta G''$ were higher for corn and wheat starches than for rice starch. At 37 ºC, rice starch led to the weakest gel with the lowest elastic modulus (872 Pa), **Table 3.1.** Meanwhile, the strongest gel (high G' value) was obtained with wheat starch (in respective sets of CV and VV gels). This property is relevant to measure the easiness of the gel to be

fragmented in small pieces under shear rates. Rheological tests confirmed that CV gels had closer values of viscous modulus. At 37 ºC, gels were subjected to two frequency sweeps (time 0 and 30 min) and the viscoelastic behavior with angular frequency was almost constant, meaning that gels maturation took mainly place during cooling and when gels achieved the lowest temperature, the maturation was practically completed (data not shown). Strong and weak gels can be classified as such based on their mechanical spectra. In all cases, $G' > G''$ from 0.1 to 10 s^{-1} with G' relatively independent of frequency (slope < 0.03) and G'' increased with increasing frequency (**Figure 3.2**). In fact, the slope of G'' with frequency varied in a narrow range (from 0.20 up to 0.25) and no significant differences ($p > 0.05$) were found between tested starch gels, **Table 3.1**. This type of spectrum is usually associated with weak gel (Feng *et al*., 2020). At small deformation, weak gels resemble strong gels, but as deformation increases, the three-dimensional networks undergo a progressive (and reversible) breakdown (Rosalina & Bhattacharya, 2002). The tan δ (G''/G') values at 0.1 Hz for VV gels were 0.033, 0.044 and 0.090 for corn, wheat, and rice gels, respectively, indicating that viscous character is low, but more relevant in rice gels. No significant differences ($p > 0.05$) between tan δ of CV gels and VV gels from same starch were observed. Therefore, some differences in the viscoelastic behavior of tested starch gels were found related to the formation of firmer (higher G') or more stable (low damping factor) structures.

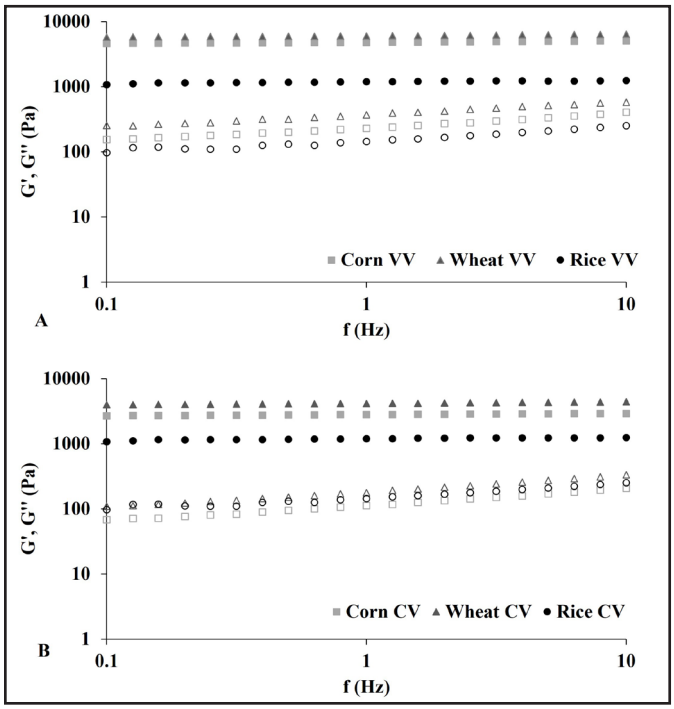

Figure 3.2: Mechanical spectra of starch gels prepared at (A) constant amount of starch giving variable viscosity (VV), or (B) different amount of starch required to reach constant viscosity (CV). Symbols: storage modulus-closed (G'); loss modulus-open (G"). Corn: ■ , wheat: ▲, rice: ●.

3.3.2 *In vitro* hydrolysis of starch gels

Starch gels were subjected to enzymatic hydrolysis with digestive enzymes (**Figure 3.3**). Intrinsic properties like amylose size and chain size distribution of amylopectin have been related to the *in vitro* digestion of native starches, but in gel state that molecular order and their contribution might no longer be crucial and be more related to new molecular organization in which the initial amorphous structure is more susceptible to enzyme hydrolysis (Martinez *et al*., 2018). Therefore, if only structural features were responsible of the starch hydrolysis kinetics, no differences would be detected due to viscosity changes.

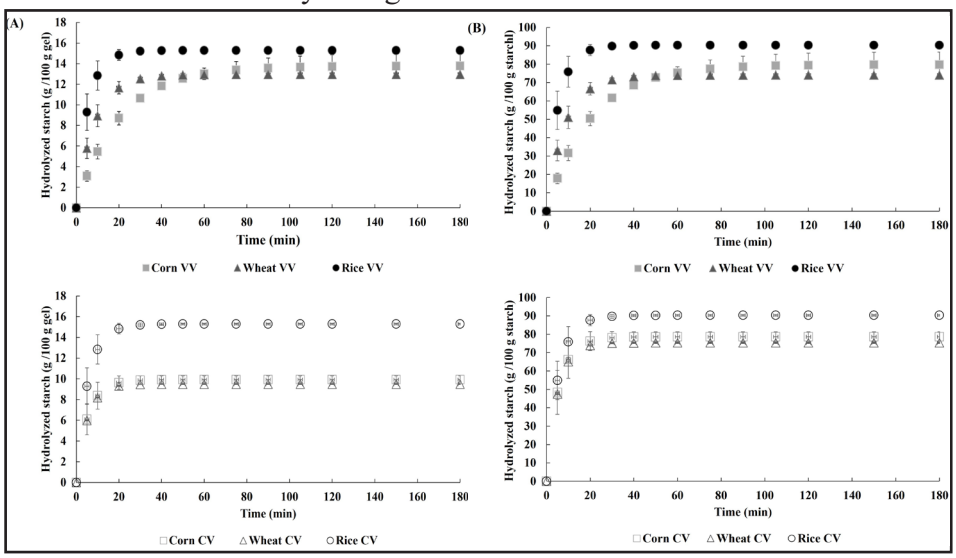

Figure 3.3: Effect of different viscosities on *in vitro* starch gels digestion. Graphs are expressed in (A): hydrolyzed starch g/100 g gel; (B) hydrolyzed starch g/100 g starch. Gels were prepared at constant amount of starch giving variable viscosity (VV, closed symbols), or different amount of starch required to reach constant viscosity (CV, open symbols). Corn: ■, wheat: ▲, rice: ●.

To assess the impact of the amount of starch, results are expressed in grams of hydrolyzed starch per 100 g of gel (**Figure 3.3-A**) and grams of hydrolyzed starch per 100 g of starch (**Figure 3.3-B**). Regarding VV gels hydrolysis, rice gel showed faster and higher hydrolysis (**Figure 3.3-A VV**), which could be related to its lower viscosity at 37 ºC (**Table 3.1**), compared to wheat and corn gels. In highly viscous systems, like wheat and corn gels, the enzyme diffusion encounters the external resistance (viscosity) of the gels, that affects the hydrolysis. Similar behavior has been observed when modulating viscosity by incorporating hydrocolloids to starch gels, and it has been attributed to limitations of the enzyme accessibility to starch (Ma *et al*., 2019; Sasaki & Kohyama, 2011). However,

when comparing gels having the same viscosity (CV) different enzymatic hydrolysis were observed (**Figure 3.3-A CV**). CV gels of wheat and corn displayed similar hydrolysis behavior, but CV rice gel showed more extensive hydrolysis. Although that trend could be initially attributed to its higher starch content, hydrolysis plots normalized to the amount of starch revealed the same trend (**Figure 3.3-B**). Therefore, results confirmed that gels hydrolysis was not only affected by starch content, and considering they had similar viscosity, gel physical properties like viscoelasticity might also influence the hydrolysis of gels. This behavior might be related either to the lower *G'* of rice gel (**Table 3.1**), which suggested a weaker gel structure, or to more porous gels, since as previously mentioned high force gels (F1 in **Table 3.1**) were related to porosity (Garzon & Rosell, 2021). Both effects would favor enzyme accessibility to the gel, explaining the more extensive hydrolysis in CV rice gels.

Starch fractions (RDS, SDS, DS and RS), according to the rate of glucose release, presented statistically significant differences ($p < 0.05$) (**Table 3.2**). The starch source significantly ($p < 0.05$) affected RDS, whereas the gels viscosity significantly ($p < 0.05$) impacted on the amount of SDS and RS. VV gels made of corn starch had the lowest amount of RDS, which agree with finding of Zhang *et al*. (2006) studying different raw cereal starches. Corn VV gel had the highest viscosity, thus the variability in the starch gel characteristics mainly affect RDS. In addition, corn VV gel had the highest amount of SDS (**Table 3.2**). Nevertheless, gels made at constant viscosity did not present statistically significant differences in SDS, and rice gel gave the highest RDS and RS.

Additionally, kinetics parameters derived from *in vitro* hydrolysis plots (**Figure 3.3-A**) are shown in **Table 3.2**. Kinetic constant (k) or hydrolysis rate was significantly ($p < 0.05$) affected by gel viscosity, being faster when decreasing the viscosity, but similar k ($p > 0.05$) was obtained with the gels obtained at CV. Therefore, the loss of the gels crystalline structure was not determining the k (Guo *et al*., 2018), but physical properties are significantly affecting hydrolysis. When variable viscosity, corn gel showed the slowest kinetic constant. A decrease in the k was accompanied by a simultaneous increase in the SDS content. For this reason, the gel viscosity could be a modulating factor, because it can limit the enzyme diffusion rate retarding the enzymatic hydrolysis. Regarding the equilibrium concentration of hydrolyzed starch (C_∞) and the area under the hydrolysis curve (AUC), they were significantly ($p < 0.05$) affected by both factors: starch source and gels viscosity. The maximum hydrolysis (C_∞) indicates the extent of the hydrolysis when the curve reaches a plateau and the area under the curve is related to the glucose release in 180 minutes of hydrolysis. As previously mentioned, rice gel presented the largest hydrolysis (**Figure 3.3-A**), even

when comparing starch gels made at constant viscosity. In samples with constant viscosity these parameters decreased, due to lower starch content in gels.

The relationship between equilibrium concentration of hydrolyzed starch and total starch content of each gel was significantly affected by the type of starch. Rice gel had higher hydrolysis percentage (90.36%), while corn and wheat gels displayed similar results. Consequently, the gel viscosity is a factor with great impact in the reaction rate (*k*) and on the starch fractions, particularly in the SDS. This result agrees with findings of Velásquez-Barreto *et al*. (2021) with tuber starches, observing positive correlations between gels viscosities and SDS amounts.

3.3.3 Correlation matrix

A correlation matrix was established to find any significant relationships between parameters recorded from pasting behaviour, viscoelastic characterization, and the *in vitro* hydrolysis of tested gels (**Table 3.3**). Viscosity at 37 ºC showed a strong positive correlation with SDS (r = 0.83) and moderate with DS (r = 0.65) and RS (r = 0.63). Therefore, results confirmed that viscosity of the gels affects the hydrolysis behaviour. Likely, viscosity of the system retards the binding of α-amylase-starch or modifies starch structure affecting α-amylase activity (Dhital *et al*., 2017). In fact, a significant negative correlation (r = -0.82) was observed between viscosity at 37 ºC and kinetic constant (*k*), confirming that viscosity limits mass transfer and affects the hydrolysis reaction rate. These results support that higher viscosity in a food matrix increases SDS content, which has been associated with lower glycemic index, greater satiety and slowing enzymatic hydrolysis (Ma *et al*., 2019; Zhu *et al*. 2013). A positive correlation was observed between the α-slope of RFA with SDS (r = 0.84) and RS (r = 0.74). Interestingly, a strong negative correlation (r = -0.87) was observed between the α-slope and kinetic constant (*k*), indicating that faster gelatinization led to gels with reduced kinetic constant. This fact is also related to gel firmness (*G´*), with also negative correlation (r = -0.73), because gels with higher gelatinization rate, give firmer gels that undergo slower hydrolysis (Garzon & Rosell, 2021). Positive moderate correlation was observed between maximum force (F1) and RS (r = 0.74). Garzon and Rosell (2021) related the force with gel structure, suggesting that higher force was required for obtaining gels with more porous structure. Breakdown was positively correlated with SDS (r = 0.83) and RS (r = 0.65) and negatively correlated with kinetic constant (r = -0.84), which agree with previous results (Gularte & Rosell, 2011). It has been reported that the loss of crystalline structure in gelatinized starch is not a determining factor for starch digestion (Guo *et al*., 2018). Nevertheless, it seems that higher breakdown, and

Table 3.2: Parameters[a] of *in vitro* starch gels hydrolysis. Gels were prepared at constant amount of starch giving variable viscosity (VV) or different amount of starch required to reach constant viscosity (CV). Gel made with rice starch was selected for defining the target viscosity at 37 ºC, because of that the same gel was used for VV and CV.

	Variable gel viscosity			Constant gel viscosity		*p*-Value	
	Corn VV	**Wheat VV**	**Rice VV Rice CV**	**Corn CV**	**Wheat CV**	Source	Viscosity
RDS (%)	8.70 ± 0.66^{c}	11.66 ± 0.60^{b}	14.84 ± 0.51^{a}	9.64 ± 0.65^{c}	9.32 ± 0.05^{c}	**0.0001**	0.4246
SDS (%)	5.02 ± 1.79^{a}	1.30 ± 0.73^{b}	0.45 ± 0.43^{b}	0.30 ± 0.31^{b}	0.18 ± 0.00^{b}	0.1190	**0.0461**
DS (%)	14.26 ± 2.76^{a}	11.51 ± 1.91^{ab}	13.26 ± 0.26^{ab}	11.83 ± 0.45^{ab}	10.26 ± 0.81^{b}	0.0756	0.1604
RS (%)	20.15 ± 1.71^{a}	17.85 ± 1.94^{a}	17.24 ± 2.79^{a}	7.76 ± 3.57^{b}	10.62 ± 1.03^{b}	0.4312	**0.0169**
k (min^{-1})	0.05 ± 0.01^{b}	0.12 ± 0.03^{ab}	0.19 ± 0.06^{a}	0.20 ± 0.07^{a}	0.20 ± 0.00^{a}	0.2488	**0.0383**
C_∞ (%)	13.77 ± 1.20^{b}	12.96 ± 0.13^{b}	15.29 ± 0.08^{a}	9.93 ± 0.34^{c}	9.50 ± 0.05^{c}	**0.0022**	**0.0063**
AUC	2194 ± 114^{b}	2215 ± 4^{b}	2661 ± 39^{a}	1729 ± 78^{c}	1656 ± 8^{c}	**0.0003**	**0.0058**
C_∞/TS (%)	79.77 ± 7.10^{b}	74.08 ± 3.36^{b}	90.36 ± 0.31^{a}	78.64 ± 3.21^{b}	75.86 ± 0.46^{b}	**0.0003**	0.9064

Means within the same row followed by different letters indicate significant differences $p < 0.05$. C_∞ and k were determined by the equation, $C = C_\infty (1 - e^{-kt})$.
[a] Rapidly digestible starch (RDS), slowly digestible starch (SDS), digestible starch (DS), resistant starch (RS), kinetic constant (k), equilibrium concentration (C_∞), area under the hydrolysis curve after 180 min (AUC), total starch content (TS) and hydrolysis percentage (C_∞/TS).

consequently lower stability during heating, allowed higher structural disorganization of the gels, which could be recrystallized during cooling giving more structured gels, that offer more resistance to hydrolysis, as indicated higher SDS and lower *k*. This assumption was also supported by the significant negative correlation observed between SDS and tan δ (*G''/G'*) values of the gels after cooling (r = -0.72), relating starch hydrolysis with the level of gel structure. Regarding the rheometric properties, those that showed the most significant correlations ($p < 0.01$) were in mechanical spectra. A significant negative correlation (r = -0.78) was observed between *G´* (0.1 Hz) and hydrolysis percentage (C_∞/TS). This could mean that a characteristic such as elasticity can influence the percentage of hydrolysis. In native starches the chain length distribution has been correlated with the starch digestibility, Martinez *et al*. (2018), but that fundamental property does not seem to explain the hydrolysis behaviour of the gels. The digestibility of the gel depends on the ability of the enzyme to penetrate into the gel, consequently, strong structures (high firmness) of gels seemed to delay the hydrolysis. Also, there was a highly correlation between tan δ (*G''/G'*) values at 0.1 Hz with RDS (r = 0.89), C_∞ (r = 0.71), AUC (r = 0.82), and C_∞/TS r = 0.69), which suggested that less structured gels (high damping factor) favoured the initial hydrolysis of starch, for the first 20 minutes, and also the extent of the gels hydrolysis.

Table 3.3: Correlation matrix among rheological properties (viscometer, RFA, and rheometer parameters) and hydrolysis parameters obtained from the different starch gels.

	RDS (%)	SDS (%)	DS (%)	RS (%)	*k*	*C*∞ (%)	AUC	*C*∞/TS (%)
η (mPa s)	-0.41	**0.83****	**0.65***	**0.63***	**-0.82****	0.30	0.14	-0.23
Onset (s)	-0.05	0.42	**0.68***	0.12	-0.25	0.31	0.24	0.49
F0 (N)	0.08	0.42	0.25	0.38	-0.31	0.44	0.38	0.20
α-Slope	-0.21	**0.84****	0.50	**0.74****	**-0.87****	0.52	0.36	-0.16
F1 (N)	0.25	0.37	0.15	**0.74***	-0.54	0.57	0.53	-0.17
F2 (N)	0.46	-0.06	-0.12	0.51	-0.14	0.41	0.46	-0.10
Breakdown (*N*)	-0.25	**0.83****	0.50	**0.65***	**-0.84****	0.46	0.31	-0.16
G′ 95 °C	0.37	0.06	-0.08	0.57	-0.30	0.42	0.44	-0.24
G″ 95 °C	0.15	0.06	-0.07	0.43	-0.34	0.19	0.20	-0.54
tan δ 95 °C	-0.33	0.01	0.09	-0.07	-0.23	-0.32	-0.34	**-0.68***
G′ 37 °C	-0.34	0.58	0.07	0.53	**-0.73***	0.16	0.03	-0.58
G″ 37 °C	0.00	0.05	-0.20	0.35	-0.30	0.04	0.03	-0.62
tan δ 37 °C	**0.66***	**-0.72***	-0.30	-0.13	**0.65***	0.04	0.21	0.18
Slope lin *G′* (0.1-10 Hz)	-0.02	-0.26	-0.47	-0.49	0.40	-0.24	-0.21	0.18
Slope lin *G″* (0.1-10 Hz)	0.52	-0.27	-0.12	0.20	0.16	0.29	0.37	0.35
G′ 0.1 Hz	-0.54	0.41	-0.17	0.28	-0.56	-0.19	-0.30	**-0.78****
G″ 0.1 Hz	0.03	0.24	0.24	0.57	-0.48	0.23	0.20	-0.43
tan δ 0.1Hz	**0.89****	-0.21	0.42	0.39	0.20	**0.71***	**0.82****	**0.69***

Bold values indicate significant correlations. ** Indicates $p < 0.01$. * Indicates $p < 0.05$.

3.4 Conclusions

Rheology performance of starch gels, besides their *in vitro* hydrolysis, allow the assessment of global starch functionality, namely technological behaviour for industrial applications and the prediction of their comportment along digestion. Viscosity plays a fundamental role on the starch gels functionality, being an important parameter to modulate those functionalities. Starch gels from different cereals have significant different viscosity when produced at constant starch concentrations, and in consequence different viscoelastic properties and *in vitro* hydrolysis kinetics. Particularly, wheat and corn gels displayed higher forces and solid like behaviour. Conversely, rice gel showed lower gelatinization rate and weak behaviour. Nevertheless, force along gelatinization and the viscoelastic properties of cereal starch gels were closer when comparing gels of similar viscosity, showing alike hydrolysis rates. Results allowed to correlate rheological properties with hydrolysis parameters, confirming the importance of gel viscosity, which was positively correlated with SDS fraction (r = 0.83), and RS (r = 0.63), and negatively with the kinetic constant (r = -0.82). Therefore, higher viscosity in the range 550-1170 mPa s slowed down the enzymatic hydrolysis. Therefore, apart from the already well-known factors (amylose/amylopectin ratio, chain length, gel structure, and so on), affecting starch digestion, gel viscosity could be a rapid indicator for estimating the starch kinetic hydrolysis. Overall, gels viscosity of cereal starches greatly affects the hydrolysis kinetics, which opens the opportunity to apply reverse engineering in the design of starch-based systems to reduce postprandial glucose level. Further on *in vivo* studies will be undertaken to confirm results obtained in model systems.

Author contributions: Credit roles: MS: Conceptualization; data curation; formal analysis; investigation; methodology; and roles/writing – original draft; LM: Investigation and methodology; RG: Methodology; supervision; and data curation; RM: Formal analysis; writing – review & editing; and funding acquisition; CMR: Conceptualization; funding acquisition; investigation; supervision; and writing – review & editing.

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Conflicts of interest: The authors declare no conflict of interest.

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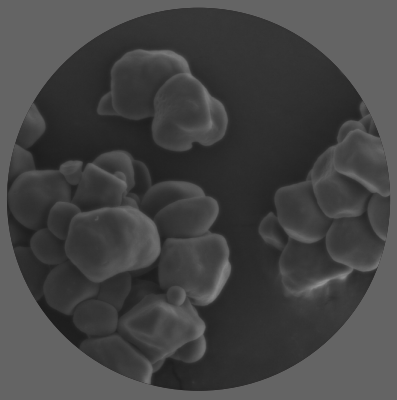

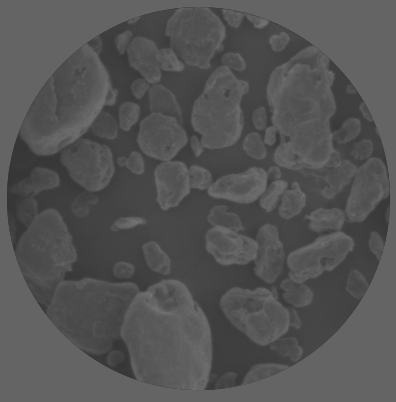

CHAPTER **FOUR**

Performance of starch gels on *in vitro* enzymatic hydrolysis assessed by rheological methodologies

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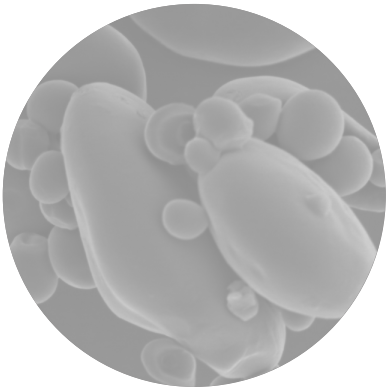

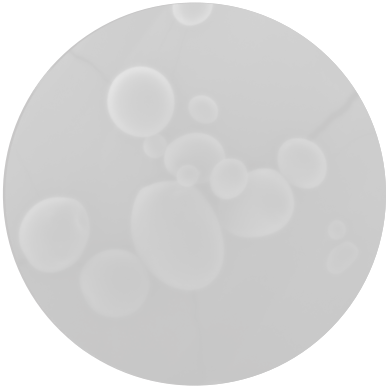

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ABSTRACT

Starch hydrolysis is attracting much attention due to its relationship to digestion and glucose release. The objective was to propose rapid and continuous analytical methods that allow measuring gels hydrolysis following apparent viscosity (μ). Three different starches (corn, wheat, and rice) were tested recording starch gelatinization followed by gels digestions (digestograms) using a rapid-visco analyzer (RVA) or a rheometer. Results were compared with those obtained by measuring glucose release along hydrolysis. A modified first-order kinetic model in the RVA ($R^2 > 0.99$) and rheometer ($R^2 > 0.99$) described the gels digestograms. Wheat gel showed higher hydrolysis rate (k), which indicated faster digestion followed by rice and corn gels. The proposed models allowed rapid analysis of starch digestograms, allowing to discriminate among hydrolysis rate of different starches. These less time-consuming methods could be an option to continuously analyze starch gelatinization followed by enzymatic digestion.

4.1 Introduction

Nowadays, one of the trend drivers for food manufacturers is the development of healthy foods, particularly addressing increase of nutrient availability, improve satiety or decrease blood glucose response (Priyadarshini *et al*., 2022). Because of that, much interest has been focused on developing *in vitro* methods that allow predicting foods and nutrients behavior along the oro-gastrointestinal digestion (Brodkorb *et al*., 2019; Havenaar & Minekus, 2019). Particularly in the case of starch digestion, the oro-gastrointestinal digestion is rather challenging due to the many dilutions that masked the kinetic changes in the starch fraction (Santamaria *et al*., 2022). Alternatively, *in vitro* starch digestion methods are the most applied ones, mainly based on enzymatic hydrolysis followed by measuring the glucose release (Dupont *et al*., 2019). However, other indirect methods for assessing starch performance along enzymatic digestion have also attracted attention, particularly following viscosity (Gee & Johnson, 1985) and the impact of different enzyme concentrations (Evans *et al*., 1986) during digestion simulation, initially using a rotary viscometer. Nowadays, there are other equipment commonly used for following rheological changes, namely rheometer and Rapid Visco Analyzer (RVA), and some authors have already used them to record rheology changes that occurred along digestion at 37 ºC (Ferry *et al*., 2005; Sorba & Sopade, 2013). Other authors followed the glucose release that occurs during the digestion period in parallel to rheology changes recorded in the rheometer (Bordoloi *et al*., 2012; Dartois *et al*., 2010; Hardacre *et al*., 2016; Hardacre *et al*., 2015). In those studies, focus has been put on the impact of shear rate (0.1, 1, 10 s^{-1}) on the *in vitro* digestion of gelatinized potato and corn starch (Hardacre *et al*., 2016) or the impact of hydrocolloids like guar gum on the digestibility of potato flour (Bordoloi *et al*., 2012) or its effect on waxy maize (Dartois *et al*., 2010). Hardacre *et al*. (2015) also studied de impact of soluble and insoluble fiber in potato and corn starches during their *in vitro* digestion.

Similarly, RVA has been used to evaluate the apparent viscosity decay produced on different wheat starch gels (6, 8 and 10%) or waxy maize starch gels (2, 4 and 6%) at 37 ºC when adding different levels of α-amylase and their relationship with volatile compounds release, but without relating those with starch digestion (Ferry *et al*., 2005). Conversely, Sorba and Sopade (2013) studied the enzymatic hydrolysis of potato and waxy maize starch gels using amylase and amyloglucosidase and recording apparent viscosity changes with RVA.

Furthermore Hódsági *et al*., (2012) found some significant correlations among glucose release during enzymatic hydrolysis of corn and wheat starches and their pasting parameters; particularly in the case of wheat starch hydrolysis rate and

peak viscosity, trough, and final viscosity, which might be useful for estimating *in vitro* digestion. However, previous studies have been conducted using rheology methods to independently evaluate gelatinization behavior of starches or to follow rheological modifications during the enzymatic hydrolysis. The aim of this study was to develop rapid methods that allow in a single test to evaluate starch performance during gelatinization followed by enzymatic digestion. For that purpose, rheological methods were developed in the RVA and rheometer using α-amylase, and result compared with the data obtained by quantifying glucose release. The inclusion of enzymatic hydrolysis into the rheological methods might provide rapid methods to predict the behavior of starch gels during enzymatic digestion.

4.2 Materials and methods

4.2.1 Materials

Starches from corn and wheat (EPSA, Valencia, Spain) and rice (Sigma Aldrich, Sigma Chemical, St. Louis, USA) were employed. Moisture content of the starches were 13.08%, 12.60% and 10.56%, for corn, wheat, and rice, respectively. The enzymes used were VI-B α-amylase from porcine pancreas (EC 3.2.1.1) from Sigma Aldrich (Sigma Chemical, St. Louis, USA) and amyloglucosidase (EC 3.2.1.3) provided by Novozymes (Bagsvaerd, Denmark). Glucose oxidase/peroxidase (GOPOD) kit (Megazyme International Ireland Ldt., Bray, Ireland) was used. All reagents were of analytical grade. Solutions and standards were prepared using deionized water.

4.2.2 Change in viscosity of gel and its hydrolysis using the Rapid Visco Analyzer

Three grams (14% moisture basis) of starch were placed into the RVA canister and dispersed in 25 mL distilled water. The pH of slurries was determined. Tests were performed in the Rapid Visco Analyzer (RVA 4500; Perten Instruments, Hägersten, Sweden) using the following settings: 50 ºC for one min, heating from 50 to 95 ºC at 10 ºC/min, holding at 95 ºC for 2.5 min, cooling down to 37 ºC at 10 ºC/min, followed by holding at 37 ºC for 36 s for adding the α-amylase solution (900 U/mL solution), and then continue recording viscosity at 37 ºC for 5 min. Preliminary assays were conducted with corn starch to select the amount of α-amylase (**Figure S 4.1**). Different concentrations of α-amylase (56, 90, 169, 225 U) were tested and the enzyme content that induced an intermediate hydrolysis rate was selected (90 U/100 µL solution that represented 30 U/g of starch). Temperature within the slurry/gel was recorded using a Comark N2014 multi-sensor temperature data logger (Comark Instruments, Norwich, Norfolk,

UK). Temperature readings were recorded every second. Rotational speed in the first 10 s was 960 rpm and then it was kept at 160 rpm along the test, except when the protocol was stopped (0 rpm) for enzyme addition. Apparent viscosity (mPa s) of starches without adding enzyme was also recorded as reference. RVA analysis were carried out at least duplicate. Pasting parameters extracted from the recorded data included: onset time (min), at which starch viscosity started to increase during heating, peak viscosity (maximum viscosity during heating), peak time (min, at which maximum viscosity is reached), trough viscosity (minimum viscosity when holding at 95 ºC), breakdown (difference between maximum and trough viscosity), setback (difference between viscosity at 37 ºC and trough viscosity), initial (after adding the enzyme) and final (at the end of the assay) viscosity during the enzymatic hydrolysis.

4.2.3 Rheology of starch gels and enzymatic hydrolysis using a rheometer

The rheological experiments were carried out with a stress-controlled rheometer (MCR 301; Anton Paar Physica, Graz, Austria) using a starch pasting cell (ST24-2D/2V/2V-30) with the following settings: measuring bob radius of 12.00 mm, cup radius of 14.46 mm and a gap of 2.46 mm. A solvent trap kit was used to minimize water evaporation during tests. A similar protocol, regarding starch concentration (3 g -14% moisture basis- in 25 mL distilled water), times and temperatures, to the one described above for the RVA, was defined to monitor in the rheometer the gel formation followed by the starch hydrolysis. A pre-shear at 100 rad/s (960 rpm), 50 ºC for 10 s was applied to achieve sample homogenization, followed by a holding time for 1 min at 50 ºC and 18 rad/s (160 rpm). This shear rate was kept for the rest of the assay. A temperature sweep was carried out from 50 to 95 ºC at 10 ºC/min to form the gel. High temperature of 95 ºC was maintained for 2.5 min. Then, a temperature sweep was made from 95 to 37 ºC at 5 ºC/min to achieve the required temperature to make the enzymatic hydrolysis. A rest time of 36 s was needed to introduce the α-amylase (as described in RVA section). Finally, apparent viscosity, μ, at 37 ºC for 10 min was monitored to assess the evolution during starch hydrolysis.

4.2.4 Starch gels digestion by *in vitro* enzymatic method

Gels from different starches were prepared in the RVA using Standard 1 method provided by supplier. Starch gels were subjected to hydrolysis digestion following the method reported (Santamaria *et al*., 2021). Experimental hydrolysis data were used to calculate rapidly digestible starch (RDS) or fraction hydrolyzed during the first 20 min, and the slowly digestible starch (SDS) hydrolyzed within 20 and 120 min (Englyst & Hudson, 1996). Data were also fitted to a first-order equation (1) to obtain the kinetic parameters of gels hydrolysis (Goñi *et al*., 1997):

$$C = C_{\infty} (1\text{-}e^{-kt}) \quad (1)$$

where was the concentration (g/100 g gel) of starch hydrolyzed at time (min), (g/ 100 g gel) was the maximum hydrolysis of starch gels, k (min^{-1}) was the kinetic constant and t was the selected time.

4.2.5 Statistical data analysis

The Microsoft Excel Solver® was used to model first-order kinetic equations. The digestion results obtained by different methodologies were correlated using Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA) by means of analysis of variance (ANOVA) with Fisher's least significant differences test (LSD). Experimental data were expressed as mean ± standard deviation and $p < 0.05$ were considered significant.

4.3 Results and discussion

4.3.1 Viscosity hydrolysis

Corn, wheat, and rice starches were selected to set up a rapid method for assessing pasting performance followed by enzymatic hydrolysis in a single assay, which were referred as digestograms. Plots of the apparent viscosities along pasting and enzymatic hydrolysis are shown in **Figure 4.1**. Parameters recorded from the apparent viscosity plots are indicated in **Table S 4.1**. Knowing the importance of temperature on the enzymatic kinetics, thermocouples were immersed in the slurries to monitor it, and values completely overlapped those recorded by the equipment. As expected, the apparent viscosity plots for corn, wheat, and rice indicate differences in their pasting performance, with corn showing an earlier swelling and major maximum apparent viscosity (2866 ± 15 mPa·s) than observed in the other starches, which agree with previously reported results (Santamaria *et al*., 2022). Moreover, Wickramasinghe *et al*., (2005) observed different viscosity peaks and swelling power among several varieties of hard or soft wheat starches. Rice showed lower apparent peak viscosity (2263 ± 93 mPa·s), with similar value to the one reported by Gelencsér *et al*., (2008). Starch granules differ in mor-

phological, and starch structure depending on botanical origin, which affect their pasting performance (Balet *et al*., 2019).

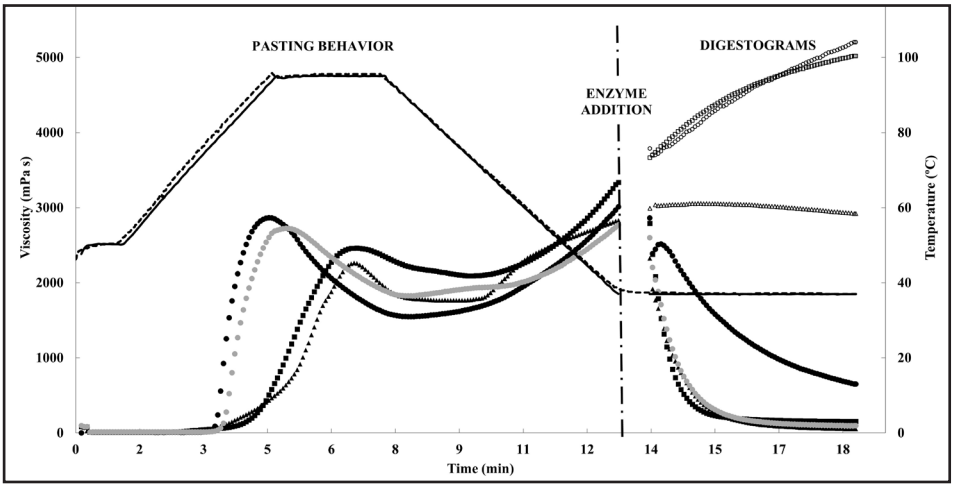

Figure 4.1: RVA method for recording the starch gelatinization and further enzymatic hydrolysis. First part records the pasting behavior of the gels, then the addition of alpha-amylase and finally the digestograms in the presence of amylase (filled symbols) and their counterparts in the absence of enzyme (empty symbols). Corn (●), corn pH 5.8 (●), wheat (■) and rice (▲) starches. Theorical (▬) and experimental (--) temperatures (ºC).

Focusing on the hydrolysis or digestogram stage, apparent viscosities of the gels in the presence and the absence of α-amylase were recorded. In the absence of α-amylase (empty symbols) a progressive increase in the apparent viscosity was observed in corn and wheat gels. Presumably, that increase in the apparent viscosity was related to their slower cooling due to their higher viscosity, which reduced the cooling rate within the gel structure. In fact, in the case of rice gel, a steady apparent viscosity was observed because its lower viscosity allowed faster heat transference within gel structure. The addition of α-amylase produced a rapid decline in the apparent viscosity, similar to that observed Gee and Johnson (1985) using a rotary viscometer. Enzymatic hydrolysis by α-amylase induces the breakdown of starch chains to the release of small fragments (dextrins) changing the starch gel behavior, from a solid gel to a weakly structured fluid gel (Sorba & Sopade, 2013). Nonetheless, comparing the digestograms of the different starches, corn gel showed lower viscosity decrease (2864 to 651 mPa·s) (**Table 4.1**). Considering the impact of pH on the enzymatic activity, first hypotheses was related to possible pH difference (Aleixandre & Rosell, 2022). In fact, corn starch slurry had pH 7.25, whereas slurries of wheat and rice starches showed pH 5.85. To confirm the impact of gel pH on α-amylase activity, corn starch gel was prepared in sodium phosphate buffer 0.01M at pH 5.8 instead of water. The digestogram obtained for corn gel with adjusted pH displayed faster hydrolysis, like the one obtained with wheat and rice gels.

Table 4.1: Gel starch viscosities (μ) obtained with RVA or rheometer before and after adding amylase, and the parameters that defined the hydrolysis kinetic (the kinetic constant and the maximum hydrolysis of starch gels).

Method	**Parameters**	Corn	Corn pH 5.8	Wheat	Rice
RVA	μ initial digestion (mPa·s)	2864 ± 90^{a}	2599 ± 146^{ab}	2793 ± 183^{a}	2324 ± 106^{b}
	μ final digestion (mPa·s)	651 ± 4^{a}	96 ± 10^{c}	154 ± 8^{b}	54 ± 3^{d}
	k_{RVA} (min^{-1})	0.40 ± 0.06^{c}	1.33 ± 0.12^{b}	1.80 ± 0.02^{a}	1.17 ± 0.11^{b}
	μ_{∞} (mPa·s)	329 ± 41^{a}	75 ± 4^{c}	137 ± 8^{b}	34 ± 6^{c}
Rheometer	μ initial digestion (mPa·s)	4975 ± 78^{a}	4670 ± 269^{ab}	4520 ± 14^{b}	2445 ± 134^{c}
	μ final digestion (mPa·s)	1810 ± 42^{a}	686 ± 15^{b}	323 ± 26^{b}	94 ± 17^{c}
	k_{Rheo} (min^{-1})	0.46 ± 0.01^{d}	0.74 ± 0.08^{c}	2.38 ± 0.07^{a}	1.04 ± 0.02^{b}
	μ_{∞} (mPa·s)	1549 ± 68^{a}	677 ± 114^{b}	336 ± 50^{c}	83 ± 16^{d}
Biochemical	k (min^{-1})	0.0334 ± 0.0009	-	0.0399 ± 0.0049	0.0335 ± 0.0012
	C_{∞} (g/100 g gel)	6.38 ± 0.35^{ab}	-	5.51 ± 0.24^{b}	6.97 ± 0.55^{a}

Means within a row followed with different letters indicate significantly different ($p < 0.05$).

Gels formation and their further hydrolysis were also carried out in the rheometer. In **Figure 4.2** it can be observed the formation of the gels and then, its maturation (empty symbols) and digestion (filled symbols). In general, same behavior than in RVA assays was observed. At the end of the gelatinization stage, it was observed that wheat starch had the highest viscosity (4520 ± 14 mPa·s), while rice starch presented the lowest viscosity (2445 ± 134 mPa·s) (**Table 4.1**). At digestion stage, a significant decrease in viscosity was seen in all samples, which agrees with results obtained with the RVA. Similar behavior was previously reported by Kim *et al*. (2015) when simulated the oro-gastrointestinal digestion of white and brown rice flours in the rheometer, and An *et al*. (2016) also reported a decrease of viscosity when wheat gels blended with increasing amounts of black rice flour were digested with pancreatin and amyloglucosidase.

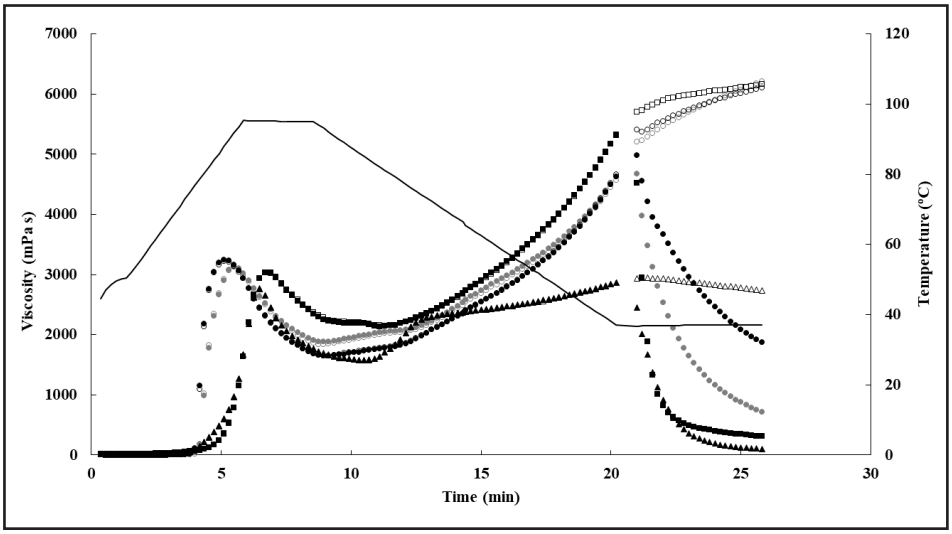

Figure 4.2: Apparent viscosity vs time recorded in a rheometer following the protocol previously described for corn (●), corn pH 5.8 (●), wheat (■) and rice (▲) starches.

4.3.2 Enzymatic hydrolysis of different starches recorded by biochemical methods

Starch gels obtained from RVA were subjected to *in vitro* digestibility to evaluate the hydrolysis kinetics of starches from different cereals, and to compare those with the results obtained in the rapid methods previously presented. In **Figure 4.3** hydrolysis plots of gels are displayed. The graphs were expressed as grams of hydrolyzed starch per 100 grams of gel. Hydrolysis pattern was different among the starches from different botanical origin. Rice gel presented higher hydrolysis, which could be related to its lower initial viscosity (2263 mPa s) that facilitates enzyme diffusion (**Table S 4.1**) (Santamaria *et al*., 2021). Consequently, rice gel reached the superior maximum hydrolysis (C_∞) (**Table 4.1**). Kinetics

parameters were satisfactorily fitted ($R^2 > 0.96$) with a fist-order kinetics-based model Eq. (1). Gels presented similar hydrolysis rate (k) and differed in the extent of the hydrolysis (C_∞), with rice gel having the highest maximum hydrolysis (**Table 4.1**). Hódsági *et al*. (2012) reported similar rate constants for gelatinized wheat and corn starches. Furthermore, although there were not significant differences, gels with lower k had higher slowly digestible starch (SDS) content. This fraction of starch is associated with satiety, less glycemic index, and prebiotic effect (Bello-Perez *et al*., 2020).

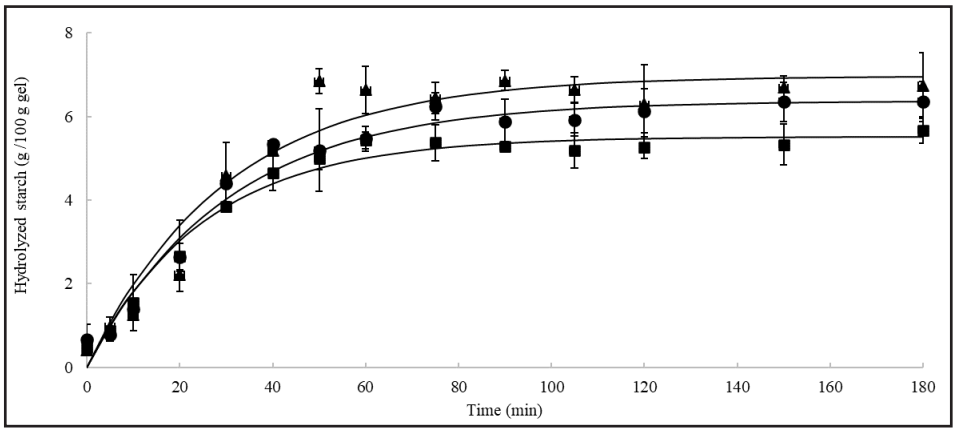

Figure 4.3*:* Enzymatic hydrolysis of different starch gels corn (●), wheat (■) and rice (▲) starches and solid lines correspond to first-order model Eq. (1) (—).

4.3.3 Modeling of digestograms

To stablish the correlation between enzymatic hydrolysis of starches by assessing glucose release and the viscosity decay measured either with RVA or rheometer, experimental data of the digestograms were mathematically fitted. **Figure 4.4** shows the starch hydrolysis by viscosity decay of gels of corn, wheat, and rice starches. The shapes of the kinetics curves were similar, but the initial (related to initial gel firmness) and final viscosities were specific for each starch. In fact, experimental apparent viscosity (mPa·s) at the beginning and end of the digestograms obtained in the RVA differed from 2864 to 651 for corn without pH adjustment, 2599 to 96 for corn at pH 5.8, 2793 to 154 for wheat and 2324 to 54 for rice (**Figure 4.4A**). Likewise, digestograms in the rheometer show that apparent viscosity (mPa·s) varied from 4975 to 1810 for corn, 4670 to 686 for corn pH 5.8, 4520 to 323 for wheat, and 2445 to 94 for rice starch gels (**Figure 4.4 B**).

A first-order kinetic model was applied to model the digestograms, Eq. (2):

$$\mu = \mu_\infty + (\mu_0 - \mu_\infty)e^{-kt} \quad (2)$$

where μ is the apparent viscosity (mPa s), μ_0 is the initial viscosity, μ_∞ is the final viscosity, k (min^{-1}) is the kinetic constant and t (min) is hydrolysis time.

The RVA experimental data presented satisfactorily fitting ($R^2 > 0.99$) to first-order kinetic model. Kinetic constant (k_{RVA}) obtained in the digestograms presented statistically differences ($p < 0.05$) depending on the starch source, as well as pH, in the case of corn starch (**Table 4.1**). The highest hydrolysis rate (k_{RVA}) was presented by wheat gel (1.80 min^{-1}), followed by corn gel after adjusting pH (1.33 min^{-1}), and rice (1.17 min^{-1}). Corn gel prepared without adjusting the pH showed the lowest k_{RVA}. Regarding $\mu_{\infty,}$ the lowest value was determined for rice starch (34 mPa·s) and the highest with corn (329 mPa·s). Higher peak viscosity has been correlated negatively with hydrolysis rate of native starches, but no correlations were observed with the enzymatic hydrolysis of the gels (Noda *et al*., 2008). Factors like source starch, enzyme type, concentration of enzyme and starch solids content affect the starch digestion rate (Sorba & Sopade, 2013).

Similar fitting was carried out with the experimental data obtained with the rheometer (**Table 4.1**) obtaining significant differences ($p > 0.95$) between k_{Rheo} and μ_∞ values for each gel were found. In **Figure 4.4B**, it can be observed the acceptable fitting quality ($R^2 > 0.99$) of the model in comparison to experimental data. Again, corn gel without adjusting the pH showed the lowest k_{Rheo} value (0.46 min^{-1}) and wheat the highest (2.38 min^{-1}). Considering the kinetics rate obtained in the RVA, the k_{Rheo} for corn gel at pH 5.8 was lower than expected, even lower than that obtained for rice. Likely differences between rotational speed of rheometer and shearing of RVA, might explain that trend. Presumably, pH equilibration of gel slurry and the enzymatic solution by the employed impellers occurred at different speed in both equipments. The slower homogenization in the rheometer would explain the lower kinetic constants obtained for corn at pH 5.8 versus rice value, in comparison with their respective RVA results. Nevertheless, independently of the specific data, the trends of the digestion kinetic constants obtained with tested starches by means of both methods (RVA and rheology) were satisfactorily in agreement. Regarding $\mu_{\infty,}$ the lowest value was determined for rice starch (83 mPa·s) and the highest with corn (1549 mPa·s). Results confirmed the viability of those test to follow enzymatic hydrolysis simulating digestion, being able to discriminate among the type of starches. Conversely, the quantification of glucose release did not show significant differences in their hydrolysis rate.

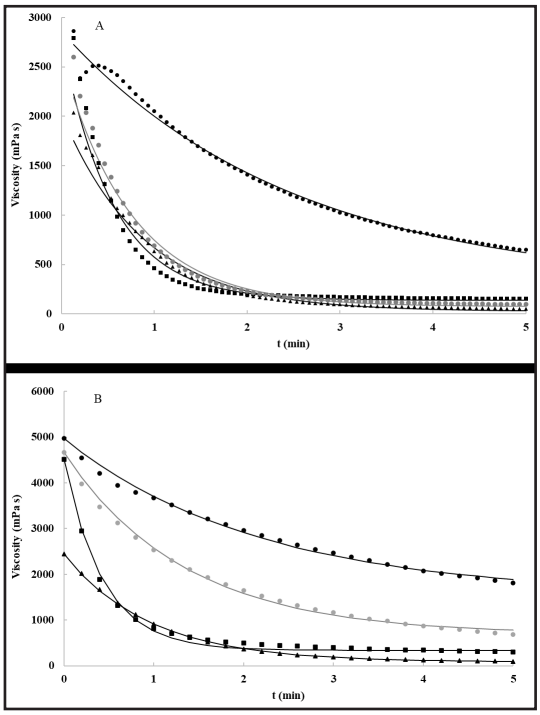

Figure 4.4: Variation of apparent viscosity during hydrolysis of corn (●), corn pH 5.8 (●), wheat (■) and rice (▲) starchy gels and their modelling by Eq. (2) (—). A: RVA digestograms and B: Rheometer digestograms.

4.3.4 Normalized digestograms

Digestograms were the results of a decrease in viscosity due to the enzymatic hydrolysis of gelatinized starch. To visualize jointly the hydrolysis kinetics of tested starchy gels, **Figure 4.5** shows the corresponding normalized curves (μ_N vs dimensionless time, t/t_{final}) of hydrolysis kinetics. Sorba and Sopade (2013) made similar adjustment for studying retrograded gels. Normalized viscosity μ_N (-) was evaluated considering μ_0 and μ_∞ values by Eq. (3), against the results of the biochemical kinetic (C/C_0) in reference to glucose content.

$$\mu_N=(\mu_t-\mu_\infty)/(\mu_0-\mu_\infty) \quad (3)$$

Regardless of the botanical origin of the starch, it can be observed the sharp drop of μ_N for wheat, intermediate one for rice and moderate drop for corn starch gels (**Figure 4.5**). These curves showed the differences in the hydrolysis time of digestible starch in the gels. Then, all curves were asymptotic at long times (all digestible starch was already hydrolyzed). Corn starch was the exception, but it was confirmed that the pH of the sample was a factor that modifies the rheological behavior, mainly in the RVA method. This indicated that the analysis had to be carried out at an optimal pH for the enzymatic activity. In the case of biochemical hydrolysis, the pH of the corn starch gel did not vary the normalized viscosity plots, that was expected since gels pH effect is negligible when diluted into the buffer solution. The models used allowed to know the rate of starch digestion (**Table 4.1**), having very good fitting RVA ($R^2 > 0.99$), rheometer ($R^2 > 0.99$) and biochemical kinetics ($R^2 > 0.96$). Differences in the fitting might be attributed to the recording time in each methodology, RVA and rheometer quantifies the viscosity every 4 s and 12 s, respectively, whereas aliquots for the biochemical analysis were withdrawn every 5, 15 or 30 min along the enzymatic assay. Most of the starch is digested, at relative high rate, for short period of time

when following the apparent viscosity. In both methodologies, wheat gel showed higher hydrolysis rate (*k*), which indicated that the digestion was faster compared to other starches.

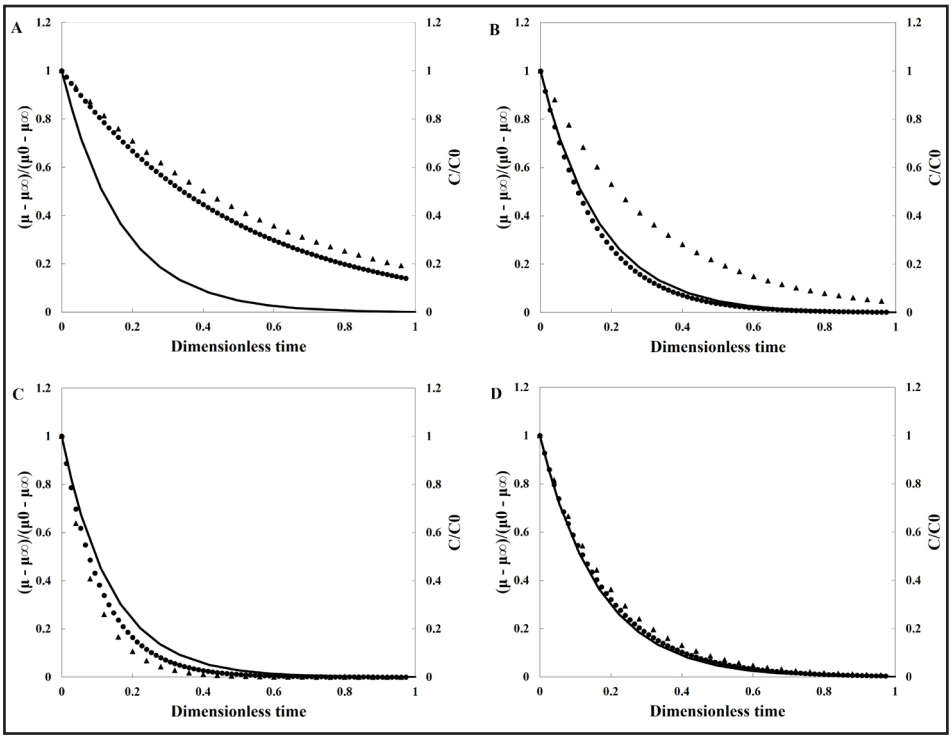

Figure 4.5: Normalized curve of apparent viscosity using Eq. (3) during different hydrolysus: biochemical (—) RVA (●) and rheometer (▲) methods. Corn (A), corn pH 5.8 (B), wheat (C) and rice (D). Biochemical hydrolysis time on the lower X-axis and digestograms time on the upper X-axis.

4.4 Conclusions

Single tests were developed to study the gelatinization performance and the digestion of different starch gels. Viscosity changes of different starches recorded with RVA or rheometer followed by amylase hydrolysis provide digestograms that were used to predict gels digestion by fitting experimental results to a first-order kinetic models. Parameters obtained from the fitting can be used for predicting starch digestion using rapid, simple and reliable methods. Those can be used to carry out preliminary studies of many samples and identify the rheological behavior with alpha-amylase addition. A preliminary discrimination for predicting starch behavior might be very useful prior to *in vitro* or *in vivo* digestions.

4.5 Supporting information

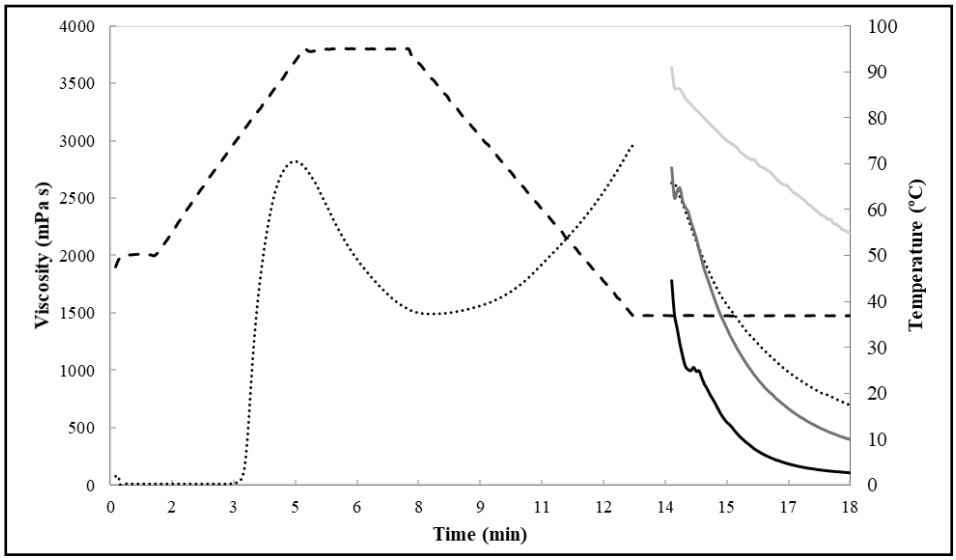

Figure S 4.1: Preliminary studies with corn starch using different amounts of α-amylase from porcine pancreas, expressed in enzyme units. Legends: 56 ▬ ,90 -- , 169 ▬ ,225 ▬, and (--) temperature (ºC).

Table S 4.1: Pasting properties of the different gels obtained with RVA parameters and hydrolysis parameters

	Corn	Corn pH 5.8	Wheat	Rice
RVA parameters				
Onset (min)	3.1 ± 0.0^{b}	3.3 ± 0.1^{a}	2.5 ± 0.0^{d}	2.7 ± 0.0^{c}
Peak viscosity (mPa·s)	2866 ± 15^{a}	2727 ± 2^{b}	2464 ± 7^{c}	2263 ± 93^{d}
Peak time (min)	4.6 ± 0.0^{c}	4.9 ± 0.1^{b}	6.5 ± 0.1^{a}	6.5 ± 0.0^{a}
Trough (mPa·s)	1549 ± 72^{c}	1825 ± 5^{b}	2091 ± 13^{a}	1763 ± 57^{b}
Breakdown (mPa·s)	1317 ± 57^{a}	902 ± 3^{b}	374 ± 21^{d}	500 ± 35^{c}
Setback (mPa·s)	1315 ± 162^{a}	775 ± 141^{b}	702 ± 197^{b}	562 ± 163^{b}
Hydrolysis parameters				
RDS (g/100 g)	3.10 ± 0.11		3.03 ± 0.38	3.40 ± 0.18
SDS (g/100 g)	3.16 ± 0.22^{ab}		2.44 ± 0.11^{b}	3.44 ± 0.34^{a}

Means within a row followed with different letters indicate significantly different ($p < 0.05$). RDS: Rapid digestible starch; SDS: Slowly digestible starch.

Author Contributions: Credit roles: MS: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft; LM: Investigation; Methodology; RG: Methodology; Supervision; Data curation; RM: Formal analysis; Writing - review & editing; Funding acquisition; CMR: Conceptualization; Funding acquisition; Investigation; Supervision; Writing - review & editing.

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Conflicts of Interest: The authors declare no conflict of interest.

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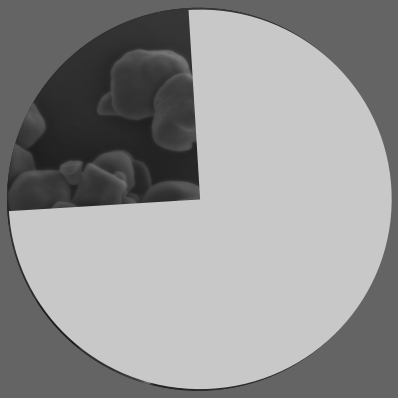
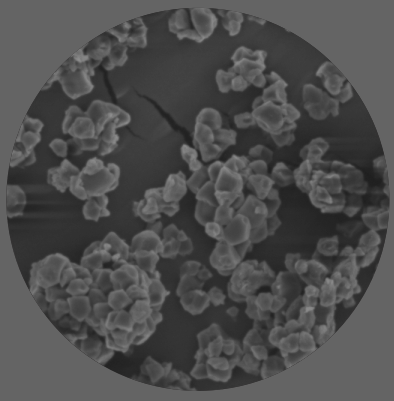
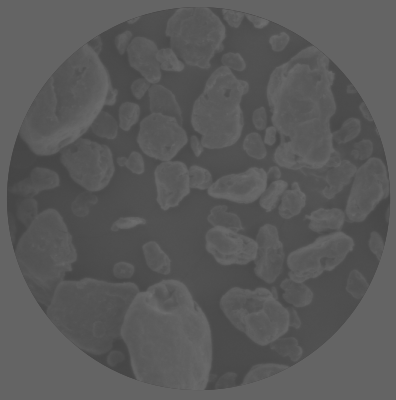




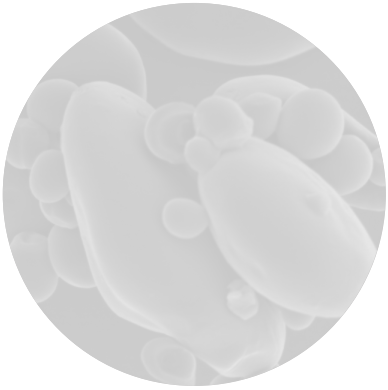


CHAPTER FIVE

Impact of starch-hydrocolloid interaction on pasting properties and enzymatic hydrolysis

Maria Santamaria[1], Raquel Garzon[1], and Cristina M. Rosell[1,2*]

Submitted



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ABSTRACT

Hydrocolloids are extensively used for food processing because their techno functional properties (emulsifier, stabilizer, and structural agent). But there is increasing interest in their role connected with nutritional improvements, particularly related to starch hydrolysis rates, which might involve the viscosity resulting from starch-hydrocolloid interaction. The objective of this research was to investigate the impact of gels viscosity on the enzymatic hydrolysis of a range of starch gels made with different starches and hydrocolloids. Heterogeneous systems (starch-hydrocolloid) were prepared with several starches (corn, wheat, rice, potato, cassava, pea) and hydrocolloids (locust bean gum, guar gum, xanthan gum, hydroxypropylmethylcellulose K4M, psyllium) at different concentrations (0% - 0.5% - 2.5%). The starch-hydrocolloid pasting behavior and their susceptibility to amylase hydrolysis was recorded with the Rapid Viscoanalyzer following a rapid method (Santamaria *et al*., 2022a). The viscosity decay due to alpha-amylase activity was modeled to obtain starch gels hydrolysis rate (*k*). A negative correlation was found among kinetic constant (*k*) and viscosity at 37 ºC (r = -0.55), setback (r = -0.50), and area under the pasting curve (r = -0.42). For instance, xanthan gum and psyllium addition showed strong negative correlation between kinetic constant and viscosity at 37 ºC (r = -0.75) and setback (r = -0.79), respectively, particularly when blended with potato starch. These correlations indicate that pasting properties of the starch-hydrocolloid systems might be predictors of the enzymatic digestion rate of the gels, allowing the design of foods with controlled postprandial glucose response.

VISCOSITY - RAPID VISCOANALYZER - ALPHA.AMYLASE - KINETIC CONSTANT - DIGESTION

5.1 Introduccion

Hydrocolloids are crucial players in food processing due to their thickening, gelling, foaming and, water-holding capacity, but also their functionality is extended to food nutrition, specifically digestion and gastrointestinal transport of nutrients (Abdel-Aal, 2009; McClements, 2021). Particularly important is the role of hydrocolloids in starch-based systems because they limit the water molecules availability and in turn the gelatinization performance of the starch. However, that effect is greatly dependent on the starch-hydrocolloid binomial (Rosell *et al*., 2011).

Regarding the role of hydrocolloids on the digestion of starch-based systems, numerous studies have been carried out. Gularte and Rosell (2011) analyzed the association between hydration and pasting properties with the *in vitro* digestibility of corn and potato starches in the presence of different hydrocolloids (high methoxylated pectin, guar gum, carboxymethylcellulose-CMC, xanthan gum, and hydroxypropylmethylcellulose -HPMC). The enzymatic hydrolysis rate was lower in guar gum - potato starch mixture, which was correlated with a viscosity increase that decrease the diffusion and activity of the amylase enzyme. Fabek *et al*. (2014) studied the impact of several hydrocolloids (guar gum, locust bean gum, fenugreek gum, flaxseed gum, xanthan gum, and soy-soluble polysaccharide) on the digestibility of waxy corn starch gels. The addition of hydrocolloids decreased glucose diffusion, and there was an inverse correlation between the digesta viscosity they induce, and the glucose amount released from starch hydrolysis. Likewise, Sasaki *et al*. (2015) analyzed the enzymatic hydrolysis of gelatinized potato starch, containing xanthan gum, guar gum, pectin, or konjac-glucomannan at different concentrations (5, 10, or 15%). Xanthan gum showed the most pronounced suppressive effect on the digestibility of gelatinized potato starch, which was attributed to xanthan gum interaction with potato amylopectin, producing a firm barrier that impedes starch hydrolysis. Conversely, the interaction hydrocolloid-amylose was mentioned by Jung *et al*. (2017), who studied high amylose rice gels made with different concentrations (0.3, 0.5, or 0.7%) of xanthan gum, Arabic gum, guar gum, or locust bean gum. Arabic and xanthan gum showed the greatest effect lowering glucose release, due to the high digesta viscosity. Nevertheless, Zhou *et al*. (2020) reported higher reduction of corn starch digestion when blending it with 2.5% guar gum, stressing the importance of the hydrocolloid concentration.

Therefore, different hypothesis have been proposed to explain the hydrocolloids effect on starch digestibility, namely hydrocolloid interaction with starch granules through amylose or amylopectin (Sasaki *et al*., 2015), the formation of

a hydrated layer encapsulating starch granules, or the viscosity increase of the digesta (Wee & Henry, 2020). It has been described that digesta viscosity has a significant impact on food digestion, since it reduces gastric emptying, decreases mass transfer and may slow down enzymatic action (Manzoor *et al*., 2020; Santamaria *et al*., 2021), but there is scarce information about potential relationship between those systems viscosity and the digestion rate.

Considering that starch-hydrocolloid interaction is dependent on both, the starch source, and the type of hydrocolloid, as well as the concentrations used, the aim of this study was to analyze the impact of gels viscosity on the enzymatic hydrolysis, of a range of starch gels made with different starches and hydrocolloids. To allow a large screening using different conditions, a simple, rapid, and reliable method reported by Santamaria *et al*. (2022a) was applied, recording the rheological behavior of starches and their performance during α-amylase hydrolysis. Several starches (corn, wheat, rice, potato, pea, and cassava) and hydrocolloids (locust bean gum, xanthan gum, guar gum, hydroxypropylmethylcellulose K4M and psyllium) at different concentrations (0% - 0.5% - 2.5%) were used to investigate their impact in the rate of starch hydrolysis.

5.2 Materials and methods

5.2.1 Materials

The starches from corn (CO), wheat (W), potato (PO) (EPSA, Valencia, Spain), green pea (PE) (Esteve Santiago, Valladolid, Spain), rice (R) and cassava (CA) (local market) were employed. Their moisture contents were 13.74%, 12.12%, 17.86%, 10.96%, 12.85% and 10.52% respectively. Regarding the hydrocolloids, locus bean gum (LBG) was generously provided by G.A Torres (Valencia, Spain), xanthan gum (XG) and guar gum (GG) were from Grupo Desarrollo (Valencia, Spain), psyllium (Isabgol, sterilized psyllium husk powder) (P) was provided by Sarda Biopolymers (Mumbai, India) and hydroxypropylmethylcellulose K4M (HPMC) was obtained from Sigma Aldrich (St Louis, Misuri, USA).

The enzyme used was VI-B α-amylase from porcine pancreas (EC 3.2.1.1) from Sigma Aldrich (Sigma Chemical, St. Louis, USA) dissolved into 0.3 M sodium maleate buffer (pH 6.9). Solutions were made using deionized water. Reagents were of analytical grade.

5.2.2 Physicochemical composition of starches and hydrocolloids

The protein content was determined according to ISO 16634-2:2016. The amylose content of starches was quantified using a commercial amylose/amylo-

pectin assay kit (K-AMYL 06/18, Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland) based on amylopectin complexes with the lectin concanavalin A. Molecular weight of starches were determined by Size-Exclusion Chromatographer (SECurity 1260, Polymer Standard Service, Mainz, Germany) coupled with Multi Angle Light Scattering (MALS). The mobile phase was DMSO with 0.1 M LiCl. Samples were dissolved at 80 ºC, then centrifugated for 10 minutes at 5000 rpm and filtered through a 0.2 µm filter before being injected into the Gel Permeation Chromatography (GPC). An analytical column (PSS-Suprema, 10 µm, 10,000 Å, ID 8.0 mm x 300 mm) was used at 70 ºC with 0.5 mL/min of flow rate.

The particle size of starches and hydrocolloids was determined by Mastersizer equipment (Scirocco 2000; Malvern Instruments Ltd., Worcestershire, UK), by laser diffraction technique and the results obtained were estimated based on volume. The volume-weighted mean diameter D (4,3) was calculated by Eq. (1). The measurement was carried out in three replicates.

$$D(4,3)=(\Sigma\, di\cdot Vi\,)/(\Sigma\, Vi) \quad (1)$$

Hydrocolloid viscosity was measured in 2% suspensions of hydrocolloid: water. The mixtures were shaken in Vibromatic (J.P Selecta S.A, Abreda, Barcelona, Spain) for 20 minutes. Then, samples were stored in a shaker incubator SKI 4 (ARGO Lab, Carpi, Italy) at 25 ºC under constant stirring at 200 rpm for 24 h. Viscosity suspensions were measured with a HAAKE viscotester 3 (Thermo Scientific, Massachusetts, US) using rotor no.1 with the measuring range (300 mPa s to 15000 mPa s).

5.2.3 Pasting behavior and digestograms of gels

Starch-hydrocolloid slurries were analyzed in the Rapid Viscoanalyzer (RVA 4500; Perten Instruments, Hägersten, Sweden). Pasting performance and digestograms were examined following the method described by Santamaria *et al*. (2022a) with minor modifications. Three grams (14% mb) of starch plus hydrocolloid at different concentrations (0% - 0.5% - 2.5%) were dissolved in 25 mL of distilled water. Slurries were exposed to heating and cooling cycles including 50 ºC for 1 min, heating from 50 to 95 ºC in 3 min 42 s, holding at 95 ºC for 2 min 30 s, then cooling down to 37 ºC in 4 min 90 s, stopping at 37 ºC for 36 s to add the α-amylase solution (900 U/mL in 0.3 M sodium maleate buffer pH 6.9), and holding at 37 ºC for 5 min. In the first stage during pasting performance, the parameters obtained were: onset (the time when viscosity started to increase), peak viscosity (highest viscosity during heating), trough (lowest viscosity when holding at 95 ºC), breakdown (difference between the maximum and minimum

viscosity), final viscosity at 37 ºC, setback (difference among final viscosity and trough), and to obtain a representative parameter of the complete pasting performance, the area under the curve (AUC) was calculated.

In the second stage during de digestograms, kinetic constant (k) was calculated using a first-order equation (Eq. 2), where μ was the apparent viscosity (mPa s), μ_0 was the initial viscosity, μ_∞ was the final viscosity, k (min^{-1}) was the kinetic constant and t (min) was hydrolysis time. The Microsoft Excel Solver® was utilized to model first-order kinetic equations.

$$\mu = \mu_\infty + (\mu_0 - \mu_\infty)e^{-kt} \quad (2)$$

5.2.4 Statistical analyses

Three replicates were made for each sample. Experimental data were statistically analyzed by Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA). Raw materials properties were examined using an analysis of variation (ANOVA). Multivariate analysis of variance (MANOVA) was used to evaluate pasting and hydrolysis parameters. The results were presented as mean ± standard deviation using Fisher's least significant differences test (LSD). Differences of $p < 0.05$ were considered significant. Furthermore, a principal component analysis (PCA) was made to explain the variability of the parameters. Pearson correlation and lineal regression were applied to identify possible correlations between pasting parameters (viscosity at 37 ºC, setback and AUC) with kinetic constant (k).

5.3 Results and discussion

5.3.1 Raw materials characterization

Starches showed significant differences ($p < 0.05$) in their physicochemical properties (**Table 5.1**). Tuber starches (potato and cassava) had the lowest protein content, followed by cereals starches. Conversely, pea starch had the highest protein content (14.63 ± 0.08%). Similar protein content in starches has been reported by Aleixandre *et al*. (2021). Amylose fraction was quantified, because it has been reported that it can interact with hydrocolloids, hindering the alpha-amylase accessibility. Amylose content ranged from 38.49% in the case of pea starch to 12.58% in rice starch. Cereals starches had lower amylose content than pulse starches (Bajaj *et al*., 2018). Corn starch, besides rice starch, showed the lowest average particle diameter of volume D (4,3), similarly to the value reported by Zhou *et al*. (2020). In opposition, cassava starch showed high average particle size value, which could be related to a less uniform milling.

These results are in accordance with a previous study (Li *et al*., 2020). Regarding, the molecular weight (Mw) of the starches, the order was potato ($18.200 \cdot 10^{-6}$ g/mol) > rice ($5.212 \cdot 10^{-6}$ g/mol) > cassava ($3.841 \cdot 10^{-6}$ g/mol) > wheat ($3.416 \cdot 10^{-6}$ g/mol) > corn ($2.769 \cdot 10^{-6}$ g/mol) > pea ($2.318 \cdot 10^{-6}$ g/mol). Similar results were found by Ong *et al*., and Harding (1994).

For the hydrocolloids' characterization, volume diameter D (4,3) and viscosity were considered (**Table 5.1**). Hydrocolloids presented higher volume diameter D (4,3). Guar gum and psyllium displayed the superior volume, 148.20 and 137.60 µm, respectively; they were also the hydrocolloids that resulted in more viscous suspensions.

Table 5.1: Physicochemical composition of starches and hydrocolloids.

Starch	Proteins [%]	Amylose [%]	D (4,3) [µm]
CO	0.79 ± 0.01^{c}	20.15 ± 0.13^{c}	18.34 ± 0.95^{e}
W	0.69 ± 0.01^{c}	23.98 ± 1.15^{b}	30.12 ± 1.05^{c}
R	1.44 ± 0.01^{b}	12.58 ± 0.29^{e}	19.06 ± 0.15^{e}
PO	0.54 ± 0.05^{d}	16.55 ± 0.18^{d}	41.82 ± 0.36^{b}
PE	14.63 ± 0.08^{a}	38.49 ± 0.80^{a}	25.37 ± 0.03^{d}
CA	0.58 ± 0.02^{d}	21.22 ± 1.48^{c}	310.53 ± 3.64^{a}
p-value	0.0000	0.0000	0.0000
Hydrocolloid	D (4,3) [µm]	Viscosity [mPa s]	
LBG	119.38 ± 4.82^{c}	2495 ± 31^{c}	
XG	54.31 ± 5.93^{e}	2521 ± 13^{c}	
GG	148.20 ± 5.44^{a}	10956 ± 435^{b}	
HPMC	108.39 ± 1.29^{d}	2166 ± 77^{c}	
P	137.60 ± 0.36^{b}	12017 ± 763^{a}	
p-value	0.0000	0.0000	

Means within the same column followed by different letters indicate significant differences $p < 0.05$. Starches: corn (CO), wheat (W), rice (R), potato (PO), pea (PE) and cassava (CA); Hydrocolloids: locust bean gum (LBG), xanthan gum (XG), guar gum (GG), hydroxypropylmethylcellulose (HPMC) and psyllium (P).

5.3.2 Starch-hydrocolloid gels and digestograms analysis

To picture the performance of the binary combinations starches-hydrocolloids during pasting and also to predict their hydrolysis susceptibility to alpha-amylase, the method reported by Santamaria *et al*. (2022a) was followed. All the experimental parameters were statistically analyzed, and a principal component analysis (PCA) used to get the full picture of those combinations (**Figure 5.1**). Two main components explained about 83.29% of the variation observed among results. Component 1 (PC1) explained 56.85% of the variation, being mainly defined by pasting parameters (peak, setback, and breakdown) and on the negative axis by the hydrolysis rate (*k*). Component 2 (PC2) explained 26.44% of the variation, being identified on the positive axis by the trough, viscosity at 37 ºC and AUC. The analysis allowed discriminating starch source impact. Potato starch (-) had greater impact on pasting properties. Gularte and Rosell (2011) found higher viscosities during heating and cooling stages in potato starch gels than in corn-based ones. Besides, potato starch (-) was in the opposite corner from the kinetic

constant (*k*), except when it was combined with xanthan gum (0.5 and 2.5%) or psyllium at 2.5%, those binary blends had lower impact than the other hydrocolloids, being characterized by trough or final viscosity at 37 ºC. However, pea (+) and cassava (●) starches were closely related to the hydrolysis rate. Cereals starches did not show a clear tendency, corn (■) and rice (▲) starches were distributed along the two axes, and wheat starch (♦) was located at the negative abscissa axis at the opposite side of the pasting properties, but closer to the kinetic constant. This PCA shows that starch source dominated the clusters aggregation and not the different hydrocolloids or their concentrations. For deeper study of the *in vitro* hydrolysis of the gels, parameters representing each of the quartiles were selected, namely the setback, the AUC, the final viscosity at 37 ºC and the kinetic constant (*k*).

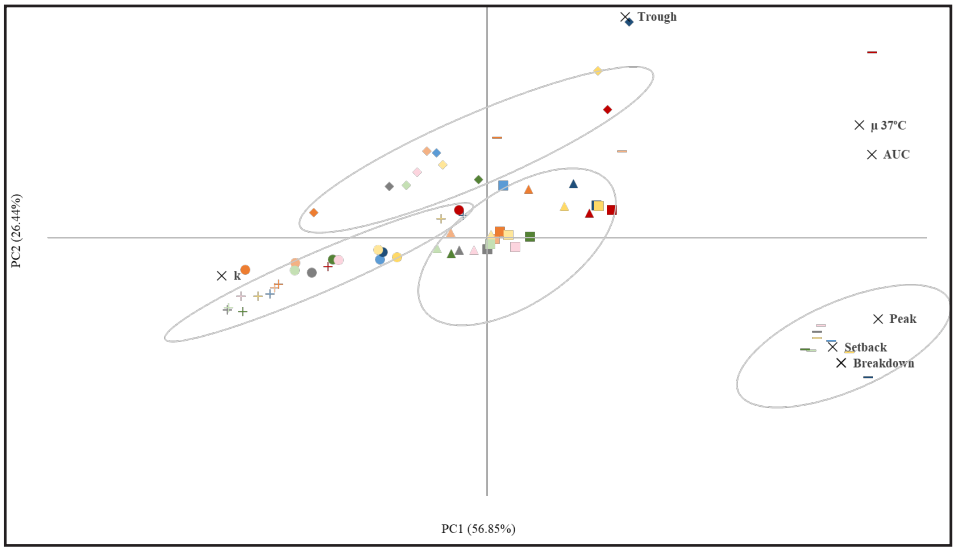

Figure 5.1: Principal component analysis of the pasting properties and in vitro hydrolysis showed by individual starches and their binary blends with hydrocolloids at different concentrations. Starches: corn (■), wheat (♦), rice (▲), potato (-), pea (+) and cassava (●). Control (grey), locust bean gum (blue), xanthan gum (orange), guar gum (yellow), Hydroxypropylmethylcellulose (green) and psyllium (red). Hydrocolloid concentration was indicated by the symbol color intensity (0.5% lighter and 2.5% darker). Parameters: peak viscosity, trough, breakdown, final viscosity (μ 37 ºC), setback, area under pasting curve (AUC) and hydrolysis rate (*k*).

Previous studies have reported the starch performance during pasting (Balet *et al*., 2019), and in some occasions how hydrocolloids affected that according to the type or level of hydrocolloid added (Gularte & Rosell, 2011). However, the rapid method applied in the present study allowed studying the influence of hydrocolloids and their concentration, on the hydrolysis of the different starches by α-amylase (**Figure 5.2**). Once the α-amylase was added to the starch gels, viscosity was rapidly decreasing due to the enzymatic action (Gasparre *et al*.,

2022). Control starch gels behave differently during the hydrolysis stage (**Figure 5.2 A**). The RVA and digestograms parameters analyzed revealed statistically significant differences ($p < 0.05$) based on the factors (starch/hydrocolloid type) and cofactor (concentration), except the constant kinetic (k) that was not influenced by the hydrocolloid level added (**Table 5.2**). The RVA parameters obtained for potato starch were higher (**Table 5.2**), which agree with previous findings (Gularte & Rosell, 2011; Liu *et al*., 2019). Sorba and Sopade (2013) explained that behavior based on the covalent binding induced by the presence of phosphorus. Cereals starches showed lower viscosities than potato starch, with corn having even lower kinetic constants than potato starch. Conversely, pea and cassava starches showed lower viscosity at 37 ºC and faster hydrolysis (**Figure 5.2 A; Table 5.2**). Santamaria *et al*. (2022a) found a negative correlation between peak viscosity and the hydrolysis rate of gelatinized starches. This inverse relationship was also observed by Fabek *et al*. (2014) in waxy corn starch matrices blended with several hydrocolloids.

The analysis of pasting parameters and gel hydrolysis of the different starches confirmed that they are dependent on the hydrocolloid type (**Table 5.2**). In general, the hydrocolloids concentration significantly affected the pasting behavior of starches but no the rate for their enzymatic hydrolysis (**Table 5.2**). Locust bean gum (LBG) at the different levels tested, increased viscosity at 37 ºC, setback and AUC of the different starches, with the exception of the setback for wheat containing 2.5% LBG, and the AUC of the potato starch (**Figure 5.2 B and Table 5.2**). Nevertheless, LBG only slowed down the hydrolysis rate of pea starch, which showed the lowest AUC, regardless the LBG concentration. The increase in the digesta viscosity induced by LBG has been used to explain the restricted accessibility of digestive enzymes and in consequence the slower digestion observed with high-amylose rice flour containing 0.5% LBG (w/w, DWB) (Jung *et al*., 2017). A low level of XG (0.5%) was enough to increase the viscosity of the starch gels after cooling (37 ºC), except of potato, but higher XG level (2.5%) only increase that viscosity in corn, rice and pea starches (**Figure 5.2 C**), which were the starches with the smaller granule size (**Table 5.1**). It is known the competency of starch granules and hydrocolloids for the water molecules (Rosell *et al*., 2011) likely, results could be related to the surface area of the starch granules. In general, XG increased the hydrolysis kinetic constant of the starch gels, except for wheat, pea and cassava, but the effect was dependent on the hydrocolloid concentration (**Table 5.2**). XG at 0.5% reduced the mean value of the hydrolysis constant of wheat, pea and cassava, meaning slower digestion respect to control samples, which might be related to its higher amylose content. Oh, Bae, and Lee (2018) observed a delay in the digestion of high amylose rice starch in the presence of 1% of XG. However, at the highest XG concentration tested (2.5%)

only cassava and pea showed slower digestion (average value of *k*); undermining the hypothesis that only amylose content could explain the starches comportment during hydrolysis. Jung *et al*. (2017) also observed that lower XG concentration (0.5%) had more impact on reducing glucose release, but at concentrations >0.7% a reverse action was observed regarding the hydrolysis rate associated to the swelling enhancement of starch granules. Therefore, the amount of XG added could play a significant role.

Overall, the addition of guar gum (GG) increased pasting behavior and in consequence, all pasting parameters for each starch (**Figure 5.2 D**). Gasparre *et al*. (2022) observed more viscous gels with the binary association of guar gum - wheat starch, associating that with an increase in the swelling capacity of starch granules, hindering the amylose leaching out. This was related to the decrease in the hydrolysis constant rate (*k*), especially in rice (0.5%), wheat (2.5%), pea, and cassava starches (**Table 5.2**). It should be highlighted that wheat starch with 2.5% GG showed higher viscosity at 37 ºC; and the increase in the GG concentration added to potato starch (from 0.5% to 2.5%) did decrease *k*, thus it could be related to the viscosity increase. In fact, gelatinized potato starch containing 5% of GG decreased blood glucose levels (Sasaki *et al*., 2015). Similarly, lower digestibility has been observed with the blends corn starch-guar, which have been related to the presence of a uniform layer covering the starch granules that could block the enzyme activity (Zhou *et al*., 2020). The addition of HPMC did barely modify the pasting behavior of starches (**Figure 5.2 E**), as observed in previous studies (Gasparre *et al*., 2022), but, the kinetic constant increased in all starches, except in pea and cassava starches that was unchanged (**Table 5.2**). Similar results have been reported with cellulose derivative (carboxymethyl cellulose), that did not impact starch hydrolysis (Oh *et al*., 2018). In the case of psyllium (P) (**Figure 5.2 F**), the addition of 2.5% increased starches viscosities. Furthermore, it was the only hydrocolloid that augmented the viscosity profile in potato and pea gels. Regarding hydrolysis, kinetic constants (*k*) of the starches were kept (corn and potato), decreased (cassava) or increased (wheat and rice) at 0.5%. It must be stressed the low *k* value obtained in potato starch that could be related to a higher viscosity at 37 ºC (**Table 5.2**). Sevilmis and Sensoy (2022) observed a decrease in slowly digestible starch when psyllium fiber was incorporated into starches (wheat, potato and cassava), impeding the accessibility and interaction of digestive enzymes with starches during digestion.

To understand possible role of the viscosity on the starch hydrolysis a correlation matrix was built to identify any significant relationship between gels parameters (viscosity 37 ºC, setback and AUC) and the hydrolysis behavior, taking the kinetic constant (*k*), of the binary starch-hydrocolloid mixtures (**Table 5.3**). The kinetic constant displayed a moderate negative correlation with viscosity

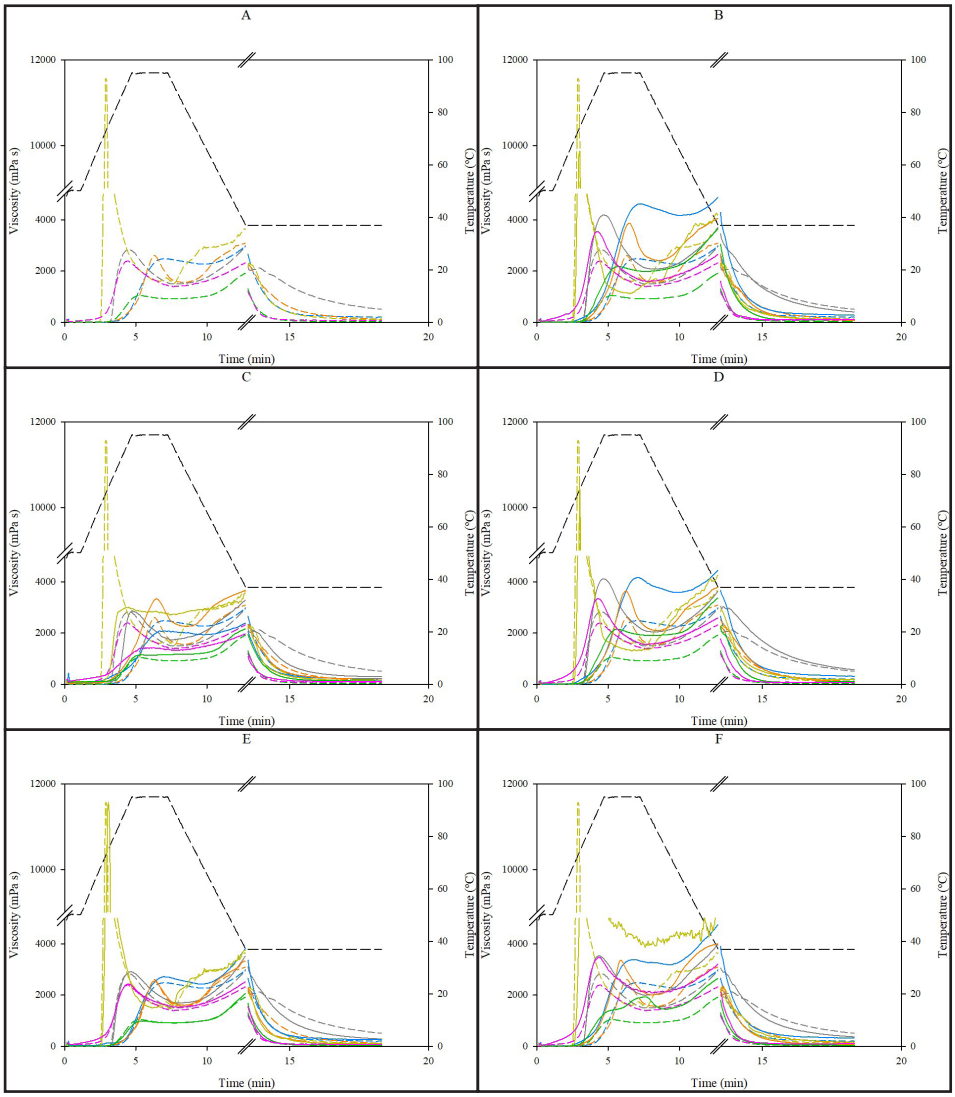

Figure 5.2: Pasting and digestograms plots for each hydrocolloid with 0% (--) and 2.5% (▬). (A) controls, (B) locust bean gum, (C) xanthan gum, (D) guar gum, (E) hydroxypropylmethylcellulose, and (F) psyllium. Starches: corn ▬ wheat ▬ rice ▬ potato ▬ pea ▬ cassava ▬.

37 ºC (r = -0.5491), setback (r = -0.5036) and AUC (r = -0.4247), considering all samples. Those correlations confirmed that the gels viscosities after cooling up to 37 ºC and their whole viscosity performance during heating and cooling, indicated by AUC, did impact on the digestion rate. The pasting parameter with the highest negative correlation with the hydrolysis kinetic was the gel viscosity at 37 ºC. This result also validates the use of the rapid test defined by Santamaria *et al*. (2022a) to predict the performance of starches on further enzymatic digestion. Furthermore, linear regressions were drawn with all the experimental results (**Figure 5.3**).

Starch	Hydrocolloid	%	Viscosity 37 ºC (mPa s)			Setback (mPa s)			AUC			k (min^{-1})		
CO		0	2963	±	59	1471	±	49	18976	±	19	0.311	±	0.020
W		0	3005	±	13	739	±	24	18464	±	99	1.363	±	0.172
R		0	3088	±	90	1528	±	92	16885	±	262	0.764	±	0.116
PO		0	3656	±	30	2156	±	204	30027	±	555	0.799	±	0.009
PE		0	1918	±	1	996	±	6	9529	±	43	1.914	±	0.055
CA		0	2316	±	80	925	±	30	17055	±	885	2.127	±	0.248
CO	LBG	0.5	3167	±	11	1518	±	5	20118	±	50	0.493	±	0.047
W	LBG	0.5	3450	±	21	767	±	97	21461	±	288	1.543	±	0.100
R	LBG	0.5	3394	±	68	1671	±	112	17719	±	490	0.815	±	0.050
PO	LBG	0.5	3869	±	95	2385	±	25	28935	±	221	0.975	±	0.519
PE	LBG	0.5	2217	±	32	1124	±	24	11121	±	45	1.795	±	0.103
CA	LBG	0.5	2656	±	62	1062	±	57	20680	±	802	2.168	±	0.043
CO	LBG	2.5	3724	±	37	1642	±	25	25736	±	295	0.655	±	0.026
W	LBG	2.5	4887	±	110	721	±	158	33050	±	538	1.457	±	0.079
R	LBG	2.5	4067	±	6	1652	±	4	24124	±	99	1.215	±	0.019
PO	LBG	2.5	4240	±	34	3114	±	21	27650	±	108	1.132	±	0.261
PE	LBG	2.5	3653	±	134	1667	±	74	19586	±	519	1.633	±	0.157
CA	LBG	2.5	2664	±	52	972	±	72	21428	±	256	2.222	±	0.033
CO	XG	0.5	3115	±	70	1478	±	49	19571	±	317	0.479	±	0.019
W	XG	0.5	3251	±	36	607	±	45	20600	±	114	1.175	±	0.218
R	XG	0.5	3135	±	45	1327	±	18	18389	±	159	1.119	±	0.154
PO	XG	0.5	3519	±	0	888	±	55	35138	±	116	0.840	±	0.185
PE	XG	0.5	2268	±	88	1094	±	49	11643	±	372	1.837	±	0.106
CA	XG	0.5	2274	±	104	840	±	52	16045	±	247	1.970	±	0.085
CO	XG	2.5	3289	±	16	1552	±	74	20193	±	94	0.706	±	0.120
W	XG	2.5	2390	±	35	454	±	21	16479	±	437	1.437	±	0.034
R	XG	2.5	3665	±	86	1412	±	148	23152	±	155	0.996	±	0.210
PO	XG	2.5	3565	±	208	867	±	30	27622	±	379	1.298	±	0.256
PE	XG	2.5	2159	±	131	1021	±	120	11682	±	499	1.481	±	0.039
CA	XG	2.5	1959	±	127	640	±	43	13895	±	975	1.913	±	0.046
CO	GG	0.5	3126	±	23	1463	±	1	20446	±	192	0.372	±	0.005
W	GG	0.5	3463	±	11	911	±	6	21067	±	19	1.521	±	0.019
R	GG	0.5	3361	±	2	1613	±	120	17616	±	312	0.691	±	0.003
PO	GG	0.5	3841	±	20	2311	±	38	29182	±	419	1.175	±	0.034
PE	GG	0.5	2156	±	6	1078	±	3	10895	±	87	1.877	±	0.066
CA	GG	0.5	2727	±	180	1073	±	116	20058	±	4	2.017	±	0.015

Table 5.2: Pasting performance parameters and hydrolysis rate based on starch, hydrocolloid, and concentration. Starches: corn (CO), wheat (W), rice (R), potato (PO), pea (PE) and cassava (CA); Hydrocolloids: locust bean gum (LBG), xanthan gum (XG), guar gum (GG), hydroxypropylmethylcellulose (HPMC) and psyllium (P). Parameters: area under pasting curve (AUC) and hydrolysis rate (k).

CO	GG	2.5	3654	±	13	1607	±	25	25067	±	57	0.363	±	0.026
W	GG	2.5	4468	±	189	887	±	86	29394	±	943	1.124	±	0.014
R	GG	2.5	3780	±	101	1680	±	33	22428	±	420	0.765	±	0.031
PO	GG	2.5	4233	±	36	2939	±	42	27862	±	1547	0.990	±	0.210
PE	GG	2.5	3368	±	52	1456	±	62	18567	±	319	1.367	±	0.088
CA	GG	2.5	2583	±	91	1027	±	37	20831	±	568	1.709	±	0.037
CO	HPMC	0.5	3122	±	52	1534	±	14	19480	±	163	0.593	±	0.112
W	HPMC	0.5	3204	±	3	888	±	8	18888	±	70	1.502	±	0.119
R	HPMC	0.5	3072	±	129	1469	±	58	16393	±	665	1.012	±	0.008
PO	HPMC	0.5	3656	±	140	2240	±	40	29680	±	116	1.230	±	0.162
PE	HPMC	0.5	1927	±	4	984	±	13	9703	±	108	1.904	±	0.008
CA	HPMC	0.5	2262	±	54	838	±	1	16690	±	846	2.255	±	0.036
CO	HPMC	2.5	3533	±	163	1831	±	132	20639	±	330	0.774	±	0.035
W	HPMC	2.5	3848	±	165	1418	±	120	20717	±	740	1.675	±	0.021
R	HPMC	2.5	3318	±	146	1709	±	207	17202	±	383	1.425	±	0.217
PO	HPMC	2.5	3737	±	49	2363	±	169	29983	±	878	1.333	±	0.283
PE	HPMC	2.5	2065	±	251	1151	±	142	9433	±	1079	1.915	±	0.161
CA	HPMC	2.5	2505	±	116	977	±	5	18247	±	798	2.136	±	0.151
CO	P	0.5	3241	±	108	1682	±	139	19853	±	115	0.484	±	0.136
W	P	0.5	3323	±	90	849	±	45	20119	±	530	1.608	±	0.007
R	P	0.5	3294	±	109	1683	±	85	17458	±	303	1.010	±	0.076
PO	P	0.5	3678	±	28	2181	±	49	32375	±	237	0.897	±	0.066
PE	P	0.5	2100	±	42	1012	±	33	11106	±	205	2.104	±	0.189
CA	P	0.5	2469	±	159	947	±	37	18441	±	1343	2.057	±	0.067
CO	P	2.5	3985	±	60	1991	±	14	24789	±	305	0.595	±	0.004
W	P	2.5	4764	±	56	1588	±	73	27789	±	22	1.476	±	0.148
R	P	2.5	3993	±	64	1986	±	122	22820	±	14	0.763	±	0.025
PO	P	2.5	5162	±	28	1579	±	170	46578	±	360	0.605	±	0.299
PE	P	2.5	2638	±	134	1187	±	60	15170	±	881	2.035	±	0.395
CA	P	2.5	3206	±	23	1133	±	7	24934	±	86	1.875	±	0.083
	p-value													
	Starch			**0.0000**			**0.0000**			**0.0000**			**0.0000**	
	Hydrocolloid			**0.0000**			**0.0000**			**0.0000**			**0.0000**	
	%			**0.0000**			**0.0041**			**0.0000**			0.6714	

Although no pasting parameters presented a strong linear adjustment, a clear overall trend could be envisaged with the starches and hydrocolloids blends, which up to know have been only reported for individual associations of starches and hydrocolloids. These findings suggested that viscosity plays an important role in starch digestion, but it is not a single effect, but other factors, such as, starch source or hydrocolloid type must be considered (Fabek *et al*., 2014).

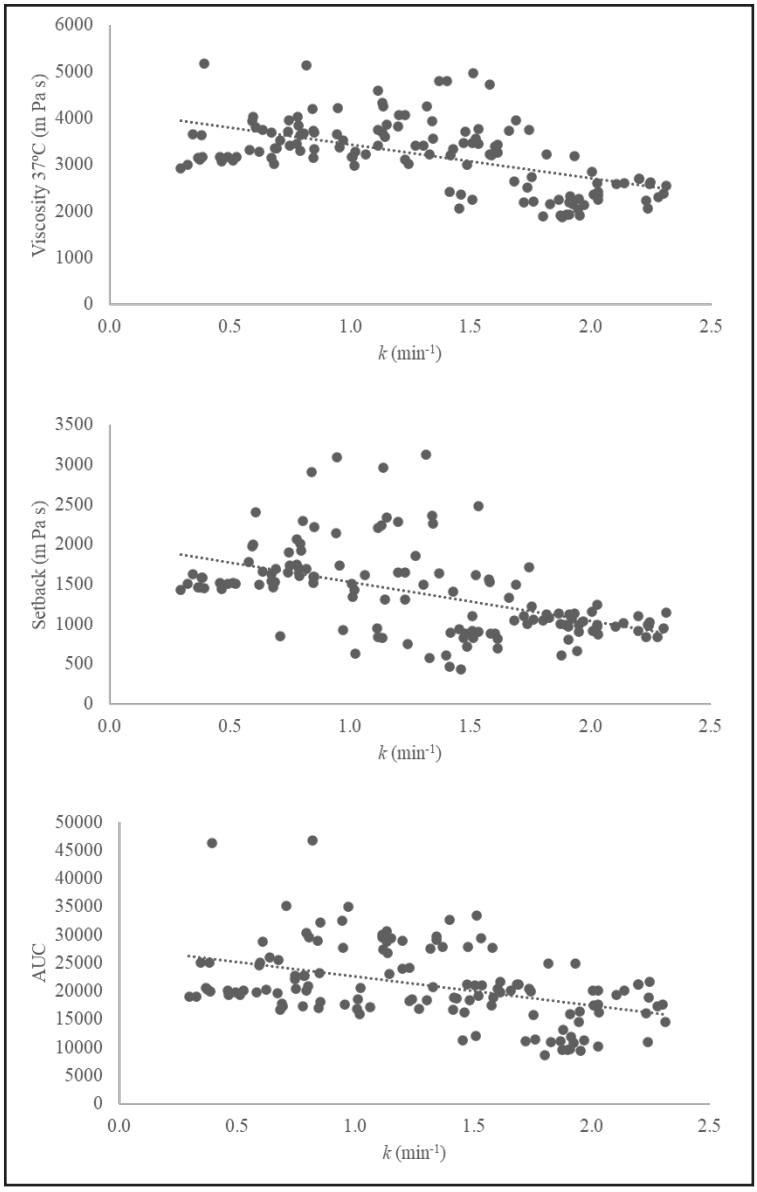

Figure 5.3: Linear regression among pasting parameters (viscosity 37 ºC, setback and AUC) and hydrolysis rate (*k*).

After the overall analysis of all the binary combinations of starches and hydrocolloids, a further analysis was carried out for the individual hydrocolloids (**Table 5.3**). Again, correlation coefficients for viscosity 37 ºC and *k* were higher than for the other pasting indicators (setback and AUC), apart from LBG and psyllium. Therefore, the negative correlation of gel viscosity after cooling down to 37 ºC with hydrolysis constant could be used as predictor of the starch gels susceptibility to enzymatic hydrolysis. This finding agrees with previously reported results, stating that viscosity greatly affects the cereal gels' digestibility (Santamaria *et al*., 2022b). Correlation coefficients between *k* and viscosity 37 ºC of starches (r = -0.7417) were decreased in the presence of the individual hydrocolloids, except for XG (**Table 5.3**). Xanthan gum addition presented a strong negative correlation (r = -0.7462) between hydrolysis rate and viscosity at 37 ºC. The starch combinations that mostly contributed to that effect were potato starch and 0.5% (XG) (viscosity of 3519 mPa s and *k* of 0.840 min^{-1}), or rice starch combined with 2.5% (XG) (viscosity of 3665 mPa s and *k* of 0.996 min^{-1}) (**Table 5.2**). Additionally, in psyllium containing gels, a strong negative correlation was observed between hydrolysis rate (*k*) and the setback (r = -0.7973). The binary combinations with utmost impact in that result were potato starch with 0.5%-P (setback of 2181 mPa s and *k* of 0.897 min^{-1}), potato starch with 2.5%-P (setback of 1579 mPa s and *k* of 0.605 min^{-1}). These results reveal that starch source and hydrocolloids type have a varied impact on alpha-amylase activity, but despite their varied functionality this study allowed drawing a relationship between viscosities and starch gels susceptibility to enzymatic hydrolysis.

Table 5.3: Correlation matrix between pasting properties (final viscosity at 37 ºC, setback, and area under pasting curve (AUC)) from gels development and hydrolysis constant (*k*). Hydrocolloids: locust bean gum (LBG), xanthan gum (XG), guar gum (GG), hydroxypropylmethylcellulose (HPMC) and psyllium (P).

	Parameters		
Kinetic constant (min^{-1})	Viscosity 37 ºC	Setback	AUC
k all samples	**-0.5491*** **	**-0.5036*** **	**-0.4247*** **
$k_{Control}$	-0.7417**	-0.6556*	
k_{LBG}	-0.4112*	-0.4690*	
k_{XG}	-0.7462***	-0.5776**	-0.5581**
k_{GG}	-0.5306**	-0.4102*	
k_{HPMC}	-0.5973**	-0.5940**	
k_{P}	-0.6404***	-0.7973***	-0.5322**

Significant correlations: *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$

5.4 Conclusions

Interactions between starches and hydrocolloids are very variable due to their different chemical structure and functionality. Because of that usually, individual interactions between starches and hydrocolloids are described. Moving forward, the present study included a variety of starches and hydrocolloids to understand possible relationship between systems viscosity and susceptibility of starches to enzymatic hydrolysis, independently on either the starch or hydrocolloid type. The principal component analysis (PCA) obtained with the pasting parameters and the enzymatic hydrolysis kinetics allowed results aggregation based on the source starch. Cereals and potato gels showed higher viscosity and lower kinetic constant, but cassava and pea gels showed the opposite performance. Gels obtained with potato starch singly or combined with hydrocolloids displayed the strongest negative impact on the hydrolysis rate. Regarding hydrocolloids, their impact on starch enzymatic hydrolysis was greatly dependent on the type of starch and hydrocolloid, even the hydrocolloids concentration. LBG reduced pea starch digestibility. XG affected the hydrolysis constant, but the effect was concentration dependent. GG influenced all starches viscosities, but the impact on their hydrolysis rate was starch dependent. HPMC did not affect starches hydrolysis. P decreased the hydrolysis rate, particularly in the case of potato gels. A correlation matrix confirmed the negative correlations between hydrolysis rate (k) of gels and their viscosity at 37 ºC, setback and AUC. This relationship could be used as predictor of either starch or starch-hydrocolloids susceptibility to enzymatic hydrolysis using a rapid viscosity test.

5.5 Supporting information

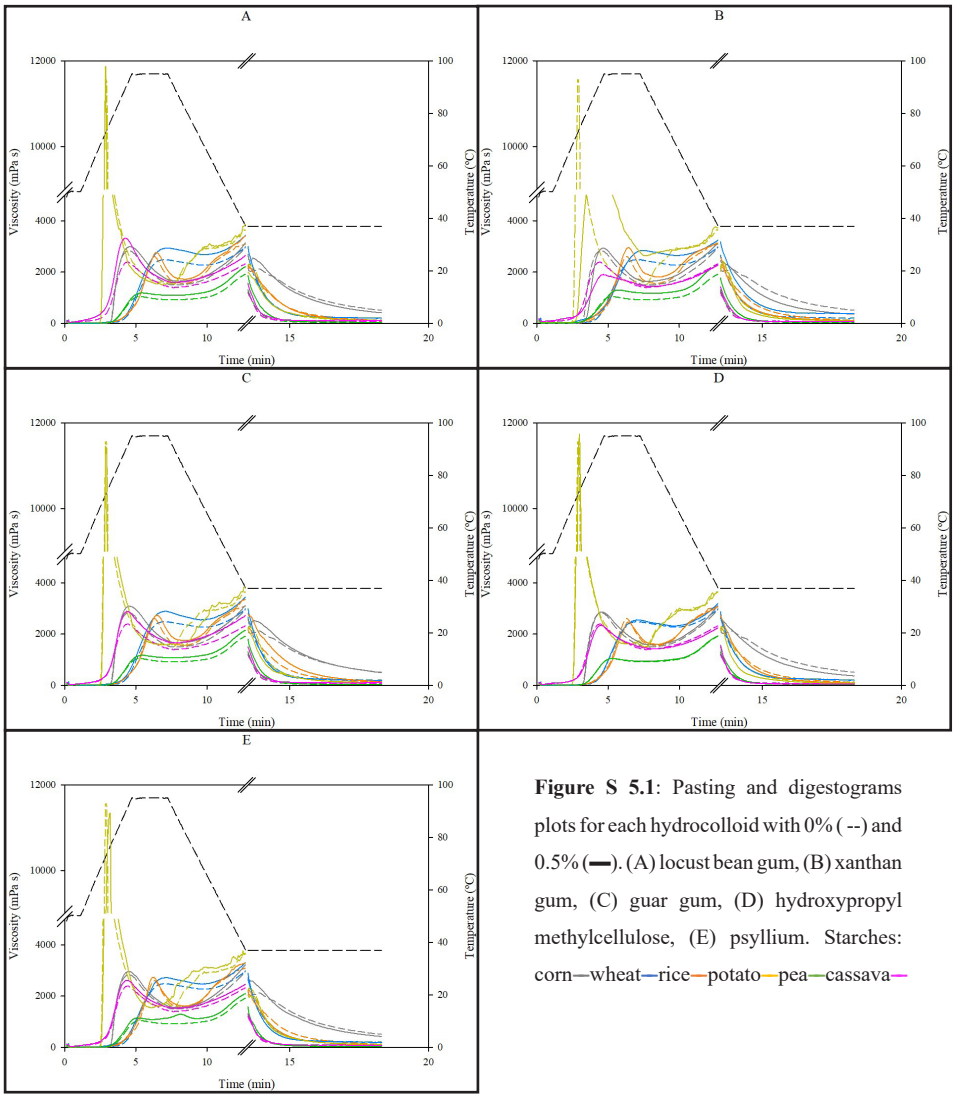

Figure S 5.1: Pasting and digestograms plots for each hydrocolloid with 0% (--) and 0.5% (—). (A) locust bean gum, (B) xanthan gum, (C) guar gum, (D) hydroxypropyl methylcellulose, (E) psyllium. Starches: corn—wheat—rice—potato—pea—cassava—

Starch	Hydrocolloid	%	Onset (min)			Peak viscosity (mPa s)			Trough (mPa s)			Breakdown (mPa s)		
CO		0	3.27	±	0.00	2834	±	1	1492	±	10	1342	±	8
W		0	3.30	±	0.42	2488	±	16	2266	±	11	222	±	27
R		0	3.70	±	0.04	2612	±	16	1560	±	2	1052	±	18
PO		0	2.46	±	0.00	11701	±	673	1501	±	175	10201	±	499
PE		0	3.33	±	0.00	1048	±	11	922	±	7	126	±	4
CA		0	2.20	±	0.09	2399	±	165	1391	±	49	1008	±	115
CO	LBG	0.5	3.20	±	0.00	2989	±	4	1649	±	6	1340	±	1
W	LBG	0.5	3.70	±	0.14	2943	±	37	2683	±	76	260	±	40
R	LBG	0.5	3.64	±	0.05	2764	±	28	1724	±	45	1040	±	17
PO	LBG	0.5	2.53	±	0.00	11877	±	643	1484	±	121	10393	±	522
PE	LBG	0.5	3.20	±	0.00	1189	±	12	1093	±	8	96	±	4
CA	LBG	0.5	1.97	±	0.05	3323	±	228	1595	±	5	1729	±	223
CO	LBG	2.5	3.30	±	0.05	4187	±	11	2083	±	12	2104	±	23
W	LBG	2.5	2.67	±	0.09	4636	±	86	4166	±	48	470	±	134
R	LBG	2.5	3.20	±	0.00	3871	±	21	2415	±	10	1456	±	11
PO	LBG	2.5	2.70	±	0.05	11486	±	235	1126	±	13	10360	±	248
PE	LBG	2.5	3.10	±	0.05	2179	±	83	1986	±	61	193	±	22
CA	LBG	2.5	1.38	±	0.45	3541	±	80	1692	±	124	1849	±	204
CO	XG	0.5	3.40	±	0.00	2935	±	44	1637	±	21	1299	±	23
W	XG	0.5	2.47	±	0.09	2847	±	8	2644	±	8	203	±	1
R	XG	0.5	3.17	±	0.05	2951	±	38	1808	±	27	1143	±	11
PO	XG	0.5	2.73	±	0.00	5596	±	179	2631	±	55	2965	±	124
PE	XG	0.5	3.13	±	0.00	1300	±	53	1174	±	40	126	±	13
CA	XG	0.5	1.73	±	0.28	1917	±	47	1434	±	52	483	±	99
CO	XG	2.5	3.57	±	0.05	2871	±	16	1737	±	57	1135	±	73
W	XG	2.5	2.46	±	0.00	2110	±	56	1936	±	14	174	±	42
R	XG	2.5	2.90	±	0.04	3342	±	10	2254	±	62	1089	±	52
PO	XG	2.5	3.13	±	0.10	3020	±	14	2699	±	177	322	±	192
PE	XG	2.5	3.20	±	0.09	1161	±	18	1138	±	11	24	±	29
CA	XG	2.5	1.20	±	0.28	1447	±	95	1319	±	83	128	±	11
CO	GG	0.5	3.27	±	0.00	3090	±	8	1664	±	23	1426	±	31
W	GG	0.5	2.57	±	0.05	2893	±	0	2552	±	6	341	±	6
R	GG	0.5	3.67	±	0.00	2777	±	24	1748	±	117	1029	±	93
PO	GG	0.5	2.53	±	0.00	11853	±	325	1530	±	18	10323	±	343
PE	GG	0.5	3.27	±	0.09	1176	±	3	1078	±	3	98	±	6

Table S 5.1: Pasting performance parameters based on starch, hydrocolloid, and concentration. Starches: corn (CO), wheat (W), rice (R), potato (PO), pea (PE) and cassava (CA); Hydrocolloids: locust bean gum (LBG), xanthan gum (XG), guar gum (GG), hydroxypropylmethylcellulose (HPMC) and psyllium (P).

CA	GG	0.5	2.17	±	0.23	2888	±	192	1654	±	64	1235	±	257
CO	GG	2.5	3.33	±	0.00	4118	±	58	2047	±	13	2071	±	71
W	GG	2.5	2.60	±	0.00	4176	±	123	3581	±	103	596	±	21
R	GG	2.5	3.10	±	0.04	3647	±	24	2100	±	68	1547	±	44
PO	GG	2.5	2.66	±	0.00	10781	±	617	1294	±	6	9488	±	623
PE	GG	2.5	3.07	±	0.09	2157	±	23	1912	±	11	245	±	13
CA	GG	2.5	1.10	±	0.14	3347	±	74	1556	±	54	1791	±	20
CO	HPMC	0.5	3.30	±	0.05	2859	±	10	1588	±	37	1272	±	28
W	HPMC	0.5	2.97	±	0.05	2558	±	27	2316	±	11	242	±	16
R	HPMC	0.5	3.73	±	0.00	2469	±	95	1603	±	71	866	±	25
PO	HPMC	0.5	2.57	±	0.05	12215	±	228	1416	±	180	10799	±	49
PE	HPMC	0.5	3.20	±	0.00	1051	±	22	943	±	16	108	±	6
CA	HPMC	0.5	2.37	±	0.05	2345	±	203	1424	±	55	921	±	148
CO	HPMC	2.5	3.33	±	0.00	2910	±	8	1702	±	31	1208	±	23
W	HPMC	2.5	2.66	±	0.00	2721	±	91	2430	±	45	291	±	45
R	HPMC	2.5	3.60	±	0.14	2548	±	106	1610	±	62	939	±	45
PO	HPMC	2.5	2.66	±	0.00	11623	±	99	1375	±	120	10249	±	21
PE	HPMC	2.5	3.26	±	0.00	994	±	131	914	±	109	80	±	22
CA	HPMC	2.5	1.80	±	0.00	2437	±	66	1529	±	111	908	±	45
CO	P	0.5	3.27	±	0.00	2961	±	11	1559	±	31	1402	±	42
W	P	0.5	2.97	±	0.23	2720	±	55	2474	±	45	247	±	11
R	P	0.5	3.43	±	0.04	2733	±	1	1611	±	24	1122	±	25
PO	P	0.5	2.60	±	0.00	11415	±	47	1498	±	21	9918	±	67
PE	P	0.5	3.17	±	0.05	1151	±	5	1088	±	9	63	±	4
CA	P	0.5	2.15	±	0.07	2614	±	192	1522	±	122	1092	±	71
CO	P	2.5	3.27	±	0.00	3530	±	67	1994	±	46	1536	±	21
W	P	2.5	2.53	±	0.10	3388	±	18	3176	±	17	212	±	1
R	P	2.5	3.03	±	0.04	3378	±	70	2008	±	59	1370	±	11
PO	P	2.5	2.73	±	0.10	6889	±	11	3742	±	25	3147	±	36
PE	P	2.5	2.93	±	0.00	1942	±	97	1451	±	74	491	±	23
CA	P	2.5	0.90	±	0.05	3482	±	109	2073	±	30	1410	±	139
	p-value													
	Starch			**0.0000**			**0.0000**			**0.0000**			**0.0000**	
	Hydrocolloid			0.0671			**0.0000**			0.0053			**0.0002**	
	% Hydrocolloid			**0.0000**			0.5997			**0.0000**			0.3978	

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GENERAL DISCUSSION

Technological and nutritional characteristics of gluten free breads

The global market of gluten free (GF) products has been changing in recent years. The statistics data still foresees an increase in the sales of GF products from 5.6 billion $US in 2020 to 8.3 billion $US in 2025 (Gao *et al*., 2018; MarketsandMarkets, 2020). Consumers who choose the GF diet are growing, either due to an increase in the incidence of gluten-related disorders (allergic, autoimmune, or immune-mediated) or its associations with a healthier diet, regardless of the lack of scientific evidence (Capriles *et al*., 2021; Monteiro *et al*., 2021; Zoghi *et al*., 2021). Gluten is a three-dimensional protein fraction, which plays an important role in cereal-based products, particularly in bakery products. The most common formulations for GF products are based on flour and starch mixtures. Starch provides a crumb-like structure, due to its gelatinization capacity, which allows holding carbon dioxide produced during dough fermentation (Sciarini *et al*., 2021; Woomer & Adedeji, 2021). The ingredients and additives frequently used in the GF industry are hydrocolloids, proteins, enzymes, or modified starches, as structuring agents to mimic gluten functionality. Hydrocolloids are the most common additives in GF formulations due to their ability to bind water molecules and to form three-dimensional structures, mimicking gluten viscoelastic properties responsible of leading cohesive doughs (Houben *et al*., 2012; Monteiro *et al*., 2021). Their incorporation results in breads with high moisture content, soft texture, higher specific volume, superior quality structure and sensory properties, and extended shelf-life (Zoghi *et al*., 2021).

There is an interest in improving the nutritional quality of GF products because they are starch-based products with high content of carbohydrates and fats, also a low quantity of proteins. Much interest is focus on reducing their starch digestibility because they have high starch content and consequently high glycemic index (GI) (Matos Segura & Rosell, 2011). Innovative strategies are currently being developed to reduce starch digestion rate in bakery products (Calle *et al*., 2020; Capriles & Arêas, 2016; Giuberti & Gallo, 2018). Some of these strategies are: (i) select the type of starches, such as legume source. Gularte *et al*. (2012) showed a reduction in the GI of cake formulates with pea and lentil flours, which could be related to the soluble dietary fiber content and its increase in viscosity paste. (ii) Apply physical treatments to the GF flours, like particle size fractionation. De la Hera *et al*. (2014) reported slower starch hydrolysis when the particle size of the rice flour used for making GF bread was higher than 150 μm. (iii) The inclusion of sourdoughs into GF bread recipes has been associated with slower starch

digestion, due to the generation of organic acids (lactic, acetic, and propionic acids) during fermentation. Wolter *et al*. (2014) presented a GI reduction after the addition of sourdough to wheat bread. (iv) Finally, the modulation of the system viscosity with the addition soluble fibers to restrict enzyme accessibility. In pumpkin-enriched bread, Ge *et al*. (2021) found that an increase in digestion viscosity hinders the α-amylase activity.

The correlation between the viscosity of the food systems with starch hydrolysis rate has been explored with complex matrices, adding soluble and insoluble dietary fibers. Nevertheless, no fundamental studies have confirmed the role of starchy food viscosity on their digestion. In this research, different model systems were defined. Then, these systems were analyzed by several *in vitro* digestion models. Finally, correlations between viscoelastic properties and their impact on starch hydrolysis were studied.

Viscosity in starch-based systems

The first system was based on a homogeneous model, removing potential influences on the starch source or hydrocolloids' presence. Therefore, corn starch gels (starch:water) were prepared using a range of starch concentrations differing from 1:4 to 1:16 in the Rapid Visco Analyzer (RVA). The highest concentrated corn starch gel had the maximum peak of apparent viscosity, as expected, because starch content was correlated to the apparent viscosity. After starch gelatinization, gels displayed a honeycomb or sponge-like structure, as observed by Aleixandre *et al*. (2021). The concentrated gels presented a more closed structure, and the number of cavities decreased from the concentration 1:8. These homogeneous systems demonstrated a positive relationship (r = 0.87) between the number of cavities and gel viscosity.

However, previous relationship was only demonstrated in corn starch gels, thus there was the remaining question if similar correlations were observed independently on the type of starch. Cereal starch gels from diverse sources (corn, wheat, and rice) were prepared controlling the level of hydration to lead two sets of gels comprising variable viscosity (VV) gels made with the same starch content (1:4) and constant viscosity (CV) gels prepared to obtain gels with the same viscosity. Starch performance during these gels gelatinization showed that corn starch had faster gelatinization and wheat starch had higher maximum force (N). Similar results were obtained by Garzon and Rosell (2021) associating higher force with a more porous structure in starch gels. Furthermore, those gels behave as solid-like ($G' > G''$) gels. Once again, during the cooling profile, the stronger gel was formed by wheat starch showing the highest elastic modulus (G'). On the

other hand, constant viscosity (CV) gels were prepared with the adjusted starch content to obtain gels with the same viscosity, but with various sources. These samples confirmed the similar physical behavior, and they obtained closer values of viscous modulus (G'').

Lastly, more complex matrices containing hydrocolloids were analyzed creating binary systems with diverse starches from cereals, tuber, and pulse sources. Hydrocolloid suspensions (2%) at 25 ºC displayed significantly difference in their viscosities: psyllium > guar gum > xanthan gum > locust bean gum > hydroxypropylmethylcellulose K4M. It must be stressed that hydrocolloids had a synergistic effect on the viscosity of potato starch during heating and cooling, and this was also observed by Gularte and Rosell (2011).

In vitro enzymatic hydrolysis of starch gels

Corn starch gels at different concentrations were analyzed by oro-gastrointestinal digestion (INFOGEST method) (Brodkorb *et al*., 2019; Minekus *et al*., 2014). However, this analysis resulted in low hydrolysis during the oral and gastric phases. Iqbal *et al*. (2021) related these results to the short time in the oral phase and the inhibition of salivary alpha-amylase in the gastric phase. Moreover, other authors indicated that samples with high starch content had slower hydrolysis due to the high viscosity of the system reduces enzyme accessibility (Sanromán *et al*., 1996; Wu *et al*., 2017). For this reason, in this research starch hydrolysis was carried out with porcine pancreatic alpha-amylase for 3 hours (Benavent-Gil & Rosell, 2017).

Owing to the starch hydrolysis *in vitro* methods measuring glucose release are time-consuming, a rapid and continuous analytical methods were designed, based on rheological measurements (RVA and rheometer). Apart from exploring the pasting behavior during heating and cooling, the digestogram phase allows measuring starch enzymatic hydrolysis following the apparent viscosity (μ). The addition of alpha-amylase and amyloglucosidase to potato and waxy corn starch gels induces the rupture of starch chains into small compounds shifting from a solid gel behavior to weakly fluid gel (Sorba & Sopade, 2013). Likewise, Gee and Johnson (1985) reported similar performance using a rotary viscometer, and An *et al*. (2016) did record the hydrolysis of wheat and rice using a rheometer. In the present research, a first-order kinetic model was used to predict the hydrolysis rate (k) of starch gels digestion and discriminate among different starch sources. Furthermore, a correlation was established between the data obtained by glucose quantification with *in vitro* hydrolysis method and the viscosity decay observed in the digestograms. Regarding starch source, rice and wheat gels presented good

fitting among the three methodologies applied, in opposition to corn starch gel, which was attributed to the pH variation that affected the enzymatic activity. For this reason, a buffer with adjusted concentration and pH was used in subsequent studies. The inclusion of enzymatic hydrolysis stage provides rapid, simple, and reliable procedures to predict the behavior of starch gels digestion.

Relationship among starch gels viscosity and their enzymatic hydrolysis

Starch fractions and hydrolysis rate were quantified in corn gels at different concentrations. Slowly digestible starch (SDS) increased in more concentrated gels. This starch fraction is related to low postprandial response, thus results indicated that gels viscosity affects alpha-amylase activity decreasing the glycemic response. The more concentrated starch gels presented slower enzymatic hydrolysis. Specifically, the extent of hydrolysis in corn starch gels, made at the ratio starch:water 1:4, was 53%. Hence, the kinetic constant (k) was lower in more concentrated gels. Nevertheless, the expected trend would be the increase in the hydrolysis rate with the amount of substrate at constant reaction conditions (enzyme concentration and temperature). The increase in viscosity induced by hydrocolloids has been related to the decrease in the enzymatic activity. These polysaccharides could generate a physical barrier that blocks the enzyme accessibility and in consequence the starch absorption in the small intestine (Chung *et al*., 2007; Dona *et al*., 2010; Gularte & Rosell, 2011; Sasaki & Kohyama, 2011). Recently, Ma *et al*. (2019) observed that the incorporation of pectin in corn starch gels slow down the starch hydrolysis rate (k). This occurrence was attributed to the creation of a pectin layer onto the surface of starch granules. However, these results demonstrated that the viscosity of starch-based system has an important role in its hydrolysis. Corn starch gels with more starch content without hydrocolloids, displayed higher apparent viscosity and lower hydrolysis kinetic, suggesting that gel properties (like starch gels' viscosity and microstructure features) could affect the enzyme-substrate interaction. In addition, this study suggested the existence of a viscosity threshold regarding its effect on the hydrolysis rate, because from 1:8 to 1:4 of starch gels concentration their effect on enzyme accessibility was higher.

After studying the impact of viscosity in corn gels, other cereals' starch gels (1:4) were hydrolyzed by alpha-amylase. Wheat and corn gels resulted in higher viscosity than rice gels and also had slower starch hydrolysis, particularly in the corn gel. Besides, corn gel displayed higher content of SDS and a slower kinetic constant (k). When measuring the hydrolysis rate by digestograms, corn gels had lower digestion showing the higher viscosity initial digestion. As discussed above, highly viscous systems could limit the amylase accessibility to starch.

In fact, when corn and wheat gels were prepared with similar viscosity (1:4, 1:5.5 and 1:5.2) alike hydrolysis behavior was observed, regarding SDS amount and hydrolysis rate. Significantly positive correlations were observed between gels viscosity and SDS (r = 0.83), and strong negative ones with the kinetic constant (r = −0.82). Velásquez-Barreto *et al*. (2021) observed the same trend among gel viscosities and SDS fraction in tuber starches. Once again, present research confirmed that the viscosity of the system affects starch-amylase interaction or produces changes in the structure of starch granules (Dhital *et al*., 2017). Moreover, the system viscosity limits the mass transfer affecting the hydrolysis rate.

When the study was extended to other starches (corn, wheat, rice, potato, pea and cassava), potato starch presented higher pasting parameters (viscosity at 37 ºC, setback, and the area under the pasting curve) than the other starches gels, which could be due to the covalent bonds resulting from the phosphate groups present in potato starch (Sorba & Sopade, 2013). Pea and cassava starches showed the opposite performance: lower viscosity, and faster hydrolysis rate (k). Furthermore, the hydrocolloids' addition affected the pasting and digestograms behavior. In general, negative correlations were found between pasting properties (viscosity at 37 ºC (r = -0.55), setback (r = -0.50), and AUC (r = -0.42)) and digestion rate. This trend was also observed in starch:hydrocolloid matrices, where digesta viscosity affected their digestibility (Fabek *et al*., 2014). In binary systems consisting of starches and diverse hydrocolloids systems, potato starch showed the greater changes in the hydrolysis rate, especially in the presence of xanthan gum or psyllium. Besides, Oh *et al*. (2018) observed the reduction of hydrolysis in high amylose rice starch modified by dry heat treatment, and this effect was greater with xanthan gum addition. Sevilmis and Sensoy (2022) reported a decrease in the content of SDS in diverse starches (wheat, potato, and cassava) when blended with psyllium fiber. This effect was associated with the hampering of enzyme-substrate interaction.

These findings confirms that the viscosity behavior of starch-based systems is correlated with their digestion and thus it could be used to design healthy foods with reduced postprandial glucose responses.

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CONCLUSIONS

Research conducted through the different chapters allows concluding that viscosity of starch gels significantly affected the rate of enzymatic hydrolysis, which could be used as an alternative strategy in the design of GF foods, particularly starch-based GF systems, that induces low postprandial glucose response.

Particularly, the following concluding remarks can be highlighted:

- Once the technological challenges have been overcome, research is required to improve the nutritional quality of GF products. Their high starch content has been an issue for its possible impact on increasing the postprandial glucose response. Studies on the structural changes in starch and their relationship with starch hydrolysis are needed to reduce the glycemic index of gluten free foods.

- Viscosity of starch gels plays a crucial role on the gels microstructure and their enzymatic digestion of starch. Using corn starch gels at different concentrations it was possible to develop a simplified model that links macrostructural properties and microstructural features to analyze hydrolysis kinetics. The structural model connects by a linear relationship ($r = 0.98$) the porous structure (cavity sizes and thickness walls) of starch gels and their viscosity. These findings could be applied in the design of food formulations aiming at postprandial glucose management.

- Viscosity plays a fundamental role in defining starch gel functionality, which it is also extended to the prediction of their comportment during digestion. Starch gels from different cereals (corn, wheat, rice) showed significantly different viscosities when produced at constant starch concentrations. Nevertheless, force along gelatinization and the viscoelastic properties of cereal starch gels were closer when comparing gels of similar viscosity, showing alike hydrolysis rates. Therefore, gel viscosity could be a rapid indicator for estimating starch kinetic hydrolysis.

- Single tests were developed to study the gelatinization performance and the digestion of different starch gels. Viscosity changes recorded with RVA or rheometer followed by amylase hydrolysis provide digestograms plots that were fitted to a first-order kinetic models to predict gels digestion. Gels made with corn, wheat and rice starches confirmed the validity of the methods.

- The relationship between viscosity and starch hydrolysis kinetics has been demonstrated using binary systems containing starches and hydrocolloids. Cereals and potato gels showed higher viscosity and lower kinetic constant, but cassava and pea gels showed the opposite performance. Regarding hydrocolloids, their impact on starch enzymatic hydrolysis was greatly dependent on the type of starch and hydrocolloid, even the hydrocolloid concentration. A correlation matrix confirmed the negative correlations between hydrolysis rate (k) of gels and their viscosity at 37 ºC. This relationship could be used as a predictor of either starch or starch-hydrocolloid susceptibility to enzymatic hydrolysis using a rapid viscosity test.

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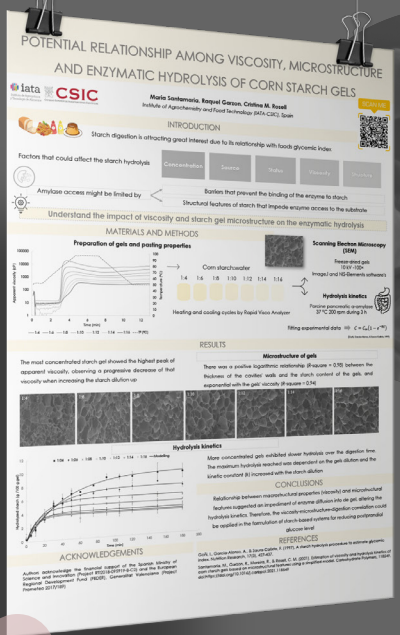

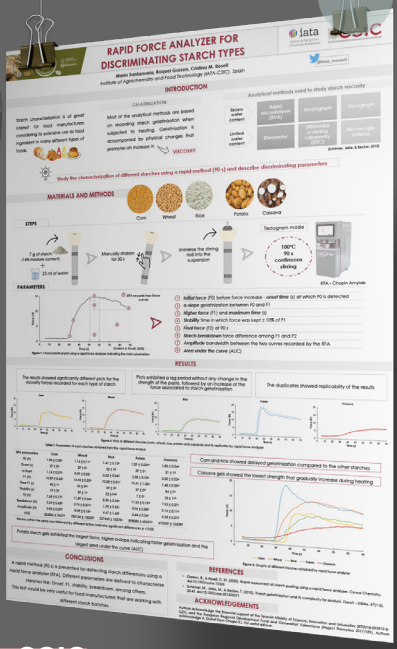

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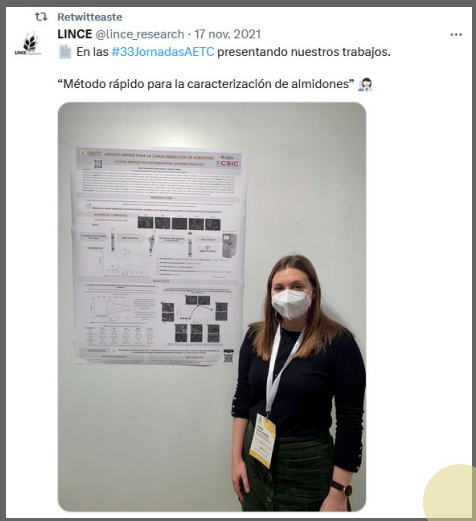

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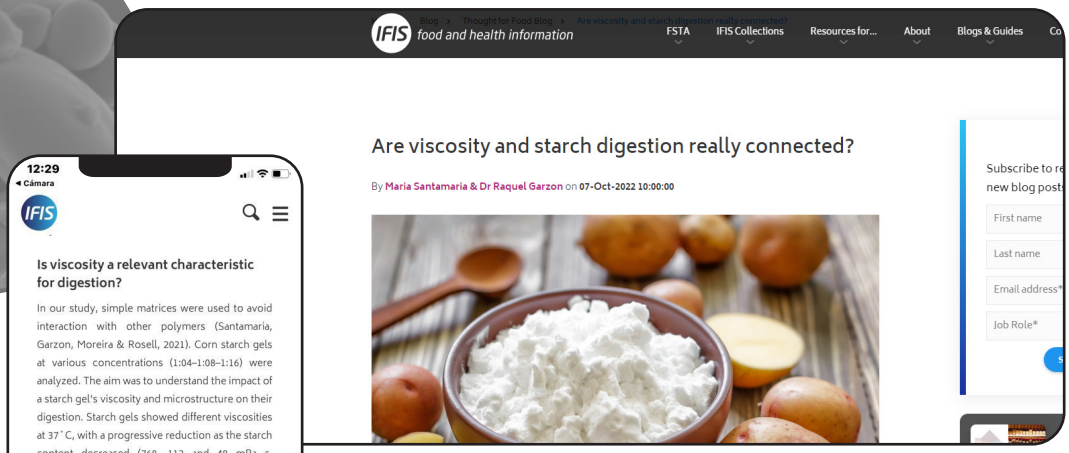

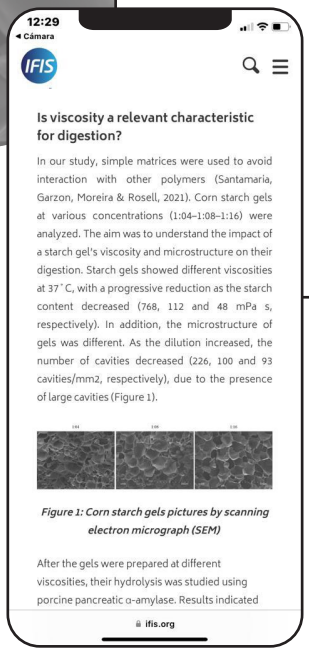

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Santamaria, M., Garzon, R., Moreira, R., & Rosell, C. M. (2021). Estimation of viscosity and hydrolysis kinetics of corn starch gels based on microstructural features using a simplified model. *Carbohydrate Polymers*, *273*, 118549.

Santamaria, M., Montes, L., Garzon, R., Moreira, R., & Rosell, C. M. (2022). Unraveling the impact of viscosity and starch type on the *in vitro* starch digestibility of different gels. *Food & Function*, *13*(14), 7582-7590.

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Estimation of viscosity and hydrolysis kinetics of corn starch gels based on microstructural features using a simplified model

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ABSTRACT

Viscosity is an important rheological property, which may have impact on the glycemic response of starchy foods. However, the relationship between starch gels viscosity on its hydrolysis has not been elucidated. The aim of this work was to assess the effect of gels viscosity on the microstructure, and the kinetics of enzymatic hydrolysis of starch. Corn starch gels were prepared from starch:water ratios varying from 1:4 to 1:16. A structural model was proposed that correlated (R-square = 0.98) the porous structure (cavity sizes, thickness walls) of gels and its viscosity. Kinetics constants of hydrolysis decreased with increasing starch content and consequently with gel viscosity. Relationships of viscosity with the microstructural features of gels suggested that enzyme diffusion into the gel was hindered, with the subsequent impact on the hydrolysis kinetics. Therefore, starch digestibility could be governed by starch gels viscosity, which also affected their microstructure.

1. Introduction

The understanding of starch hydrolysis is attracting much research owing its relationship with the metabolic processes occurring along human digestion, particularly the postprandial blood glucose levels (Hardacre, Lentle, Yap, & Monro, 2016). Previous to the glucose absorption in small intestine, starch is hydrolyzed by salivary and pancreatic α-amylase in the mouth and small intestine, respectively, generating short oligomers, such as maltose or maltotriose (Dona, Pages, Gilbert, & Kuchel, 2010). According to the rate of hydrolysis, starch is commonly categorized into three fractions (Englyst & Hudson, 1996): rapidly digestible starch (RDS) associated with a fast increase in blood glucose level, slowly digestible starch (SDS) slowly hydrolyzed in the small intestine, and resistant starch (RS), which is not digested by the enzymes in the superior gastrointestinal tract, but microorganisms can ferment it to short chain fatty acids (SCFA) in the large intestine (Dura, Rose, & Rosell, 2017; Zhou et al., 2020).

Despite the interest in starch digestion, there is uncertainty about the factors that could affect the hydrolysis of starch catalyzed by α-amylase. The starch concentration, its botanical origin, or the starch status as native or gelatinized form are important properties that may influence the hydrolysis. Previous studies suggested that cereal flours are digested more rapidly than tubers and legume flours, due to their difference in starch microstructure and chemical composition (Gularte & Rosell, 2011; Liu, Donner, Yin, Huang, & Fan, 2006). Furthermore, Dhital, Warren, Butterworth, Ellis, and Gidley (2017) described that mechanisms limiting enzymatic activity are related to binding or blocking the access of α-amylase. Those authors differentiated when enzymatic hydrolysis is in aqueous solution as occurs in the gelatinized starch or in slurry as the case of granular starch. In both cases the amylase hydrolysis might be limited by, first the barriers that prevent the binding of the enzyme to starch and secondly, the structural features of starch that impede amylase access to the substrate. Consequently, physical characterization of the starch granule as size, pores in the granular surface or the supramolecular structure are properties that can impact the adsorption and binding of the α-amylase. Besides starch structure, viscosity of the system has been incorporated as one important element in the starch digestion (Hardacre, Lentle, Yap, & Monro, 2016). However, studies investigating viscosity have been focused on the impact of soluble and insoluble dietary fiber, but not on the role of gels viscosity produced as a result of starch gelatinization. The addition of hydrocolloids (usually labelled as non-starch polysaccharides, NPS) modifies the gelatinization/gelation process of the starch (Brennan, Suter, Luethi, Matia-Merino, & Qvortrup, 2008; Tomoko & Kaoru, 2011). A study

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carried out with corn and potato starches and different hydrocolloids (pectin, guar gum, xanthan gum and soluble cellulose derivatives CMC and HPMC) confirmed that hydrocolloids affected the hydrolysis rate to different extent, depending on the hydrocolloid and type of starch (Gularte & Rosell, 2011). Authors observed an increase in initial rate of starch amylolysis in the presence of hydrocolloids, with the exception of guar gum that decreased the kinetic constant in potato gels (Gularte & Rosell, 2011). Yuris, Goh, Hardacre, and Matia-Merino (2019) studied the digestibility of wheat starch gels in the presence of several polysaccharides (xanthan, guar, agar) and explained the reduction in the starch digestibility by the increase in gel hardness that limits the enzyme accessibility to starch. Similarly, guar and xanthan gums added to high-amylose corn starch affected starch viscosity and retarded starch hydrolysis leading to lower estimated glycemic response (Chung, Liu, & Lim, 2007; Zhang, Li, You, Fang, & Li, 2020). The different studies discussed the relationship between the extent of starch hydrolysis and the system viscosity, but divergences on the role of viscosity accelerating or slowing down the starch hydrolysis have been encountered, which might be attributed to a possible viscosity threshold required for that enzymatic inhibition. Additionally, some studies analyzed the relation between insoluble fiber like cellulose and the α-amylase activity. Nsor-atindana, Yu, Goff, Chen, and Zhong (2020) reported that amylase can bind cellulose and act as a reversible and non-specific inhibitor, and the inhibition becomes more apparent as the particle size of the polymer decreases (Dhital, Gidley, & Warren, 2015; Nsor-atindana, Yu, Goff, Chen, & Zhong, 2020).

Therefore, although it has been found out that the viscosity of exogenous sources of hydrocolloids impacts the rate of digestive hydrolysis of starch to our best knowledge there are no studies regarding the viscosity effect of starch gels on their hydrolysis by digestive enzymes. Based on this, we initially hypothesized that starch gels viscosity could affect their digestion, and furthermore, that their structural features also might influence the enzymes accessibility to the starch. The aim of this study was to unravel the impact of viscosity and gel microstructure on the enzymatic hydrolysis of starch gels, using homogeneous gels prepared only with starch, in order to avoid possible artifacts derived from the interaction between heterologous polymers as it occurs in the presence of different hydrocolloids. Corn starch gels were prepared with different starch concentrations leading to gels with different properties and microstructure. To simulate starch digestion, the oro-gastrointestinal digestion (Minekus et al., 2014) and a direct *in vitro* enzymatic hydrolysis (Benavent-Gil & Rosell, 2017) were applied to the different gels.

2. Materials and methods

2.1. Materials

Corn starch EPSA (Valencia, Spain) of 95% purity (20.25% amylose content) and 13.22% moisture content was used. The enzymes used were type VI-B α-amylase from porcine pancreas (EC 3.2.1.1), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas (EC 232.468.9), bile salts and 3,5-dinitrosalicylic acid (DNS) were acquired from Sigma Aldrich (Sigma Chemical, St. Louis, USA). Amyloglucosidase (EC 3.2.1.3) was provided by Novozymes (Bagsvaerd, Denmark). Glucose oxidase/peroxidase (GOPOD) kit (Megazyme International Ireland Ltd., Bray, Ireland) was used. Solutions and standards were prepared by using deionized water. All reagents were of analytical grade.

2.2. Preparation of gels and pasting properties

The preparation of starch gels and the pasting performance of each samples was determined by Rapid Visco Analyzer (RVA 4500; Perten Instruments, Hägersten, Sweden). Corn starch gels were prepared at different concentrations with deionized water (w:w, 1:4; 1:6; 1:8; 1:10; 1:12; 1:14; 1:16). Slurries were subjected to heating and cooling cycles consisting of: 50 °C for 1 min, heating from 50 to 95 °C in 3 min 42 s, holding at 95 °C for 2 min 30 s, then cooling down to 50 °C in 3 min 48 s and holding at 50 °C for 2 min. The pasting parameters evaluated included the peak viscosity (maximum viscosity during heating), breakdown (viscosity difference between peak viscosity and trough), and the pasting rate calculated as the slope of the apparent viscosity during heating until 95 °C. The apparent viscosity of the formed gels was measured at 37 °C with a vibrational viscometer VL7-100B-d15 (Hydramotion Ltd., Malton, UK). This apparatus measures viscosity at high shear rate where the strong shear-thinning behavior of samples is less relevant. Moisture of gels was determined in two steps using an infrared balance (KERN, Balingen, Germany). Three different batches for each gel were prepared.

2.3. Total starch

The amount of total starch of the gels was quantified using a commercial assay kit (Megazyme International Ireland Ltd., Bray, Ireland). Two replicates were measured for each sample.

2.4. Scanning Electron Microscopy (SEM)

Fresh gels were immersed in liquid nitrogen and then freeze-dried. The microstructure of the different freeze-dried gels was observed using scanning electron microscopy (S-4800, Hitachi, Ibaraki, Japan). Samples were examined at an accelerating voltage of 10 kV and 100× magnification. Micrographs (1.3 × 0.98 mm) were captured. The microstructure analysis was carried out using the ImageJ analysis program (ImageJ, National Institutes of Health, Bethesda, Maryland, USA) and NIS-Elements software (Nikon Instruments Inc., Tokyo, Japan). An auto local thresholding was applied using ImageJ software and measured the wall thickness, and then the measurement of gel cavities or holes was carried out with Nis-Elements software. Parameters assessed were number of cavities/mm^2, mean cavity area (μm^2), porosity (%) calculated as ratio of total area of cavities and total image area, and wall thickness (μm) as previously described by Garzon and Rosell (2021). Three images were used to calculate the average of previous parameters.

2.5. In vitro oro-gastrointestinal digestion

The oro-gastrointestinal digestion was carried out following the standardized static digestion method described by Minekus et al. (2014) and adapted by Aleixandre, Benavent-Gil, and Rosell (2019). Minor modifications included the use of five grams of gel prepared in the Rapid Visco Analyzer (RVA) and 27 U/mL of α-amylase solution. Aliquots were withdrawn along digestion. Specifically, at the end of oral and gastric digestion and during the three hours of intestinal digestion. Aliquots were immediately heated to 100 °C for 5 min to stop enzyme hydrolysis. Hydrolysis was quantified with 3,5-dinitrosalicylic acid (DNS) spectrophotometrically using an SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany) at 540 nm, using maltose as standard. Resistant starch was determined at the end of the digestion.

2.6. Hydrolysis kinetics and expected glycemic index

Hydrolysis kinetics of starch gels were determined following the method described by Benavent-Gil and Rosell (2017) with minor modifications. One gram of gel was suspended into 4 mL of 0.1 M sodium maleate buffer (pH 6.9) with porcine pancreatic α-amylase (0.9 U/mL) and incubated in a shaker incubator SKI 4 (ARGO Lab, Carpi, Italy) at 37 °C under constant stirring at 200 rpm during 3 h. Aliquots (100 μL) were taken during incubation and mixed with 100 μL ethanol (96%) to stop the enzymatic hydrolysis. Then, it was centrifuged for 5 min (10,000 ×*g*, 4 °C). The pellet was suspended in 100 μL of ethanol (50%)

and centrifuged as described before. Supernatants were pooled together and kept at 4 °C. Supernatant (100 μL) was diluted with 885 μL of 0.1 M sodium acetate buffer (pH 4.5) and incubated with 15 μL amyloglucosidase (214.5 U/mL) at 50 °C for 30 min in a shaking incubator, before quantifying glucose content.

The remnant starch after 24 h hydrolysis was solubilized with 2 mL of cold 1.7 M NaOH. The mixture was homogenized with Polytron Ultra-Turrax T18 (IKA-Werke GmbH and Co. KG, Staufen, Germany) for 5 min at 14,000 rpm in an ice bath. The homogenate was diluted with 8 mL 0.6 M sodium acetate pH 3.8 containing calcium chloride (5 mM) and incubated with 100 μL AMG (143 U/mL) at 50 °C for 30 min in a shaking water bath. Afterwards, the glucose content was measured using a glucose oxidase–peroxidase (GOPOD). The absorbance was measured at 510 nm. Starch was calculated as glucose (mg) × 0.9.

The hydrolysis results allowed to calculate the amount of starch fractions. Rapidly digestible starch (RDS) was the starch fraction hydrolyzed within 20 min of incubation, slowly digestible starch (SDS) was the fraction hydrolyzed within 20 and 120 min, total digestible starch (DS) the amount of hydrolyzed starch after 24 h of incubation and resistant starch (RS) was the starch fraction that remained unhydrolyzed after 24 h of incubation (Calle, Benavent-Gil, & Rosell, 2020). The *in vitro* digestion kinetics were calculated fitting experimental data to a first-order equation (Eq. 1) (Goñi, Garcia-Alonso, & Saura-Calixto, 1997):

$$C = C_{\infty}\left(1 - e^{-kt}\right) \quad (1)$$

where C was the percentage of starch hydrolyzed at t time, C_{∞} was the equilibrium concentration or maximum hydrolysis of starch gels, k was the kinetic constant and t was the time chosen. In addition, the time required to reach 50% of C_{∞} (t_{50}) was calculated. The hydrolysis index (HI) was obtained by dividing the area under hydrolysis curve (0–180 min) of the sample by the area of the sample more concentrated (1:4) over the same period. The expected glycemic index (*e*GI) was calculated with the proposed Eq. (2) (Granfeldt, Björck, Drews, & Tovar, 1992).

$$e\mathrm{GI} = 8.198 + 0.862\ \mathrm{HI} \quad (2)$$

2.7. Statistical analyses

Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as mean ± standard deviation, using Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA). Fisher's least significant differences test (LSD) was used to estimate significant differences among experimental mean values. Differences of $P < 0.05$ were considered significant. Furthermore, Pearson correlation analysis was used to identify possible relationships among experimental parameters.

3. Results and discussion

3.1. Formation process of gel

The pasting properties were recorded to identify the impact of starch concentration on the gel performance. Rapid Visco Analyzer (RVA) registered the apparent viscosity during heating and cooling cycle; the logarithmic scale for the apparent viscosity was used for comparison purposes (Fig. 1). The pasting behavior in RVA cycle was different among samples. At high starch content the maximum peak viscosity was reached earlier with higher slope (pasting rate) during heating, indicating faster increase of apparent viscosity. Peak viscosity is considered the equilibrium point between swelling and rupture of starch granules (Balet, Guelpa, Fox, & Manley, 2019). Therefore, at low starch content the granules can swell more freely, without the contact of other swollen granules. In consequence the rupture was delayed and reached at higher temperatures. As a result, the peak temperature decreased from 95 to 84 °C with increasing starch content. Eerlingen, Jacobs, Block, and Delcour (1997) reported similar performance when different concentrations of potato starch were subjected to different hydrothermal treatments. At low concentrations, the starch particles are completely swollen, but the space is rather limited at a higher starch concentration and swollen granules can only fill up the available space referred as close packing concentration. At the lowest concentration, a shoulder was visible before reaching the maximum peak viscosity, likely evidencing differences in swelling rate of starch granules associated to their particle size distribution. It has been reported that the average size of individual corn starch granules ranged within 1–7 μm for small and 15–20 μm for large granules (Singh, Singh, Kaur, Singh Sodhi, & Singh Gill, 2003). Mishra and Rai (2006) observed that corn starch exhibited polyhedral granules with size ranging from 3.6 to 14.3 μm. Differences in the granular size led to diverse surface area that could interact with water, and in consequence modifying the swelling rate. Nevertheless, the viscosity shoulder was only visible in the more diluted system, probably at higher concentration the predominant granules size population masked the swelling of the less abundant one.

Regarding the maximum apparent viscosity, as expected, the most concentrated starch gel (starch:water, 1:4) showed the highest peak of

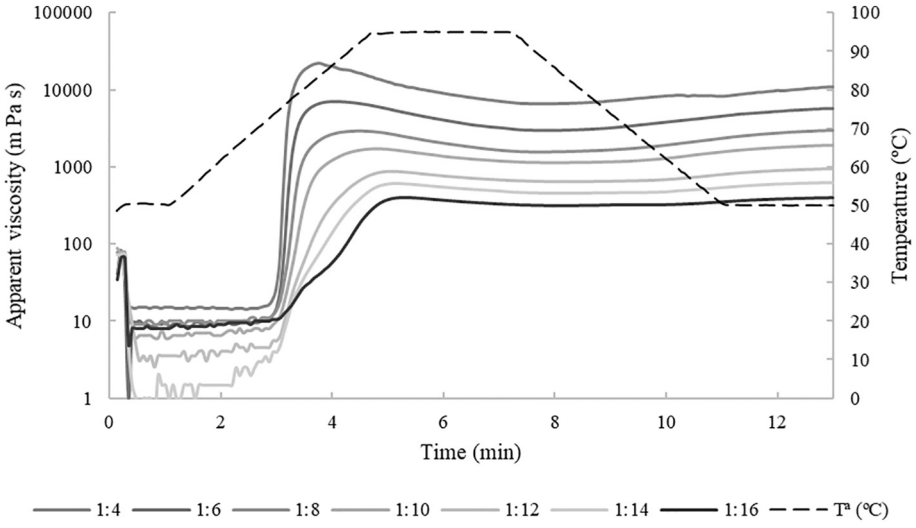

Fig. 1. RVA pasting profiles of corn starch gels prepared with different starch concentrations. Values in the legend are referred to the ratio starch:water (w:w). Discontinuous line shows the temperature applied during the heating-cooling cycle.

apparent viscosity (21,727 mPa s), observing a progressive decrease of that viscosity when increasing the starch dilution up to 1:16. Similar trend was observed in the final viscosity. This result was expected based on the amount of starch added in each slurry, because the apparent viscosity was directly related to the amount of starch.

The viscosity decay observed along holding at 95 °C (breakdown), associated with the disintegration degree of starch granules, exhibited also differences among samples. Major differences were observed within the most concentrated gels up to 1:8, at higher dilution changes in apparent viscosity were less visible, even during cooling. Standard methods for recording apparent viscosity of starches are usually carried out with starch:water slurries of 1:8, obtaining pasting profiles similar to the present study (Calle, Benavent-Gil, & Rosell, 2021; Mishra & Rai, 2006). Nevertheless, no previous study showed the apparent viscosity of gels with different starch concentration and how it impacts on the starch digestibility.

3.2. Characterization of the gels

Considering the potential impact of gels characteristics on their hydrolysis performance, a thorough analysis of the gels was carried out. Viscosity at 37 °C and the content of total starch in tested gels are presented in Table 1. The total starch content decreased as the dilution increased. The wide range of gels concentrations, from 4.5% to 18.6%, could cover the concentration existing in very diverse starch foods, from soups to salad dressings (4–15%). As expected, starch concentration had a significant impact on the gels' viscosity (R-square = 0.97). Sample with the highest content of total starch (18.6%) also showed the highest viscosity (768 mPa s). Conversely, the viscosity of the more diluted gel was 48 mPa s. A significant power law correlation was observed between the starch content and the resulting gels viscosities, which was related to the change on flow resistance when modifying the amount of solid per volume unit (Moreira, Chenlo, Torres, & Glazer, 2012).

The structural impact of starch concentration on the resulting gels was evaluated by analyzing the SEM micrographs (Fig. 2). The gels morphology considerably varied with the starch content. Gel microstructure resembled a network with small cavities. As the starch dilution increased, an enhancement in the size of cavities was observed with samples 1:4 and 1:6 having more closed structures (Fig. 2a and b). The disintegration of granules during heating, as indicated the breakdown observed for those gels in the RVA, might be responsible for that tight structure. The results of the image analysis (Table 1) confirmed significant differences ($P < 0.05$) in the microstructure of the gels, except for porosity. The number of cavities or holes in the gels showed a steady decrease as the starch dilution increased up to 1:8. Further dilutions did not induce significant differences in the number of cavities/mm^2. Simultaneously, the mean area of the cavities progressively increased with the starch dilution in the gels, again until sample 1:8, with no additional changes at higher dilution values. There was a significant positive relationship between number of cavities with viscosity (R-square = 0.87) and total starch (R-square = 0.82). Conversely, negative significant relationships were obtained between the mean area of the cavities with viscosity (R-square = −0.84) and total starch (R-square = −0.84). When the median area of the cavities was used for comparing gels, the same trend was observed, except for the gel with the highest dilution (1:16) that exhibited significantly larger cavities.

Possible relationships among starch content, gels microstructure and their viscosity were analyzed. There was a positive logarithmic relationship (R-square = 0.98) between the thickness of the cavities' walls and the starch content of the gels, and exponential with the gels' viscosity (R-square = 0.94). It was expected that the apparent viscosity of the gels depends mainly on the solid content, but viscosity values (Table 1) suggested that the 3-D network of the gel and its spatial distribution also must be considered. The gel structures shown in Fig. 2 were modelled as follows: pores (with an equivalent radius, r_{eq}) given by the median cavity area (A) and walls whose thickness (e) can be

Table 1
Characterization of corn gels: total starch, viscosity at 37 °C and microstructure parameters.

Sample	Total starch (g/100 g gel)	Viscosity (mPa s)	No. cavities/mm^2	Mean cavity area (μm^2)	Median cavity area (μm^2)	Porosity (%)	Wall thickness (μm)	W_{eq} [a]
1:4	18.6 ± 0.1^{a}	768 ± 23^{a}	226 ± 9^{a}	2591 ± 119^{b}	1027 ± 134^{d}	59.9 ± 3.7^{ab}	9.1 ± 1.1^{a}	24.9 ± 1.8^{a}
1:6	11.2 ± 0.2^{b}	422 ± 27^{b}	175 ± 60^{ab}	3221 ± 1432^{b}	1613 ± 946^{cd}	58.0 ± 4.7^{ab}	7.3 ± 0.2^{b}	14.8 ± 2.1^{b}
1:8	8.7 ± 0.2^{c}	112 ± 30^{c}	100 ± 16^{bc}	6259 ± 685^{a}	3321 ± 130^{bc}	61.6 ± 3.8^{ab}	5.8 ± 0.3^{cd}	6.5 ± 0.7^{c}
1:10	7.2 ± 0.1^{d}	111 ± 15^{c}	88 ± 22^{c}	7709 ± 2155^{a}	5493 ± 2371^{ab}	66.3 ± 2.2^{a}	4.8 ± 0.0^{de}	3.4 ± 1.3^{d}
1:12	5.7 ± 0.1^{e}	74 ± 1^{d}	93 ± 14^{c}	7650 ± 246^{a}	5209 ± 520^{b}	69.8 ± 8.5^{a}	3.5 ± 0.3^{ef}	2.7 ± 0.6^{de}
1:14	5.4 ± 0.1^{e}	62 ± 7^{de}	122 ± 4^{bc}	7050 ± 1750^{a}	4691 ± 117^{b}	65.5 ± 7.7^{ab}	2.7 ± 0.2^{fg}	2.4 ± 0.4^{de}
1:16	4.5 ± 0.0^{f}	48 ± 4^{e}	93 ± 33^{c}	8806 ± 930^{a}	7668 ± 871^{a}	65.8 ± 3.6^{ab}	1.8 ± 0.2^{g}	1.0 ± 0.3^{e}
P-value	0.0001	0.0001	0.0050	0.0012	0.0009	0.2623	0.0001	0.0001

Means within the same column followed by different letters indicate significant differences $P < 0.05$.
[a] W_{eq} was obtained from Eq. (4): $W_{eq} = A_{TP(1:16)}/A_{TP} \cdot e/e_{1:16}$.

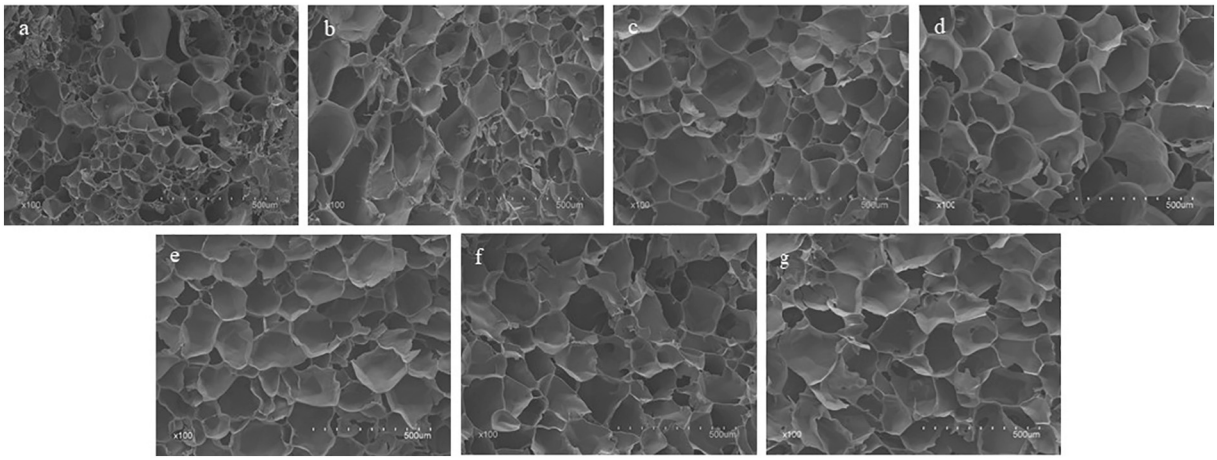

Fig. 2. Scanning electron micrograph of corn starch gels. Magnification 100×. The starch:water ratio is: 1:4 (a); 1:6 (b); 1:8 (c); 1:10 (d); 1:12 (e); 1:14 (f); 1:16 (g).

considered as two semi-thicknesses by the contribution of each neighboring pore covering. The area occupied by starch walls (A_{TP}) in relation to porous area can be evaluated by:

$$\frac{A_{TP}}{A} = \frac{A_e + A_s - A}{A} = \frac{A_e + A_s}{A} - 1 = \frac{(\pi + \sqrt{3} - \pi/2)\,(r_{eq} + e/2)^2}{A} - 1 \tag{3}$$

where A_e is the area of the circle with radius given by the sum of r_{eq} and e; A_s is the area between three tangent circles with area A_e.

Spatial distribution of the starch and the thickness of the wall depended on the starch gel content. As r_{eq} was in all cases longer than e, the highest A_{TP} (Eq. 3) was obtained with the highest cavity area (in this case 1:16). A_{TP} is employed to evaluate the number of cavities equivalent to contain the same amount of starch than in other gels. Nevertheless, these cavities have thicker walls and the number of equivalent walls, W_{eq}, regarded to the reference wall (thinnest wall, $e_{1:16}$) must be evaluated by means of:

$$W_{eq} = \frac{A_{TP(1:16)}}{A_{TP}}\,\frac{e}{e_{1:16}} \tag{4}$$

Eq. (4) allows the determination of the number of the walls with the same thickness (1.8 μm) per unit of starch gel. Introducing the corresponding data collected in Table 1 and by evaluation of Eq. (3), the number of walls increased with increasing starch content from 1 (1:16) up to 24.9 (1:4). A linear relationship (R-square = 0.98) between number of equivalent walls (W_{eq}) and viscosity (μ, mPa s) was found, Eq. (5), achieving a structural model that involves the porous characteristics of starchy gels and a physical property such as viscosity.

$$\mu = 30.46\, W_{eq} - 14.97 \tag{5}$$

3.3. *In vitro* digestion and hydrolysis of gels

The method INFOGEST was used to simulate the digestion of corn starch gels in the oro-gastrointestinal tract (Fig. 3). Experimental results are displayed as g of hydrolyzed starch per 100 g of gel, since the *in vitro* method is directly based on the amount of food ingested, in this case gels. Starch hydrolysis during oral and gastric phase presented very low hydrolysis considering the percentage of starch hydrolyzed. This was already reported by Iqbal, Wu, Kirk, and Chen (2021) because of a short residence time during oral phase and the inhibition of α-amylase at low pH in the gastric phase. In the intestinal phase, there was only an initial increase in the amount of hydrolyzed starch, but no further changes were observed along the intestinal digestion time. The oro-gastrointestinal digestion did not show a trend with the different starch gels, although the most concentrated gel (1:4) exhibited the lowest level of starch hydrolysis (1.5 g of hydrolyzed starch/100 g gel).

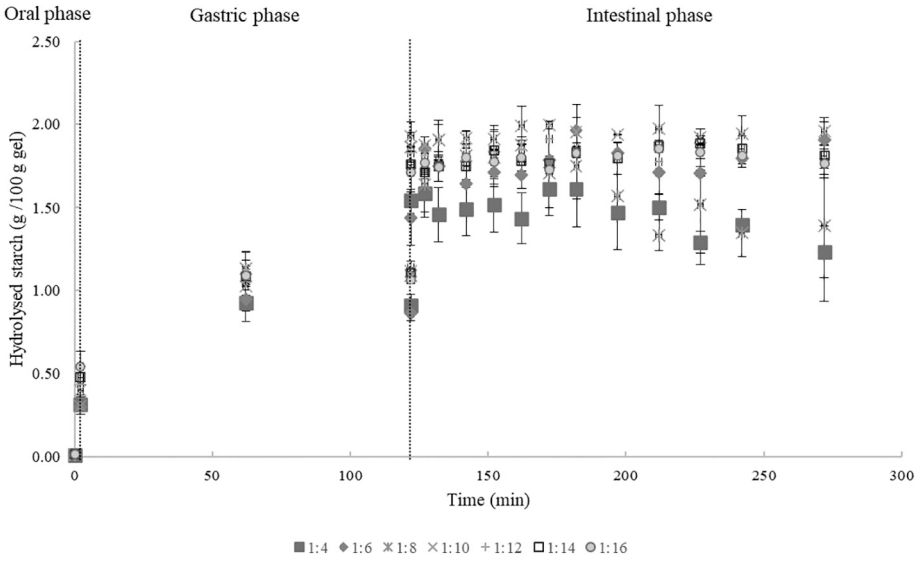

Fig. 3. *In vitro* oro-gastrointestinal digestion of gels prepared with different starch concentration. Legend is indicating the ratio starch:water used to prepare the gels.

Some authors indicated that samples with high starch content underwent slow hydrolysis, which has been related with the viscosity impeding the diffusion of enzymes, and in consequence, the enzymes accessibility and their binding to their substrate (Sanromán, Murado, & Lema, 1996; Wu et al., 2017).

Overall, the application of the oro-gastrointestinal *in vitro* digestion to starch gels did not allow us to identify the possible impact of gels viscosity and microstructure on the enzymatic hydrolysis, since the progressive dilution of the samples in each digestion phase masked differences associated to intrinsic characteristics of the gels. For this reason, the starch hydrolysis was directly carried out with porcine pancreatic α-amylase following methodology previously reported (Benavent-Gil & Rosell, 2017).

According to the rate and extent of *in vitro* digestion of starch, rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) were quantified, obtaining significant differences ($P < 0.05$) among the gels (Table 2). RDS, starch digested in the first 20 min, is the fraction that causes rapid increase in blood glucose after digestion of carbohydrates (Dona, Pages, Gilbert, & Kuchel, 2010). In this study, RDS did not present a linear correlation with the starch concentration. Sample 1:8 showed the highest amount of RDS. According to Dhital, Warren, Butterworth, Ellis, and Gidley (2017), the hydrolytic activity of the amylase could be reduced when the enzyme access to the starch is limited. In the present system, a decrease of the RDS might be expected when increasing gel viscosity, and thus the starch concentration of the gel. Nevertheless, that decrease was only observed at higher starch concentrations until 1:8, which suggests that a viscosity threshold was required in order to affect the enzyme accessibility. Conversely, SDS, related to low postprandial glycemic peak, showed steady decrease with the starch concentration, and the more diluted samples led to lower SDS. Chung, Liu, and Lim (2007) found that the incorporation of hydrocolloids increased the SDS, but without any clear trend on RDS content. Namely, samples with higher content of starch (1:4; 1:6) showed greater differences. Predictably, as the starch content in the gels was reduced, DS and RS decreased. Differences in DS were narrowed from sample 1:8 to 1:16, probably related to their viscosity differences at 37 °C (Table 1). Concerning RS, the amount of this fraction was directly related to the total starch amount of the gels.

For the more concentrated samples greater difference in viscosity was observed and the same trend was seen in the *in vitro* digestion parameters. Again, significant relationships were encountered with viscosity and the hydrolysis fractions SDS (R-square = 0.95) and RS (R-square = 0.96); and also the area of the cavities with SDS (R-square = −0.87) and RS (R-square = −0.84). The fraction of RDS content in relation to the initial starch content of the gel, *RDS*(%), decreased from 79.8% (1:16) up to 18.9% (1:4) with increasing starch content. It is worthy to mention that *RDS%* could be satisfactorily related with the structural parameter, W_{eq}, Eq. (4), by means of:

$$RDS\% = 74.45 - 16.73\ log\left(W_{eq}\right) \quad (6)$$

Table 2
Parameters of *in vitro* corn starch gels digestibility: rapidly digestible starch (RDS), slowly digestible starch (SDS), digestible starch (DS), resistant starch (RS).

Sample	RDS (g/100 g)	SDS (g/100 g)	DS (g/100 g)	RS (g/100 g)
1:4	3.51 ± 0.49^{bcd}	5.68 ± 1.16^{a}	9.99 ± 0.55^{a}	3.63 ± 0.24^{a}
1:6	3.77 ± 0.04^{ab}	3.64 ± 0.04^{b}	7.73 ± 0.17^{b}	2.41 ± 0.17^{b}
1:8	4.05 ± 0.22^{a}	1.95 ± 0.36^{c}	5.58 ± 0.69^{c}	1.59 ± 0.24^{c}
1:10	3.46 ± 0.18^{bcd}	1.57 ± 0.02^{c}	5.24 ± 0.67^{cd}	1.32 ± 0.13^{cd}
1:12	3.07 ± 0.07^{d}	1.43 ± 0.20^{cd}	4.17 ± 0.49^{de}	0.98 ± 0.06^{de}
1:14	3.14 ± 0.08^{cd}	0.86 ± 0.10^{cd}	4.23 ± 0.50^{de}	0.85 ± 0.15^{e}
1:16	3.59 ± 0.06^{abc}	0.27 ± 0.05^{d}	3.96 ± 0.14^{e}	0.70 ± 0.12^{e}
P-value	0.0110	0.0001	0.0001	0.0001

Values within the same column followed by different letters indicate significant differences $P < 0.05$.

This relationship (R-square = 0.95) indicates that the presence of a high number of equivalent walls of starch results in a decrease of the initial amount of starch that is accessible by enzymes.

Starch hydrolysis of gels prepared with different concentration of corn starch is presented in Fig. 4. Results have been plotted as both the amount of hydrolyzed starch per 100 g of gels *vs* time and the amount of hydrolyzed starch per 100 g of starch *vs* time. Those two different graphs for expressing results were chosen to understand the role of starch concentration in the gels. Hydrolysis plots confirmed the different behavior of the gels depending on the starch concentration. Fig. 4A showed the initial starch hydrolysis with minor differences in the rate of hydrolysis but the maximum hydrolysis reached was dependent on the gels dilution. A progressive reduction in the maximum hydrolyzed starch was observed when increasing gels dilution. Samples with higher dilution (1:12; 1:14; 1:16) had a rapid initial hydrolysis but reached a plateau after hydrolyzing low amount of starch (*ca.* 4%) (Fig. 4A). Regarding the starch content of the gels, when hydrolysis was followed recording the amount of hydrolyzed starch per starch amount on the gels (g starch/100 g of starch) (Fig. 4B) the pattern was completely different. There was a slower hydrolysis in the more concentrated gels and faster hydrolysis in the diluted ones, which also reached higher hydrolysis extension (up to 86%), compared to the 53% hydrolysis observed in the gel 1:4. Other studies (Tomoko & Kaoru, 2011), reported the impact of viscosity, provided by the addition of different gums, on the decrease of the starch hydrolysis. Likewise, Ma et al. (2019) reported that the incorporation of pectin increased the viscosity in the gut lumen and showed slower rate of starch hydrolysis. This could be attributed to the formation of a pectin layer around starch granules that limited the access of enzymes. Conversely, in the present study, a homogenous system comprising only starch has been used and results confirm the real impact of viscosity on the starch hydrolysis.

The starch hydrolysis in all the gels showed a very good fitting (R-square = 0.96) to a first order kinetics model. The kinetics parameters derived from hydrolysis of gels including kinetics constant (k), equilibrium concentration of hydrolyzed starch (C_∞), area under the hydrolysis curve after 180 min (AUC 180), hydrolysis index (HI) and estimated glycemic index (*e*GI) are summarized in Table 3. These parameters were significantly ($P < 0.05$) different depending on the gel concentration. The kinetics constant (k) increased with the starch dilution and the time to reach 50% of the hydrolysis (t_{50}) showed a progressive decrease with the dilution. Therefore, more concentrated gels exhibited slower hydrolysis over the digestion time. At constant enzyme concentration and temperature of reaction, an increase of enzymatic reaction rate would be expected when increasing the substrate concentration. However, in the present gels, there is an increase of reaction rate when diluting the starch and therefore, when decreasing the amount of starch in the gels, suggesting that the formation of enzyme-substrate complexes depended on the own structural gel features. High starch content hinders the enzyme diffusion into the gel and macroscopically this resistance associated to the mass transport can be related to gel viscosity (previously related to microstructural gel features with the proposed model). In fact, the hydrolysis kinetics constant depended inversely on the gel viscosity (Fig. 5). Two different trends could be determined, associated with high (>100 mPa s) and low (<100 mPa s) viscosities corresponding to high (>7 g starch/100 g gel) and low (<7 g starch/100 g gel) amount of starch in the gels. At low viscosity range, the kinetics constant value drops linearly (R-square = 0.98) with gel viscosity. This regression allows the empirical prediction of enzymatic kinetics constant value (k_1 = 0.22 min^{-1}) at very low starch amount present in the gel (very low substrate concentration and gel viscosity assumed equal to water viscosity at 37 °C, 0.692 mPa s) (Lide, 2005). This kinetics constant value could be interpreted like the kinetics constant in absence of mass transfer resistances within gel. In fact, the kinetics constant values collected in Table 3 must be considered like a global kinetics coefficient where enzymatic reaction constant value (k_1, min^{-1}) and mass transfer coefficient (k_m, min^{-1}) are involved and the simplified relationship,

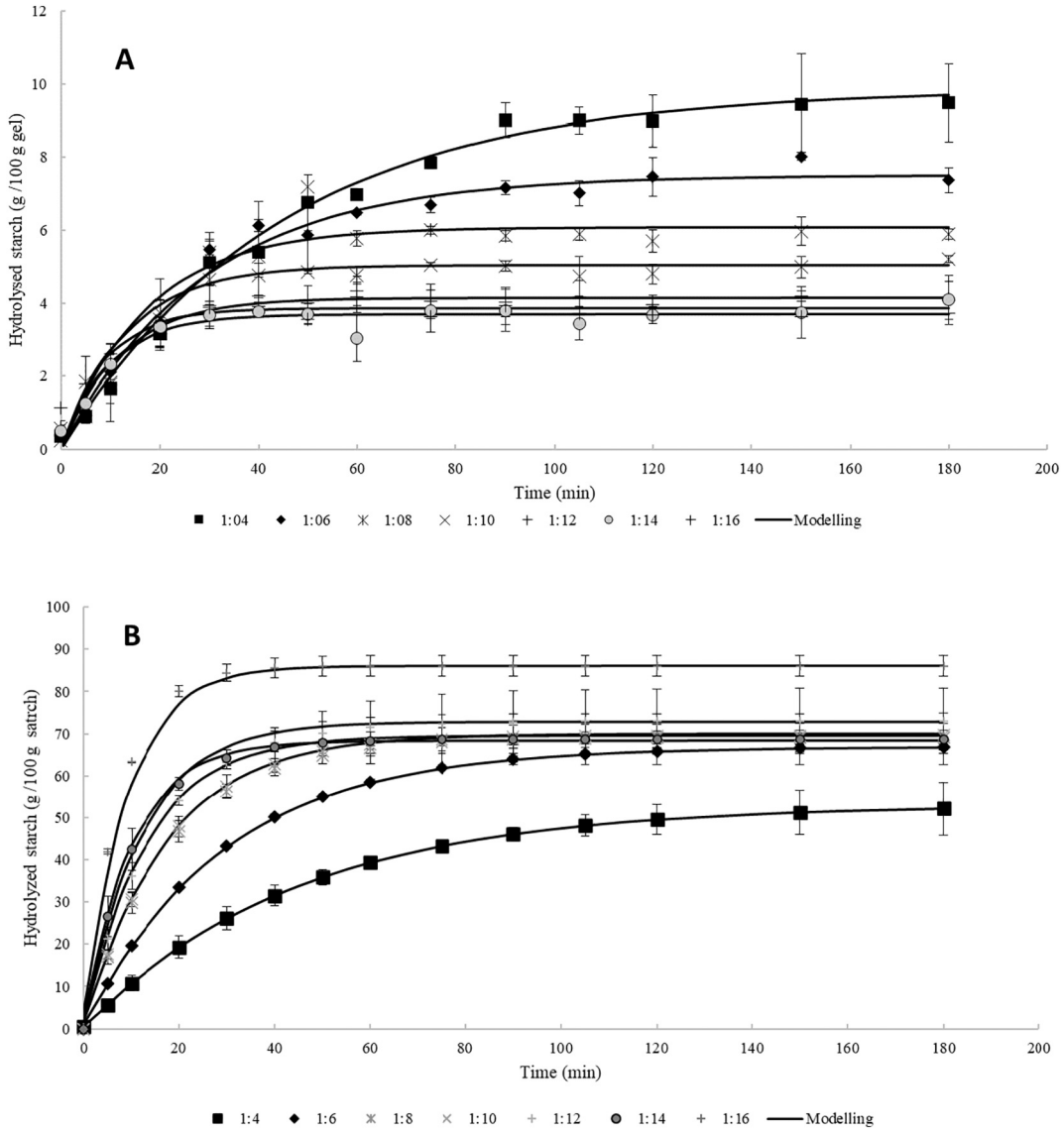

Fig. 4. Enzymatic starch hydrolysis of different corn starch gels prepared with different starch concentration. Legend is indicating the ratio starch:water used to prepare the gels. Hydrolysis plots are expressed as: g/100 g gel (A) and g/100 g starch (B). Solid lines correspond to first-order model with kinetics constant evaluated by Eq. (8).

Table 3
Kinetic parameters resulting from the enzymatic hydrolysis of corn gels with different starch concentrations. Kinetic parameters include: kinetic constant (k), time required to reach 50% of C_∞ (t_{50}); equilibrium concentration (C_∞), area under the hydrolysis curve after 180 min (AUC), hydrolysis index (HI) and estimated glycemic index (eGI) for corn gels with different concentration. Expressed per 100 g of gels (Fig. 4A).

Sample	k (min^{-1})	t_{50} (min)	C_∞ [a]	AUC	HI	*e*GI [b]	k_m [c] (min^{-1})
1:4	0.02 ± 0.01^{e}	35 ± 7^{a}	10.10 ± 1.53^{a}	1335.00 ± 49.50^{a}	100.00 ± 2.99^{a}	94.40 ± 2.58^{b}	0.02 ± 0.01^{e}
1:6	0.03 ± 0.00^{de}	20 ± 0^{b}	7.52 ± 0.08^{b}	1136.00 ± 12.73^{b}	85.09 ± 0.77^{b}	81.55 ± 0.66^{c}	0.04 ± 0.01^{de}
1:8	0.06 ± 0.01^{cd}	10 ± 0^{c}	6.01 ± 014^{c}	971.75 ± 8.27^{c}	72.79 ± 0.50^{c}	70.94 ± 0.43^{d}	0.07 ± 0.02^{cd}
1:10	0.06 ± 0.00^{cd}	10 ± 0^{c}	5.03 ± 0.20^{cd}	818.05 ± 34.29^{d}	61.28 ± 2.07^{d}	61.02 ± 1.79^{e}	0.08 ± 0.02^{cd}
1:12	0.07 ± 0.02^{bc}	10 ± 0^{c}	4.14 ± 0.44^{d}	683.65 ± 52.68^{e}	51.21 ± 3.18^{e}	52.34 ± 2.74^{f}	0.10 ± 0.02^{c}
1:14	0.10 ± 0.03^{ab}	8 ± 4^{c}	3.72 ± 0.33^{d}	628.00 ± 42.00^{e}	47.04 ± 2.54^{e}	48.75 ± 2.19^{f}	0.18 ± 0.03^{b}
1:16	0.13 ± 0.01^{a}	5 ± 0^{c}	3.86 ± 0.11^{d}	663.45 ± 17.04^{e}	49.70 ± 1.03^{e}	51.04 ± 0.89^{f}	0.34 ± 0.04^{a}
P-value	0.0004	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Values followed by different letters within a column denote significant differences ($P < 0.05$).
[a] C_∞ and k were determined by the equation, $C = C_\infty (1 - e^{-kt})$.
[b] *e*GI was quantified following the equation proposed by Granfeldt, Björck, Drews, and Tovar (1992).
[c] Obtained from Eq. (7): $1/k = 1/k_1 + 1/k_m$.

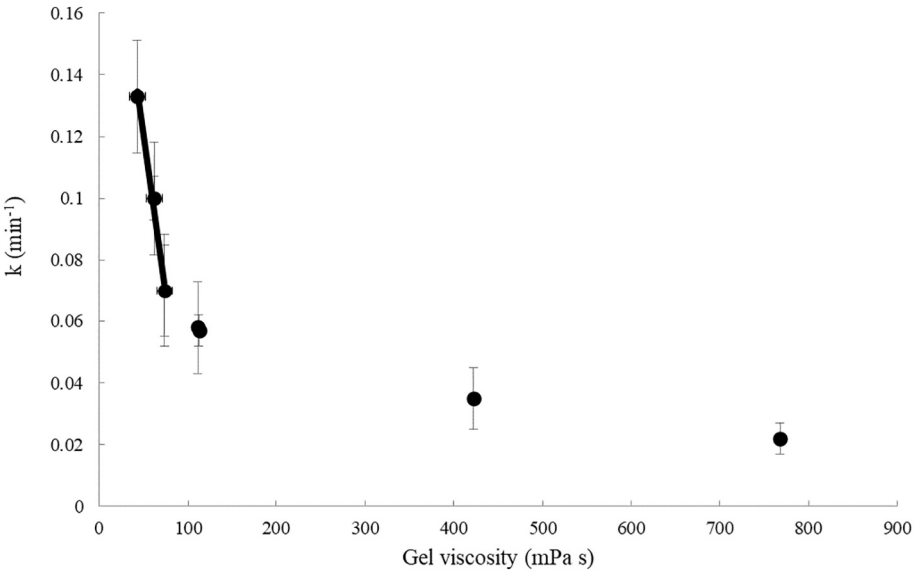

Fig. 5. Relationship of the kinetics constant of first order model with gel viscosity.

after several assumptions for a model of resistances in series, is given by the Eq. (7) (Levenspiel, 1998):

$$\frac{1}{k} = \frac{1}{k_1} + \frac{1}{k_m} \tag{7}$$

Eq. (7) allows the estimation of k_m of enzyme into the gels with different starch content and the corresponding values are shown in Table 3. The mass transfer coefficients value strictly depends on the characteristics of compound diffusing, turbulence conditions on the surface and properties of the fluid. In our case, in a simplified way, it was found a power relationship between k_m and viscosity (R-square = 0.996) and Eq. (7) can be written after substitution:

$$\frac{1}{k} = \frac{1}{0.22} + 0.196\,\eta^{0.8} \tag{8}$$

A very high correlation (R-square > 0.94) was obtained between experimental kinetics constant data and estimated values employing Eq. (8). The goodness of the first order model with the kinetics constant evaluated by Eq. (8) can be observed in the Fig. 4A and B. These results confirmed that the viscosity of starch gels must be considered to evaluate the hydrolysis rates. Previous hydrolysis studies dealing with changes in viscosity have been carried out with diverse hydrocolloids, and the slowdown of the enzymatic activity has been explained based on the hydrocolloid coating of the starch surface that block the enzyme accessibility to the substrate (Chung, Liu, & Lim, 2007; Gularte & Rosell, 2011). However, the present research confirmed the role of the apparent viscosity of the gels on the enzymatic hydrolysis.

In addition, the maximum hydrolysis (C_∞) reached with the different gels (Fig. 4A, Table 3) showed a significant decrease when increasing gels dilution. A similar trend was observed for the total area under the hydrolysis curve (AUC), which is related to the glucose released over a hydrolysis period of 180 min (Goñi, Garcia-Alonso, & Saura-Calixto, 1997). To estimate the glycemic index (*e*GI), the hydrolysis index (HI) of each gel was calculated taking the sample 1:4 as a reference (HI = 100%). The *e*GI showed a steady decrease until 51% in the most diluted sample. Glycemic index is used to describe how the food starch is hydrolyzed in the digestive system and absorbed into the bloodstream (Dona, Pages, Gilbert, & Kuchel, 2010). Some authors reported that the high viscosity induced by hydrocolloids might form a physical barrier for the α-amylase access, which would explain the decrease in glucose released and its absorption in the intestine (Dartois, Singh, Kaur, & Singh, 2010; Gularte & Rosell, 2011). Here, the same behavior was observed regarding the reduction in the hydrolysis rate, but now it is related to the increase of viscosity by the increase of starch content in the gels.

4. Conclusions

This study investigated for the first time the role of the viscosity of starch gels on the digestion of starch. Corn starch gels of varying starch concentration resulted in a range of different viscosities and microstructures. A structural model is proposed that connects by a linear relationship (R-square = 0.98) the porous structure (cavity sizes and thickness walls) of starch gels and their viscosity. The viscosity showed a linear relationship with the number of starch walls per area and its thickness (equivalent walls). The kinetics constant values of the starch hydrolysis decreased when increasing gel viscosity. Hydrolysis constants, considering mass transfer resistance within the gel, were successfully correlated with gel viscosity by means of a simple model, confirming the initial formulated hypothesis. Overall, the proposed simplified model links macrostructural properties (viscosity) and microstructural features (median cavity area and wall thickness) to analyze hydrolysis kinetics. It could also be extended to other physical and chemical processes where starch gels are involved and validated with other gels formed with starches from other sources. From the technological point of view, these findings could be applied in the design of food formulations aiming at postprandial glucose management.

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CRediT authorship contribution statement

Maria Santamaria: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Raquel Garzon:** Methodology, Supervision, Data curation. **Ramón Moreira:** Formal analysis, Writing – review & editing, Funding acquisition. **Cristina M. Rosell:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

None.

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Unraveling the impact of viscosity and starch type on the *in vitro* starch digestibility of different gels

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Starch is one of the most important carbohydrates that is present in many foods. Gelatinization is an important property of starch, associated with physical changes that promote an increase in viscosity. The objective of this research was to understand how the viscosity of starch gels affects their hydrolysis and whether that effect was dependent on the type of starch. Different gels (corn, wheat, and rice) with variable or constant viscosity were analyzed using diverse methodologies to determine the changes in the pasting behavior. A rapid force analyzer, a vibration viscometer and a rheometer were used to differentiate the gels based on the starch source and concentration. At a fixed starch concentration, corn gel displayed the highest viscosity, slowing the enzymatic starch hydrolysis. The higher viscosity of those gels prepared with a fixed starch concentration significantly enhanced the slowly digestible starch (SDS) and reduced the kinetic constant (k). Nevertheless, gels with constant viscosity (550 mPa s) showed comparable hydrolysis kinetics, obtaining similar SDS, total hydrolyzed starch and k. The correlation matrix confirmed the relationship between k and gel viscosity (r = −0.82), gelatinization rate (α-slope) (r = −0.87), breakdown (r = −0.84) and elastic modulus (G' 37 °C) (r = −0.73). Therefore, these parameters could be used as predictors of the hydrolysis performance of starch gels as well as in reverse engineering for the design of healthy foods.

Introduction

Starch is a polysaccharide extensively used as a functional ingredient in many foods due to its applications as a thickener, stabilizer, gelling agent, and water retention agent.[1] Because of that, besides intrinsic properties like amylose content, granule size, length of amylopectin branches and crystallinity, the pasting properties or viscosity performance (peak viscosity, final viscosity, breakdown and setback viscosity) of the slurries during heating and cooling are always reported as key properties for starch characterization.[2]

Consumers' health concerns have prompted the evaluation of food-related properties that could contribute to human well-being and prevent diseases. In that scenario, starch hydrolysis plays a fundamental role pertaining to postprandial glucose levels and in consequence the glycemic index of the foods.[3] Starch digestion by the action of enzymes in the small intestine and the subsequent rate of absorption of the released glucose have been used to categorize starch into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS).[4] These facts have pointed out the importance of starch hydrolysis kinetics. Thus, besides the intrinsic features of starch previously mentioned, the digestive performance of different starches is usually included in the studies of starch characterization.[5] Different strategies have been developed to modulate carbohydrate digestion, which include reducing the amount of available carbohydrates, reducing the rate of digestion or reducing the glucose absorption rate.[6] In response to that, starches with low digestibility have been developed, like those rich in resistant starch either present in the native starch or obtained after chemical modification or processing.[7]

Nevertheless, the digestion of starch is not only affected by its features but also by the physical properties of the media which can modulate the rate of enzyme diffusion to starch substrates.[7] Literature studies have confirmed the role of bulk viscosity in gastric emptying and the reduction of glycemic index, thus opening the opportunity to modulate digestion with compounds that affect viscosity. This has been explored with diverse starches and hydrocolloids, which might restrict enzyme accessibility to starch by interacting with the surface of starch granules or creating a hydrated network surrounding that encapsulates the granule, or increasing the bulk viscosity.[8,9] In fact, results with different polysaccharides (guar gum and chitosan) indicated a negative correlation between the peak viscosity (11 814–14 535 mPa s) and the SDS fraction

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of potato starches, suggesting that the effect might be more related to physical properties than chemical interactions.[10] Nevertheless, very limited studies have correlated the viscosity of starch gels with the digestion parameters. For instance, a higher peak viscosity (480–5076 mPa s) and viscosity breakdown, defined as the difference between the peak viscosity and the lowest viscosity of potato starches during the holding stage at 95 °C (24–3540 mPa s), were correlated with lower hydrolysis rates of native starches but that correlation was not observed with gelatinized starches.[11] Bajaj *et al.* (2018)[2] reported a reverse relationship between gel hardness and gelatinization temperatures with the RS amount, but no relationship with the peak viscosity in the range of 2183 to 8387 mPa s. Velásquez-Barreto *et al.* (2021)[12] have recently reported the positive relationship of SDS, obtained in *in vitro* digestibility studies, with the Rapid Visco Analyzer (RVA) peak viscosity of gels (290–370 mPa s) and the viscosity upon cooling the starch gels isolated from unconventional Peruvian tubers up to 60 ○C (92–180 mPa s). Furthermore, other researchers used rheometric techniques to relate starch rheological behavior with its hydrolysis.[13] Yield stress (σ_0) or the minimum force required to initiate the flow of starch paste was positively correlated with the peak viscosity (4647–8303 mPa s) in pearl millet starches and negatively correlated with the RS amount.[13] Overall, although previous research has characterized the rheological properties of different starch gels and their hydrolysis, the results do not allow the identification of the potential role of viscosity in explaining the encountered divergences.

Recently, the authors studied the impact of the viscosity of corn starch gels, obtained by varying the starch concentration, on *in vitro* hydrolysis and observed that the hydrolysis kinetics constant is inversely dependent on gel viscosity due to enzyme diffusion limitation.[14] Specifically, a positive significant relationship was defined between gel viscosity and the starch fraction SDS (R^2 = 0.95) and RS (R^2 = 0.96). In the case of RDS, the results suggested that a viscosity threshold is required to affect enzyme accessibility. Nevertheless, that impact of viscosity was only tested with corn starch gels, and thus what happens with other cereal starches remains to be investigated.

The possible correlation between starch gel characteristics and starch digestion might contribute to reverse engineering in the design of starch-based systems. In this way, foods could be designed based on the knowledge of the targeted final food characteristics. For this reason, the present study aims to validate the relationship of gel characteristics with the *in vitro* hydrolysis of starch gels obtained from different cereals. Starch gels from corn, wheat, and rice with variable viscosity (VV) or constant viscosity (CV) were rheologically characterized and their properties were correlated with the *in vitro* hydrolysis parameters.

Materials and methods

Materials

Commercial food grade starches, having similar amylose content, from corn (20.15% amylose content and 12.43% moisture content) and wheat (23.98% amylose content and 12.72% moisture content) were supplied by EPSA (Valencia, Spain) and rice starch (20.71% amylose content and 10.30% moisture content) was purchased from Sigma Aldrich (Sigma Chemical, St Louis, USA). The enzymes used were type VI-B α-amylase from porcine pancreas (EC 3.2.1.1) from Sigma Aldrich (Sigma Chemical, St Louis, USA) and amyloglucosidase (EC 3.2.1.3) from Novozymes (Bagsvaerd, Denmark). A D-Glucose Assay Kit (GOPOD) was provided by Megazyme (Megazyme International Ireland Ltd., Bray, Ireland). Other chemicals were of analytical grade.

Preparation of starch gels with constant amounts of starch (variable viscosity) or constant viscosity

Two sets of gels were prepared: the first one using a fixed amount of starch; those gels were referred to as variable viscosity (VV), and the second one by varying the amount of starch to obtain constant viscosity (CV). For gels under VV notation, 5 g of starch (based on 14% moisture content) was suspended in 20 g of water. Starches (corn, wheat, and rice) were manually dispersed in deionized water and the slurries were heated in a boiling water bath for 20 minutes and manual stirring was applied every five minutes. The resulting gels were cooled down to 37 ○C for further analysis.

The viscosity of the rice gel, prepared as previously described, was measured at 37 ○C using a vibration viscometer VL7-100B-d15 (Hydramotion Ltd, Malton, United Kingdom). Although the viscosity is measured at high shears, when reaching the Newtonian plateau, the complexity associated with shear-thinning materials is removed. Preliminary assays were conducted with corn and wheat starches to identify the amount of starch required to obtain a viscosity similar to the one obtained with the rice gel. Afterwards, the second set of gels was prepared with starch: water, setting up the ratio for rice, corn, and wheat at 1 : 4, 1 : 5.5 and 1 : 5.2, respectively, to obtain gels with similar viscosities, referred to as constant viscosity (CV).

The amount of total starch (TS) in the gels was quantified using a commercial assay kit (K.TSTA) (Megazyme International Ireland Ltd., Bray, Ireland) following the determination of the total starch content of the samples containing resistant starch (RTS-NaOH procedure is recommended).

Rapid force analyzer

The force changes during starch gelatinization were studied using a rapid force analyzer (RFA, Amylab® Chopin Technologies, Villeneuve-la-Garenne, Cedex, France), as previously described by Garzon and Rosell *et al.* (2021).[15] Briefly, the starch slurry was placed into the precision test tubes of the device and manually shaken 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. The rapid test consisted of heating the sample at 100 ○C for 90 s and subjecting it to continuous shearing. The plots recorded the force, expressed in Newtons, of the slurry/gel under continuous heating/shearing. The parameters defined include the onset

time indicating the start of gelatinization, the initial (F0) and maximum force (F1), the α-slope among F0 and F1, the final force at 90 s (F2) and the force difference between F1 and F2 related to starch breakdown.

Gels viscoelastic behavior

The viscoelastic characterization was made using a stress-controlled rheometer (MCR 301; Anton Paar, Graz, Austria) using a starch pasting cell (ST24-2D/2V/2V-30, gap 2.460 mm, bob radius 12 mm) with a solvent trap kit to minimize water evaporation during the tests. Different starches (corn, wheat, and rice) were dispersed in water (total weight 20 g) with constant and variable gel viscosity and poured into the rheometer cuvette at 95 °C. First, a pre-shear of 100 s^{-1} was made for 1 min to homogenize the sample at 95 °C. Secondly, a time sweep was carried out at 30 Pa, 1 Hz and 95 °C for 19 min (previous assays were performed to ensure that frequency sweeps were carried out inside the linear viscoelastic region of tested gels). Then, a cooling profile was made from 95 °C to 37 °C at 3 °C min^{-1} with a constant stress of 30 Pa and a constant frequency of 1 Hz. The frequency sweep was carried out from 0.1 to 10 Hz at 1% strain and 37 °C. Afterwards, a time sweep was carried out at 30 Pa, 1 Hz and at 37 °C for 30 min to observe the maturation of the gel. A second frequency sweep was made under the same conditions as the first one.

In vitro digestibility

The digestibility of the starch gels was determined following the method described by Santamaria *et al.* (2021),[14] with a few modifications. A fresh gel (200 mg) was mixed with 4 mL of 0.1 M sodium maleate buffer (pH 6.9) containing porcine pancreatic α-amylase (0.9 U mL^{-1}) by using an Ultra Turrax T18 basic homogenizer (IKA-Werke GmbH and Co. KG, Staufen, Germany). The slurry was incubated in a shaker incubator (SKI 4; ARGO Lab, Carpi, Italy) at 37 °C for 3 h under constant stirring (200 rpm). Aliquots were taken to quantify glucose release. The remnant starch after the 24 h hydrolysis was solubilized with 2 mL of 1.7 M NaOH using an Ultra-Turrax T18 homogenizer (IKA-Werke GmbH and Co. KG, Staufen, Germany) for 5 min at 14 000 rpm in an ice bath and hydrolyzed with amyloglucosidase (143 U mL^{-1}) at 50 °C for 30 min in a shaking water bath for its complete hydrolysis. Glucose determination was performed using a glucose oxidase-peroxidase (GOPOD) kit. The absorbance was measured using a SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany) at 510 nm. Starch was calculated as glucose (mg) × 0.9.

From the hydrolysis results, rapidly digestible starch (RDS) or the percentage of total starch hydrolyzed within 20 min of incubation, slowly digestible starch (SDS) or the starch fraction hydrolyzed within 20 and 120 min, digestible starch or total starch hydrolyzed after 24 h (DS), and resistant starch (RS) that remained after 24 h of incubation were calculated.

The *in vitro* hydrolysis data were fit to a first-order equation (eqn (1)) to describe the kinetic parameters of starch hydrolysis as reported by Goñi *et al.* (1997).[16]

$$C = C_\infty\ (1 - e^{-kt}) \quad (1)$$

where C was the concentration at time t, C_∞ was the equilibrium concentration or maximum hydrolysis extent, k was the kinetic constant and t was the time chosen. Moreover, the area under the hydrolysis curve in 180 min (AUC) was calculated and the hydrolysis percentage was the relation between C_∞ and the total starch content of each gel. All hydrolysis parameters were calculated in relation to 100 g of gel.

Statistical analysis

All experiments were carried out in triplicate and the experimental data were statistically analyzed by the Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA). Data were subjected to multivariate analysis of variance (MANOVA) and the values were expressed as a mean ± standard deviation. Fisher's least significant differences test (LSD) was used to estimate the significant differences among experimental mean values with a significance level of $p \leq 0.05$. Furthermore, Pearson correlation analysis was used to identify the possible relationship between the rheological and hydrolysis parameters.

Results and discussion

Two different types of gels were prepared using corn, wheat or rice starches to identify the role of viscosity in the pasting properties, viscoelastic properties, and digestibility performance. The first set of gels was prepared with the same amount of starch and thus variable viscosity (VV). The initial amount of starch selected for those gels was based on a previous study,[14] where the concentration (1 : 4 starch : water) for corn starch gels was the most limiting one regarding the relationship among the closed gel structure, the higher viscosity, and the slowest and more limited starch hydrolysis. In contrast, the second set was prepared with varying amounts of starch for obtaining gels with the same viscosity (CV). The amount of total starch in samples with variable gel viscosity was 17.20 ± 0.20 g per 100 g. On the other hand, the constant viscosity was 12.63 ± 0.08 g per 100 g, 12.60 ± 0.18 g per 100 g and 16.93 ± 0.15 g per 100 g of starch for corn, wheat, and rice gels, respectively.

The viscosity of the gels prepared at VV was significantly ($p < 0.05$) influenced by the starch source (Table 1). The corn gel presented the highest viscosity (1170 mPa s) at 37 °C, followed by the wheat gel (834 mPa s), and finally the rice gel (525 mPa s). The viscosity of the rice starch was selected as the target to obtain CV gels.

Starch performance during gelatinization and the viscoelastic properties of gels

After setting up the conditions to obtain the two types of gels, their textural performance during gelatinization was recorded using a rapid force analyzer (RFA).[15] It uses a rapid (90 s) thermal method under continuous shearing. The force required to stir the slurries during gelatinization was different for each starch gel (Fig. 1). A very low force was detected at the

Table 1 Rheological parameters of starch gels prepared at constant amount of starch giving variable gel viscosity (VV) or different amount of starch required to reach constant gel viscosity (CV). Gel development was recorded with a Rapid Force Analyzer and rheometric behaviour in the stages of cooling and mechanical spectra were evaluated with a rheometer. Gel made with rice starch was selected for defining the target viscosity at 37 °C, because of that the same gel was used for VV and CV

		Variable gel viscosity (VV)			Constant gel viscosity (CV)		*p*-Value	
		Corn VV 1 : 4	Wheat VV 1 : 4	Rice VV, Rice CV 1 : 4	Corn CV 1 : 5.5	Wheat CV 1 : 5.2	Source	Viscosity
η adjustment	Vibration viscosimeter							
	η (mPa s)	1170 ± 293^{a}	834 ± 81^{b}	525 ± 15^{c}	542 ± 88^{c}	553 ± 55^{c}	**0.0297**	**0.0044**
Gel development	RFA parameters							
	Onset (s)	36 ± 1^{a}	28 ± 0^{b}	34 ± 2^{a}	34 ± 1^{a}	28 ± 3^{b}	**0.0005**	0.7310
	F_0 (N)	2.10 ± 0.28	1.98 ± 0.49	1.90 ± 0.76	1.72 ± 0.12	1.51 ± 0.62	0.8749	0.3515
	α-Slope	1.23 ± 0.00^{a}	0.99 ± 0.01^{b}	0.57 ± 0.02^{c}	0.52 ± 0.04^{c}	0.39 ± 0.02^{d}	0.1314	**0.0043**
	F1 (N)	11.39 ± 0.30^{b}	15.29 ± 0.55^{a}	9.93 ± 0.86^{b}	6.11 ± 0.26^{d}	8.08 ± 0.68^{c}	0.1626	**0.0060**
	F2 (N)	6.74 ± 0.25^{c}	11.99 ± 1.14^{a}	8.78 ± 1.03^{b}	4.54 ± 0.02^{d}	7.92 ± 0.62^{bc}	**0.0030**	**0.0189**
	Breakdown (N)	4.65 ± 0.05^{a}	3.19 ± 0.44^{b}	1.16 ± 0.17^{c}	1.57 ± 0.28^{c}	0.15 ± 0.06^{d}	**0.0394**	**0.0046**
Gel behavior	Rheometric parameters							
	Cooling profile (initial and end values, at 1 Hz)							
	G' 95 ○C (Pa)	301 ± 2^{c}	575 ± 7^{a}	340 ± 8^{b}	171 ± 6^{d}	293 ± 16^{c}	**0.0134**	**0.0102**
	G'' 95 ○C (Pa)	108 ± 39^{b}	233 ± 42^{a}	81 ± 21^{b}	73 ± 19^{b}	79 ± 0^{b}	0.1073	**0.0488**
	tan δ 95 ○C	0.359 ± 0.125^{ab}	0.405 ± 0.069^{ab}	0.237 ± 0.057^{b}	0.428 ± 0.095^{a}	0.269 ± 0.016^{ab}	0.0824	0.6637
	G' 37 ○C (Pa)	3025 ± 49^{b}	3580 ± 141^{a}	872 ± 4^{e}	1380 ± 85^{d}	1580 ± 99^{c}	**0.0049**	**0.0045**
	G'' 37 ○C (Pa)	155 ± 31^{b}	344 ± 4^{a}	99 ± 12^{c}	92 ± 9^{c}	173 ± 5^{b}	**0.0022**	**0.0175**
	tan δ 37 ○C	0.051 ± 0.011^{b}	0.096 ± 0.003^{a}	0.113 ± 0.013^{a}	0.067 ± 0.011^{b}	0.109 ± 0.004^{a}	**0.0001**	0.1211
	Mechanical spectra							
	Slope linear G' (0.1–10 Hz)	0.020 ± 0.001	0.022 ± 0.002	0.026 ± 0.008	0.019 ± 0.003	0.023 ± 0.002	0.6419	0.1769
	Slope linear G'' (0.1–10 Hz)	0.213 ± 0.035	0.195 ± 0.074	0.235 ± 0.042	0.247 ± 0.019	0.246 ± 0.002	0.1919	0.9474
	G' (0.1 Hz)	4620 ± 71^{b}	5775 ± 7^{a}	1075 ± 35^{e}	2675 ± 148^{d}	3955 ± 92^{c}	**0.0000**	**0.0042**
	G'' (0.1 Hz)	154 ± 61^{ab}	255 ± 87^{a}	97 ± 24^{b}	68 ± 6^{b}	109 ± 14^{b}	0.1148	**0.0387**
	tan δ (0.1 Hz)	0.033 ± 0.013^{b}	0.044 ± 0.015^{b}	0.090 ± 0.020^{a}	0.025 ± 0.001^{b}	0.028 ± 0.004^{b}	**0.0003**	0.3128

Values followed by different letters within the same row denote significant differences $p < 0.05$. Parameters: η (viscosity), onset (starch gelatinization initial time), F0 (initial force), α-slope (between F0 and F1), F1 (maximum force), F2 (final force), breakdown (difference between F1 and F2), G' (storage modulus) G'' (loss modulus), and tan δ (damping factor).

beginning of the test, which was high enough till heating to promote the onset of starch swelling with a simultaneous increase in the stirring force. The pasting performance of the gels was dependent on the source of starch and, obviously, on the amount of starch. However, the observed changes in the plots revealed not only the starch dilution but also the changes in the force pattern of the gels. The parameters defined to analyze the gel performance in the RFA are shown in Table 1. Upon adapting viscosity (CV), to have constant gel viscosity, differences within the RFA plots were reduced, particularly during gelatinization. Regarding specific parameters, the starch source significantly ($p < 0.05$) affected the onset of gelatinization, force at 90 s (F2) and breakdown, whereas the gel viscosity (CV or VV gels) factor affected significantly ($p < 0.05$) the α-slope, maximum (F1) and final force (F2), and breakdown. Wheat gels showed the lowest onset indicating that gelatinization began at lower temperatures.[15] Among the VV gels made with the same amount of starch, the corn gel showed a higher α-slope, indicating faster gelatinization, and the wheat gel displayed the highest maximum force (F1). Garzon and Rosell *et al.* (2021)[15] observed the same trend and correlated higher force with more porous gels, revealing thicker walls and big holes. The corn gel presented a higher breakdown, indicating lower resistance to physical rupture during starch granule swelling. A similar result was reported using the RVA when comparing corn and rice starches and it was related to the higher swelling of granules.[17] When adapting gels to obtain CV, corn and wheat gels showed lower forces with respect to rice gel along gelatinization. The starches showed significant differences with regard to F1 but the onset, α-slope and breakdown of the rice and corn starches were similar, confirming the proximity of the physical behavior of the starch gels when adapting viscosity.

All starch gels, after fully developing a stable network structure, showed a solid like behavior ($G' > G''$) (Table 1). During the cooling profile from 95 to 37 ○C, both moduli increased, but greater differences were observed on G' than G''. In VV gels, $\Delta G'$ and $\Delta G''$ were higher for corn and wheat starches than for rice starch. At 37 ○C, the rice starch led to the weakest gel with the lowest elastic modulus (872 Pa), Table 1. Meanwhile, the strongest gel (high G' value) was obtained with wheat starch (in respective sets of CV and VV gels). This prop-

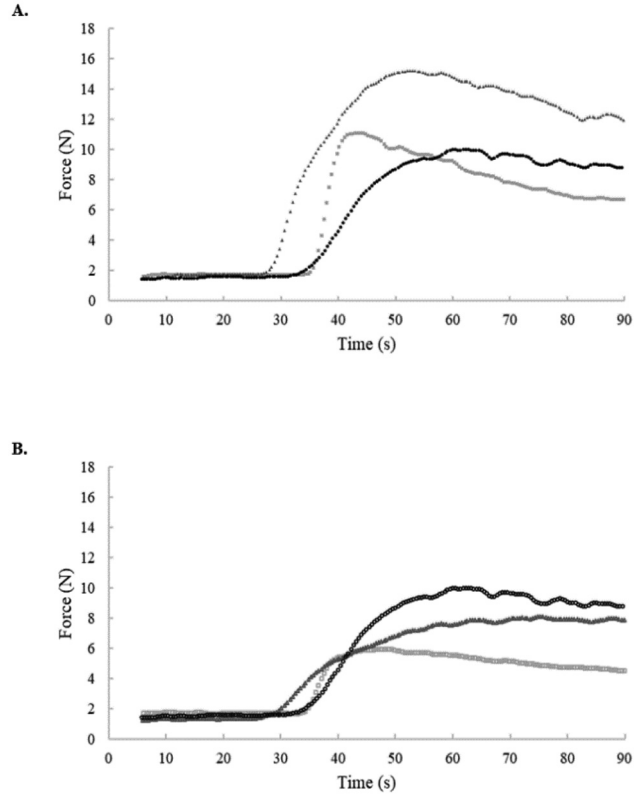

Fig. 1 Plots of gel force during gelatinization of different starches using a rapid force analyser. (A) Gels were prepared with a constant amount of starch giving variable viscosity (VV, closed symbols) or (B) different amounts of starch required to reach the constant viscosity (CV, open symbols). Corn: ■, wheat: ▲, and rice: ●.

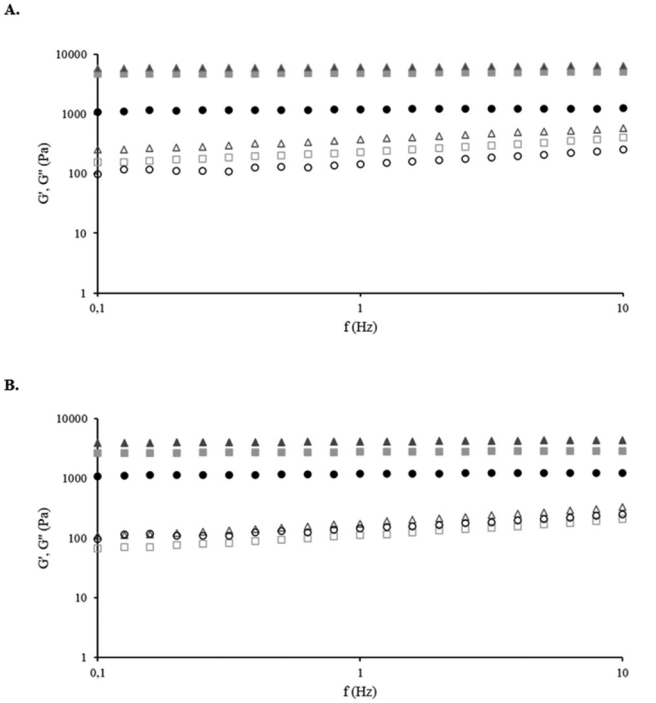

Fig. 2 Mechanical spectra of starch gels prepared at (A) constant amount of starch giving variable viscosity (VV) or (B) different amounts of starch required to reach constant viscosity (CV). Symbols: storage modulus-closed (G'); loss modulus-open (G''). Corn: ■, wheat: ▲, and rice: ●.

erty is relevant to measuring the easiness of the gel to be fragmented into small pieces under shear rates. The rheological tests confirmed that CV gels had closer values of viscous modulus. At 37 ○C, the gels were subjected to two frequency sweeps (time 0 and 30 min) and the viscoelastic behavior with angular frequency was almost constant, meaning that gel maturation took place mainly during cooling and when the gel achieved the lowest temperature, the maturation was practically complete (data not shown). Strong and weak gels can be classified as such based on their mechanical spectra. In all the cases, $G' > G''$ from 0.1 to 10 s^{-1}, with G' being relatively independent of frequency (slope <0.03) and G'' increasing with increasing frequency (Fig. 2). In fact, the slope of G'' with frequency varied in a narrow range (from 0.20 up to 0.25) and no significant difference ($p > 0.05$) was found between the tested starch gels, Table 1. This type of spectrum is usually associated with a weak gel.[18] Upon small deformations, weak gels resemble strong gels, but as the deformations increase, the three-dimensional networks undergo a progressive (and reversible) breakdown.[19] The $\tan\delta$ (G''/G') values at 0.1 Hz for VV gels were 0.033, 0.044 and 0.090 for corn, wheat, and rice gels, respectively, indicating that the viscous character is low, but more relevant in rice gels. No significant difference ($p > 0.05$) between the $\tan\delta$ values of CV gels and VV gels from the same starch was observed. Therefore, some differences in the viscoelastic behavior of the tested starch gels were found in relation to the formation of firmer (higher G') or more stable (low damping factor) structures.

In vitro hydrolysis of starch gels

Starch gels were subjected to enzymatic hydrolysis with digestive enzymes (Fig. 3). Intrinsic properties like amylose size and chain size distribution of amylopectin have been related to the *in vitro* digestion of native starches, but in the gel state that molecular order and their contribution might no longer be crucial and be more related to the new molecular organization in which the initial amorphous structure is more susceptible to enzyme hydrolysis.[20] Therefore, if only structural features were responsible for the starch hydrolysis kinetics, no differences would be detected due to viscosity changes.

To assess the impact of the amount of starch, the results are expressed in grams of hydrolyzed starch per 100 g of gel (Fig. 3A) and grams of hydrolyzed starch per 100 g of starch (Fig. 3B). Regarding VV gel hydrolysis, the rice gel showed faster and higher hydrolysis (Fig. 3A VV), which could be related to its lower viscosity at 37 ○C (Table 1), compared to the wheat and corn gels. In highly viscous systems, like wheat and corn gels, enzyme diffusion encounters the external resistance (viscosity) of the gels that affects the hydrolysis. A similar behavior has been observed when modulating the vis-

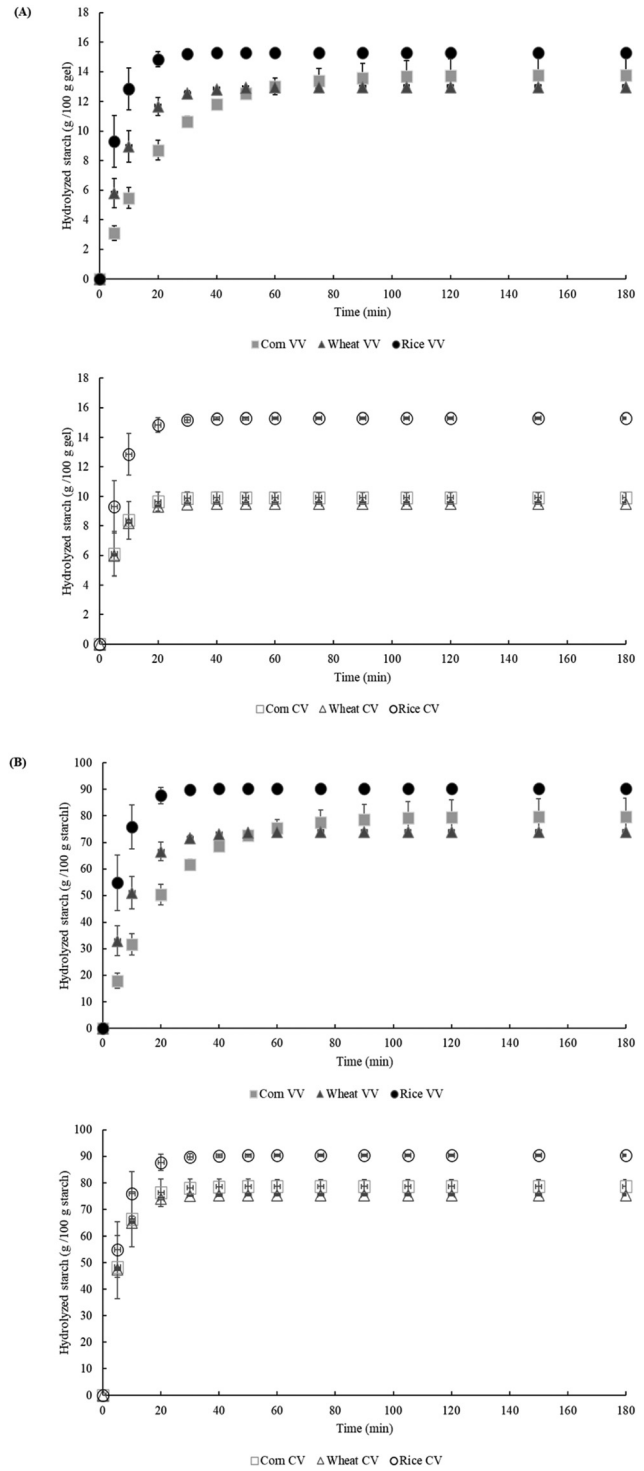

Fig. 3 Effect of different viscosities on *in vitro* starch gel digestion. Graphs are expressed in (A): hydrolyzed starch g per 100 g gel; (B) hydrolyzed starch g per 100 g starch. Gels were prepared at a constant amount of starch giving variable viscosity (VV, closed symbols) or different amounts of starch required to reach constant viscosity (CV, open symbols). Corn: ■, wheat: ▲, and rice: ●.

cosity by incorporating hydrocolloids in starch gels and it has been attributed to the limitations of the enzyme accessibility to starch.[21,22] However, when comparing gels having the same viscosity (CV) different enzymatic hydrolyses were observed (Fig. 3A CV). The CV gels of wheat and corn displayed a similar hydrolysis behavior but the CV gel of rice showed more extensive hydrolysis. Although that trend could be initially attributed to its higher starch content, the hydrolysis plots normalized to the amount of starch revealed the same trend (Fig. 3B). Therefore, the results confirmed that gel hydrolysis was not only affected by starch content, and considering they had similar viscosity, gel physical properties like viscoelasticity might also influence the hydrolysis of gels. This behavior might be related either to the lower G' of the rice gel (Table 1), which suggested a weaker gel structure, or to more porous gels, as previously mentioned high force gels (F1 in Table 1) were related to porosity as reported by Garzon and Rosell *et al.* (2021).[15] Both effects would favor enzyme accessibility to the gel, explaining the more extensive hydrolysis of CV rice gels.

Starch fractions (RDS, SDS, DS and RS), according to the rate of glucose release, presented statistically significant differences ($p < 0.05$) (Table 2). The starch source significantly ($p < 0.05$) affected the RDS, whereas gel viscosity significantly ($p < 0.05$) impacted the amounts of SDS and RS. VV gels made of corn starch had the lowest amount of RDS, which agrees with the findings of Zhang *et al.* (2006)[23] by studying different raw cereal starches. Corn VV gel had the highest viscosity and thus the variability in the starch gel characteristics mainly affect the RDS. In addition, the corn VV gel had the highest amount of SDS (Table 2). Nevertheless, gels made at constant viscosity did not present statistically significant differences in SDS, and rice gel gave the highest RDS and RS.

In addition, the kinetic parameters derived from *in vitro* hydrolysis plots (Fig. 3A) are shown in Table 2. The kinetic constant (k) or the hydrolysis rate was significantly ($p < 0.05$) affected by gel viscosity, being faster when decreasing the viscosity, but a similar k ($p > 0.05$) was obtained with the gels obtained at CV. Therefore, the loss of the gel crystalline structure did not determine the k,[24] but the physical properties are significantly affecting hydrolysis. With regard to variable viscosity, the corn gel showed the slowest kinetic constant. A decrease in the k was accompanied by a simultaneous increase in the SDS content. For this reason, gel viscosity could be a modulating factor as it can limit the enzyme diffusion rate and slow down the enzymatic hydrolysis. Regarding the equilibrium concentration of the hydrolyzed starch (C_∞) and the area under the hydrolysis curve (AUC), they were significantly ($p < 0.05$) affected by both factors: starch source and gel viscosity. The maximum hydrolysis (C_∞) indicates the extent of the hydrolysis when the curve reaches a plateau and the area under the curve is related to the glucose release in 180 minutes of hydrolysis. As previously mentioned, the rice gel presented the largest hydrolysis (Fig. 3A), even when comparing the starch gels made at constant viscosity. In samples with constant viscosity, these parameters decreased due to the lower starch content of the gels.

Table 2 Parameters[a] of *in vitro* starch gel hydrolysis. Gels were prepared with a constant amount of starch giving variable viscosity (VV) or different amounts of starch required to reach constant viscosity (CV). Gel made with rice starch was selected for defining the target viscosity at 37 °C because the same gel was used for VV and CV

	Variable gel viscosity			Constant gel viscosity		*p*-Value	
	Corn VV	Wheat VV	Rice VV, Rice CV	Corn CV	Wheat CV	Source	Viscosity
RDS (%)	8.70 ± 0.66^{c}	11.66 ± 0.60^{b}	14.84 ± 0.51^{a}	9.64 ± 0.65^{c}	9.32 ± 0.05^{c}	**0.0001**	0.4246
SDS (%)	5.02 ± 1.79^{a}	1.30 ± 0.73^{b}	0.45 ± 0.43^{b}	0.30 ± 0.31^{b}	0.18 ± 0.00^{b}	0.1190	**0.0461**
DS (%)	14.26 ± 2.76^{a}	11.51 ± 1.91^{ab}	13.26 ± 0.26^{ab}	11.83 ± 0.45^{ab}	10.26 ± 0.81^{b}	0.0756	0.1604
RS (%)	20.15 ± 1.71^{a}	17.85 ± 1.94^{a}	17.24 ± 2.79^{a}	7.76 ± 3.57^{b}	10.62 ± 1.03^{b}	0.4312	**0.0169**
k (min^{-1})	0.05 ± 0.01^{b}	0.12 ± 0.03^{ab}	0.19 ± 0.06^{a}	0.20 ± 0.07^{a}	0.20 ± 0.00^{a}	0.2488	**0.0383**
C_∞ (%)	13.77 ± 1.20^{b}	12.96 ± 0.13^{b}	15.29 ± 0.08^{a}	9.93 ± 0.34^{c}	9.50 ± 0.05^{c}	**0.0022**	**0.0063**
AUC	2194 ± 114^{b}	2215 ± 4^{b}	2661 ± 39^{a}	1729 ± 78^{c}	1656 ± 8^{c}	**0.0003**	**0.0058**
C_∞/TS (%)	79.77 ± 7.10^{b}	74.08 ± 3.36^{b}	90.36 ± 0.31^{a}	78.64 ± 3.21^{b}	75.86 ± 0.46^{b}	**0.0003**	0.9064

Means within the same row followed by different letters indicate significant differences $p < 0.05$. C_∞ and k were determined by the equation, $C = C_\infty (1 - e^{-kt})$. [a] Rapidly digestible starch (RDS), slowly digestible starch (SDS), digestible starch (DS), resistant starch (RS), kinetic constant (k), equilibrium concentration (C_∞), area under the hydrolysis curve after 180 min (AUC), total starch content (TS) and hydrolysis percentage (C_∞/TS).

The relationship between the equilibrium concentration of hydrolyzed starch and the total starch content of each gel was significantly affected by the type of starch. The rice gel had a higher hydrolysis percentage (90.36%), while the corn and wheat gels displayed similar results. Consequently, gel viscosity is a factor with a great impact on the reaction rate (k) and on the starch fractions, particularly the SDS. This result agrees with the findings of Velásquez-Barreto *et al.* (2021)[12] who studied tuber starches and observed positive correlations between gel viscosities and SDS amounts.

Correlation matrix

A correlation matrix was established to find any significant relationship between the parameters recorded from the pasting behaviour, the viscoelastic characterization, and the *in vitro* hydrolysis of tested gels (Table 3). The viscosity at 37 ○C showed a strong positive correlation with SDS (r = 0.83) and moderate correlations with DS (r = 0.65) and RS (r = 0.63). Therefore, the results confirmed that the viscosity of the gels affects the hydrolysis behaviour. Likely, the viscosity of the system retards the binding of α-amylase-starch or modifies the starch structure thus affecting the α-amylase activity.[25] In fact, a significant negative correlation (r = −0.82) was observed between the viscosity at 37 ○C and the kinetic constant (k), thus confirming that viscosity limits the mass transfer and affects the hydrolysis reaction rate. These results support that higher viscosity in a food matrix increases SDS content, which has been associated with a lower glycemic index, greater satiety and slower enzymatic hydrolysis.[22,26] A positive correlation was observed between the α-slope of RFA with SDS (r = 0.84) and RS (r = 0.74). Interestingly, a strong negative correlation (r = −0.87) was observed between the α-slope and kinetic constant (k), indicating that faster gelatinization led to gels with reduced kinetic constant. This fact is also related to gel

Table 3 Correlation matrix among the rheological properties (viscometer, RFA, and rheometer parameters) and hydrolysis parameters obtained from the different starch gels

	RDS (%)	SDS (%)	DS (%)	RS (%)	k	C_∞ (%)	AUC	C_∞/TS (%)
η (mPa s)	**−0.41**	**0.83****	**0.65***	**0.63***	**−0.82****	**0.30**	**0.14**	**−0.23**
Onset (s)	−0.05	0.42	**0.68***	0.12	−0.25	0.31	0.24	0.49
F0 (N)	0.08	0.42	0.25	0.38	−0.31	0.44	0.38	0.20
α-Slope	−0.21	**0.84****	0.50	**0.74****	**−0.87****	0.52	0.36	−0.16
F1 (N)	0.25	0.37	0.15	**0.74***	−0.54	0.57	0.53	−0.17
F2 (N)	0.46	−0.06	−0.12	0.51	−0.14	0.41	0.46	−0.10
Breakdown (N)	−0.25	**0.83****	0.50	**0.65***	**−0.84****	0.46	0.31	−0.16
G' 95 ○C	0.37	0.06	−0.08	0.57	−0.30	0.42	0.44	−0.24
G'' 95 ○C	0.15	0.06	−0.07	0.43	−0.34	0.19	0.20	−0.54
tan δ 95 ○C	−0.33	0.01	0.09	−0.07	−0.23	−0.32	−0.34	**−0.68***
G' 37 ○C	−0.34	0.58	0.07	0.53	**−0.73***	0.16	0.03	−0.58
G'' 37 ○C	0.00	0.05	−0.20	0.35	−0.30	0.04	0.03	−0.62
tan δ 37 ○C	**0.66***	**−0.72***	−0.30	−0.13	**0.65***	0.04	0.21	0.18
Slope lin G' (0.1–10 Hz)	−0.02	−0.26	−0.47	−0.49	0.40	−0.24	−0.21	0.18
Slope lin G'' (0.1–10 Hz)	0.52	−0.27	−0.12	0.20	0.16	0.29	0.37	0.35
G' 0.1 Hz	−0.54	0.41	−0.17	0.28	−0.56	−0.19	−0.30	**−0.78****
G'' 0.1 Hz	0.03	0.24	0.24	0.57	−0.48	0.23	0.20	−0.43
tan δ 0.1Hz	**0.89****	−0.21	0.42	0.39	0.20	**0.71***	**0.82****	**0.69***

Bold values indicate significant correlations. ** Indicates $p < 0.01$. * Indicates $p < 0.05$.

firmness (G') and negative correlation ($r = -0.73$), because gels with a higher gelatinization rate give firmer gels that undergo slower hydrolysis.[15] A positive moderate correlation was observed between maximum force (F1) and RS ($r = 0.74$). Garzon and Rosell *et al.* (2021)[15] related the force with gel structure, suggesting that higher force was required for obtaining gels with a more porous structure. Breakdown was positively correlated with SDS ($r = 0.83$) and RS ($r = 0.65$) and negatively correlated with kinetic constant ($r = -0.84$), which agree with previous results.[8] It has been reported that the loss of crystalline structure in gelatinized starch is not a determining factor for starch digestion.[24] Nevertheless, it seems that higher breakdown, and consequently lower stability during heating, allowed higher structural disorganization of the gels, which could be recrystallized during cooling giving more structured gels that offer more resistance to hydrolysis as indicated by the higher SDS and lower k. This assumption was also supported by the significant negative correlation observed between the SDS and $\tan\delta$ (G''/G') values of the gels after cooling ($r = -0.72$), relating starch hydrolysis with the level of the gel structure. Regarding the rheometric properties, those that showed the most significant correlations ($p < 0.01$) were in mechanical spectra. A significant negative correlation ($r = -0.78$) was observed between G' (0.1 Hz) and the hydrolysis percentage (C_∞/TS). This could mean that a characteristic such as elasticity can influence the percentage of hydrolysis. In native starches, the chain length distribution has been correlated with the starch digestibility,[20] but that fundamental property does not seem to explain the hydrolysis behaviour of the gels. The digestibility of the gel depends on the ability of the enzyme to penetrate into the gel; consequently, strong structures (high firmness) of gels seem to delay the hydrolysis. In addition, there was a high correlation between the $\tan\delta$ (G''/G') values at 0.1 Hz with RDS ($r = 0.89$), C_∞ ($r = 0.71$), AUC ($r = 0.82$), and C_∞/TS ($r = 0.69$), which suggested that less structured gels (high damping factor) favoured the initial hydrolysis of starch, for the first 20 minutes, and also the extent of the gels hydrolysis.

Conclusions

The rheological performance of starch gels, besides their *in vitro* hydrolysis, allows the assessment of global starch functionality, namely the technological behaviour for industrial applications and the prediction of their comportment during digestion. Viscosity plays a fundamental role in starch gel functionality, being an important parameter that modulates those functionalities. Starch gels from different cereals have significantly different viscosities when produced at constant starch concentrations, and as a consequence, different viscoelastic properties and *in vitro* hydrolysis kinetics. Particularly, wheat and corn gels displayed higher forces and solid like behaviour. Conversely, rice gel showed a lower gelatinization rate and weak behaviour. Nevertheless, force along gelatinization and the viscoelastic properties of cereal starch gels were closer when comparing gels of similar viscosity, showing alike hydrolysis rates. The results allowed the correlation of the rheological properties with the hydrolysis parameters, thus confirming the importance of gel viscosity, which was positively correlated with the SDS fraction ($r = 0.83$) and RS ($r = 0.63$), and negatively correlated with the kinetic constant ($r = -0.82$). Therefore, a higher viscosity in the range of 550–1170 mPa s will slow down enzymatic hydrolysis. Therefore, apart from the already well-known factors (amylose/amylopectin ratio, chain length, gel structure, and so on) that affect starch digestion, gel viscosity could be a rapid indicator for estimating starch kinetic hydrolysis. Overall, the gel viscosity of cereal starches greatly affects the hydrolysis kinetics, which opens the opportunity to apply reverse engineering in the design of starch-based systems to reduce postprandial glucose levels. Further *in vivo* studies will be undertaken to confirm the results obtained from the model systems.

Author contributions

Credit roles: MS: Conceptualization; data curation; formal analysis; investigation; methodology; and roles/writing – original draft; LM: Investigation and methodology; RG: Methodology; supervision; and data curation; RM: Formal analysis; writing – review & editing; and funding acquisition; CMR: Conceptualization; funding acquisition; investigation; supervision; and writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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Performance of Starch Gels on In Vitro Enzymatic Hydrolysis Assessed by Rheological Methodologies

*Maria Santamaria, Leticia Montes, Raquel Garzon, Ramón Moreira, and Cristina M. Rosell**

Starch hydrolysis is attracting much attention due to its relationship to digestion and glucose release. The objective is to propose rapid and continuous analytical methods that allow measuring gels hydrolysis following apparent viscosity (μ). Three different starches (corn, wheat, and rice) are tested recording starch gelatinization followed by gels digestions (digestograms) using a rapid-visco analyzer (RVA) or a rheometer. Results are compared with those obtained by measuring glucose release along hydrolysis. A modified first-order kinetic model in the RVA ($R^2 > 0.99$) and rheometer ($R^2 > 0.99$) describes the gels digestograms. Wheat gel shows a higher hydrolysis rate (k), which indicates faster digestion followed by rice and corn gels. The proposed models allow rapid analysis of starch digestograms, allowing to discriminate among hydrolysis rate of different starches. These less time-consuming methods can be an option to continuously analyze starch gelatinization followed by enzymatic digestion.

1. Introduction

Nowadays, one of the trend drivers for food manufacturers is the development of healthy foods, particularly addressing increase of nutrient availability, improve satiety, or decrease blood glucose response.[1] Because of that, much interest has been focused on developing in vitro methods that allow predicting foods and nutrients behavior along the oro-gastrointestinal digestion.[2,3] Particularly in the case of starch digestion, the oro-gastrointestinal digestion is rather challenging due to the many dilutions that masked the kinetic changes in the starch fraction.[4] Alternatively, in vitro starch digestion methods are the most applied ones, mainly based on enzymatic hydrolysis followed by measuring the glucose release.[5] However, other indirect methods for assessing starch performance along enzymatic digestion have also attracted attention, particularly following viscosity[6] and the impact of different enzyme concentrations[7] during digestion simulation, initially using a rotary viscometer. Nowadays, there are other equipment commonly used for following rheological changes, namely rheometer and rapid visco analyzer (RVA), and some authors have already used them to record rheology changes that occurred along digestion at 37 °C.[8,9] Other authors followed the glucose release that occurs during the digestion period in parallel to rheology changes recorded in the rheometer.[10–13] In those studies, focus has been put on the impact of shear rate (0.1, 1, 10 s^{-1}) on the in vitro digestion of gelatinized potato and corn starch[12] or the impact of hydrocolloids like guar gum on the digestibility of potato flour[11] or its effect on waxy maize.[13] Hardacre et al.[10] also studied de impact of soluble and insoluble fiber in potato and corn starches during their in vitro digestion.

Similarly, RVA has been used to evaluate the apparent viscosity decay produced on different wheat starch gels (6%, 8%, and 10%) or waxy maize starch gels (2%, 4%, and 6%) at 37 °C when adding different levels of α-amylase and their relationship with volatile compounds release, but without relating those with starch digestion.[8] Conversely, Sorba et al.[9] studied the enzymatic hydrolysis of potato and waxy maize starch gels using amylase and amyloglucosidase and recording apparent viscosity changes with RVA.

Furthermore, Hódsági et al.[14] found some significant correlations among glucose release during enzymatic hydrolysis of corn and wheat starches and their pasting parameters; particularly in the case of wheat starch hydrolysis rate and peak viscosity, trough, and final viscosity, which might be useful for estimating in vitro digestion. However, previous studies have been conducted using rheology methods to independently evaluate gelatinization behavior of starches or to follow rheological modifications during the enzymatic hydrolysis. The aim of this study was to develop rapid methods that allow in a single test to evaluate starch performance during gelatinization followed by enzymatic digestion. For that purpose, rheological methods were developed in the RVA and rheometer using α-amylase, and result compared with the data obtained by quantifying glucose release. The inclusion

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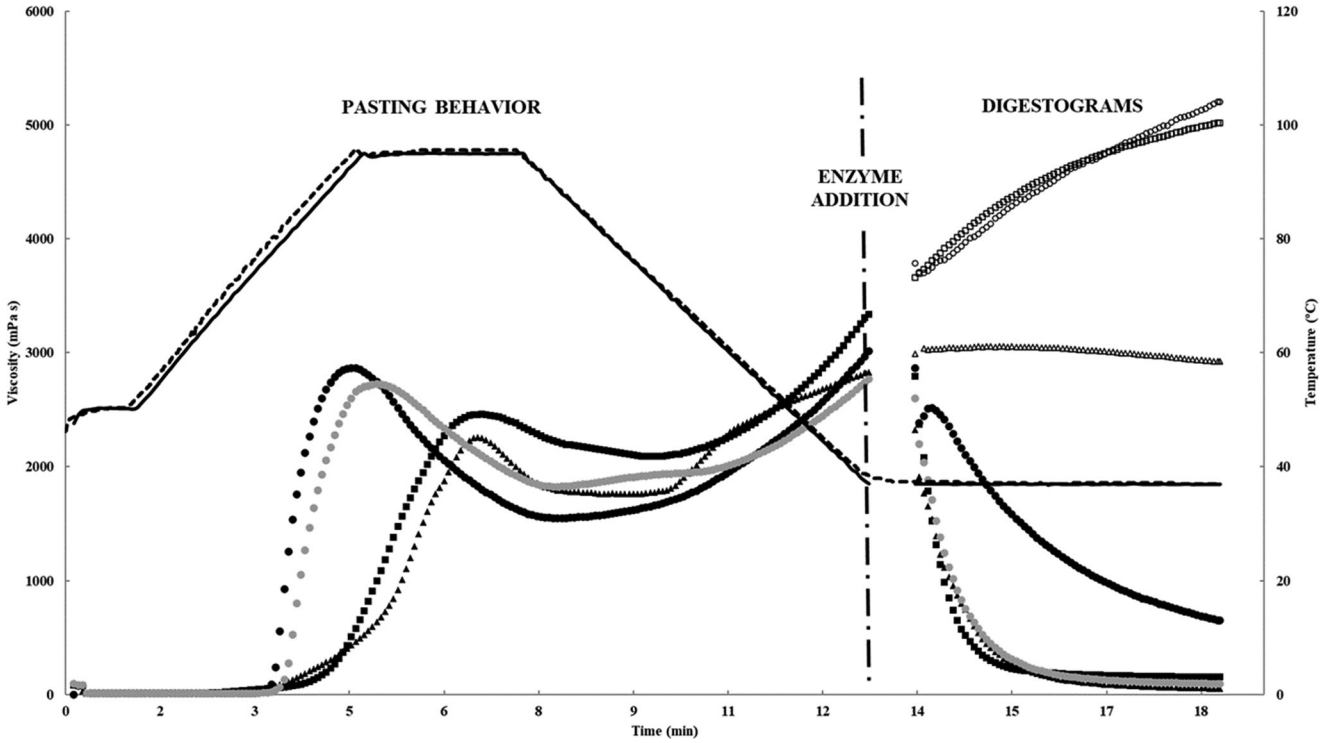

Figure 1. RVA method for recording the starch gelatinization and further enzymatic hydrolysis. First part records the pasting behavior of the gels, then the addition of alpha-amylase and finally the digestograms in the presence of amylase (filled symbols) and their counterparts in the absence of enzyme (empty symbols). Corn (●), corn pH 5.8 (●), wheat (■), and rice (▲) starches. Theorical (▬) and experimental (-----) temperatures (°C).

of enzymatic hydrolysis into the rheological methods might provide rapid methods to predict the behavior of starch gels during enzymatic digestion.

2. Results and Discussion

2.1. Viscosity Hydrolysis

Corn, wheat, and rice starches were selected to set up a rapid method for assessing pasting performance followed by enzymatic hydrolysis in a single assay, which were referred as digestograms. Plots of the apparent viscosities along pasting and enzymatic hydrolysis are shown in **Figure 1**. Parameters recorded from the apparent viscosity plots are indicated in Table S1, Supporting Information. Knowing the importance of temperature on the enzymatic kinetics, thermocouples were immersed in the slurries to monitor it, and values completely overlapped those recorded by the equipment. As expected, the apparent viscosity plots for corn, wheat, and rice indicate differences in their pasting performance, with corn showing an earlier swelling and major maximum apparent viscosity (2866 ± 15 mPa s) than observed in the other starches, which agree with previously reported results.[4] Moreover, Wickramasinghe et al.[15] observed different viscosity peaks and swelling power among several varieties of hard or soft wheat starches. Rice showed lower apparent peak viscosity (2263 ± 93 mPa s), with similar value to the one reported.[16] Starch granules differ in morphological, and starch structure depending on botanical origin, which affect their pasting performance.[17]

Focusing on the hydrolysis or digestogram stage, apparent viscosities of the gels in the presence and the absence of α-amylase were recorded. In the absence of α-amylase (empty symbols) a progressive increase in the apparent viscosity was observed in corn and wheat gels. Presumably, that increase in the apparent viscosity was related to their slower cooling due to their higher viscosity, which reduced the cooling rate within the gel structure. In fact, in the case of rice gel, a steady apparent viscosity was observed because its lower viscosity allowed faster heat transference within gel structure. The addition of α-amylase produced a rapid decline in the apparent viscosity, similar to that observed Gee et al.[6] using a rotary viscometer. Enzymatic hydrolysis by α-amylase induces the breakdown of starch chains to the release of small fragments (dextrins) changing the starch gel behavior, from a solid gel to a weakly structured fluid gel.[9] Nonetheless, comparing the digestograms of the different starches, corn gel showed lower viscosity decrease (2864–651 mPa s) (**Table 1**). Considering the impact of pH on the enzymatic activity, first hypotheses was related to possible pH difference.[18] In fact, corn starch slurry had pH 7.25, whereas slurries of wheat and rice starches showed pH 5.85. To confirm the impact of gel pH on α-amylase activity, corn starch gel was prepared in sodium phosphate buffer 0.01 M at pH 5.8 instead of water. The digestogram obtained for corn gel with adjusted pH displayed faster hydrolysis, like the one obtained with wheat and rice gels.

Gels formation and their further hydrolysis were also carried out in the rheometer. In **Figure 2** it can be observed the formation of the gels and then, its maturation (empty symbols) and

Table 1. Gel starch viscosities (μ) obtained with RVA or rheometer before and after adding amylase, and the parameters that defined the hydrolysis kinetic (the kinetic constant and the maximum hydrolysis of starch gels).

Method	Parameters	Corn	Corn pH 5.8	Wheat	Rice
RVA	μ initial digestion [mPa s]	2864 ± 90^{a}	2599 ± 146^{ab}	2793 ± 183^{a}	2324 ± 106^{b}
	μ final digestion [mPa s]	651 ± 4^{a}	96 ± 10^{c}	154 ± 8^{b}	54 ± 3^{d}
	k_{RVA} [min^{-1}]	0.40 ± 0.06^{c}	1.33 ± 0.12^{b}	1.80 ± 0.02^{a}	1.17 ± 0.11^{b}
	μ_{∞} [mPa s]	329 ± 41^{a}	75 ± 4^{c}	137 ± 8^{b}	34 ± 6^{c}
Rheometer	μ initial digestion [mPa s]	4975 ± 78^{a}	4670 ± 269^{ab}	4520 ± 14^{b}	2445 ± 134^{c}
	μ final digestion [mPa s]	1810 ± 42^{a}	686 ± 15^{b}	323 ± 26^{b}	94 ± 17^{c}
	k_{Rheo} [min^{-1}]	0.46 ± 0.01^{d}	0.74 ± 0.08^{c}	2.38 ± 0.07^{a}	1.04 ± 0.02^{b}
	μ_{∞} [mPa s]	1549 ± 68^{a}	677 ± 114^{b}	336 ± 50^{c}	83 ± 16^{d}
Biochemical	k [min^{-1}]	0.0334 ± 0.0009	–	0.0399 ± 0.0049	0.0335 ± 0.0012
	C_{∞} [g 100 g^{-1} gel]	6.38 ± 0.35^{ab}	–	5.51 ± 0.24^{b}	6.97 ± 0.55^{a}

Means within a row followed with different letters indicate significantly different ($p < 0.05$).

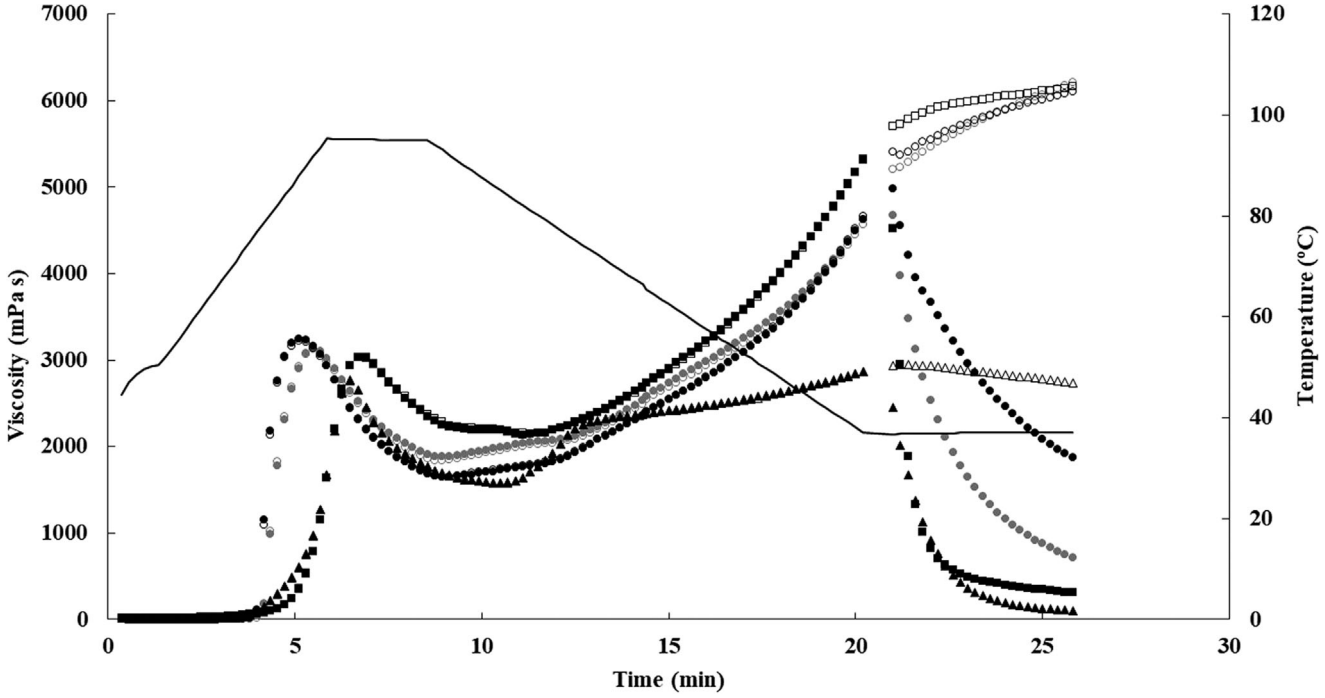

Figure 2. Full assay in rheometer where it is represented the apparent viscosity versus time following the protocol previously described for corn (●), corn pH 5.8 (●), wheat (■), and rice (▲) starches.

digestion (filled symbols). In general, same behavior than in RVA assays was observed. At the end of the gelatinization stage, it was observed that wheat starch had the highest viscosity (4520 ± 14 mPa s), while rice starch presented the lowest viscosity (2445 ± 134 mPa s) (Table 1). At digestion stage, a significant decrease in viscosity was seen in all samples, which agrees with results obtained with the RVA. Similar behavior was previously reported by Kim et al.[19] when simulated the oro-gastrointestinal digestion of white and brown rice flours in the rheometer, and An et al.[20] also reported a decrease of viscosity when wheat gels blended with increasing amounts of black rice flour were digested with pancreatin and amyloglucosidase.

2.2. Enzymatic Hydrolysis of Different Starches Recorded by Biochemical Methods

Starch gels obtained from RVA were subjected to in vitro digestibility to evaluate the hydrolysis kinetics of starches from different cereals, and to compare those with the results obtained in the rapid methods previously presented. In **Figure 3** hydrolysis plots of gels are displayed. The graphs were expressed as grams of hydrolyzed starch per 100 g of gel. Hydrolysis pattern was different among the starches from different botanical origin. Rice gel presented higher hydrolysis, which could be related to its lower initial viscosity (2263 mPa s) that facilitates

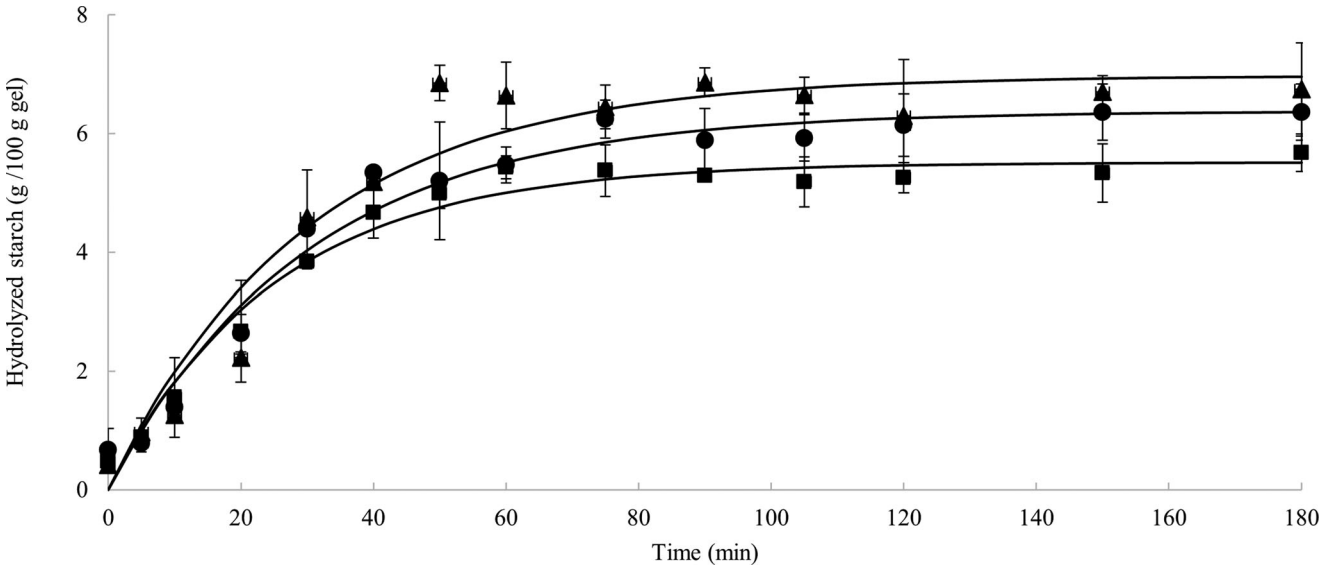

Figure 3. Enzymatic hydrolysis of different starch gels corn (●), wheat (■), and rice (▲) starches, and solid lines correspond to first-order model Equation (3) (▬).

enzyme diffusion (Table S1, Supporting Information).[21] Consequently, rice gel reached the superior maximum hydrolysis (C_∞) (Table 1). Kinetics parameters were satisfactorily fitted ($R^2 > 0.96$) with a fist-order kinetics-based model Equation (3). Gels presented similar hydrolysis rate (k) and differed in the extent of the hydrolysis (C_∞), with rice gel having the highest maximum hydrolysis (Table 1). Hódsági et al.[14] reported similar rate constants for gelatinized wheat and corn starches. Furthermore, although there were not significant differences, gels with lower k had higher slowly digestible starch (SDS) content. This fraction of starch is associated with satiety, less glycemic index, and prebiotic effect.[22]

2.3. Modeling of Digestograms

To establish the correlation between enzymatic hydrolysis of starches by assessing glucose release and the viscosity decay measured either with RVA or rheometer, experimental data of the digestograms were mathematically fitted. **Figure 4** shows the starch hydrolysis by viscosity decay of gels of corn, wheat, and rice starches. The shapes of the kinetics curves were similar, but the initial (related to initial gel firmness) and final viscosities were specific for each starch. In fact, experimental apparent viscosity (mPa s) at the beginning and end of the digestograms obtained in the RVA differed from 2864 to 651 for corn without pH adjustment, 2599–96 for corn at pH 5.8, 2793–154 for wheat, and 2324–54 for rice (Figure 4A). Likewise, digestograms in the rheometer show that apparent viscosity (mPa s) varied from 4975 to 1810 for corn, 4670–686 for corn pH 5.8, 4520–323 for wheat, and 2445–94 for rice starch gels (Figure 4B).

A first-order kinetic model was applied to model the digestograms, Equation (1):

$$\mu = \mu_\infty + \left(\mu_0 - \mu_\infty\right) e^{-kt} \quad (1)$$

where μ is the apparent viscosity (mPa s), μ_0 is the initial viscosity, μ_∞ is the final viscosity, k (min^{-1}) is the kinetic constant, and t (min) is hydrolysis time.

The RVA experimental data presented satisfactorily fitting ($R^2 > 0.99$) to first-order kinetic model. Kinetic constant (k_{RVA}) obtained in the digestograms presented statistically differences ($p < 0.05$) depending on the starch source, as well as pH, in the case of corn starch (Table 1). The highest hydrolysis rate (k_{RVA}) was presented by wheat gel (1.80 min^{-1}), followed by corn gel after adjusting pH (1.33 min^{-1}), and rice (1.17 min^{-1}). Corn gel prepared without adjusting the pH showed the lowest k_{RVA}. Regarding μ_∞, the lowest value was determined for rice starch (34 mPa s) and the highest with corn (329 mPa s). Higher peak viscosity has been correlated negatively with hydrolysis rate of native starches, but no correlations were observed with the enzymatic hydrolysis of the gels.[23] Factors like source starch, enzyme type, concentration of enzyme, and starch solids content affect the starch digestion rate.[9]

Similar fitting was carried out with the experimental data obtained with the rheometer (Table 1) obtaining significant differences ($p > 0.95$) between k_{Rheo} and μ_∞ values for each gel were found. In Figure 4B, it can be observed the acceptable fitting quality ($R^2 > 0.99$) of the model in comparison to experimental data. Again, corn gel without adjusting the pH showed the lowest k_{Rheo} value (0.46 min^{-1}) and wheat the highest (2.38 min^{-1}). Considering the kinetics rate obtained in the RVA, the k_{Rheo} for corn gel at pH 5.8 was lower than expected, even lower than that obtained for rice. Likely differences between rotational speed of rheometer and shearing of RVA, might explain that trend. Presumably, pH equilibration of gel slurry and the enzymatic solution by the employed impellers occurred at different speed in both equipments. The slower homogenization in the rheometer would explain the lower kinetic constants obtained for corn at pH 5.8 versus rice value, in comparison with their respective RVA results. Nevertheless, independently of the specific data, the trends of the digestion kinetic constants obtained with tested starches by means of both

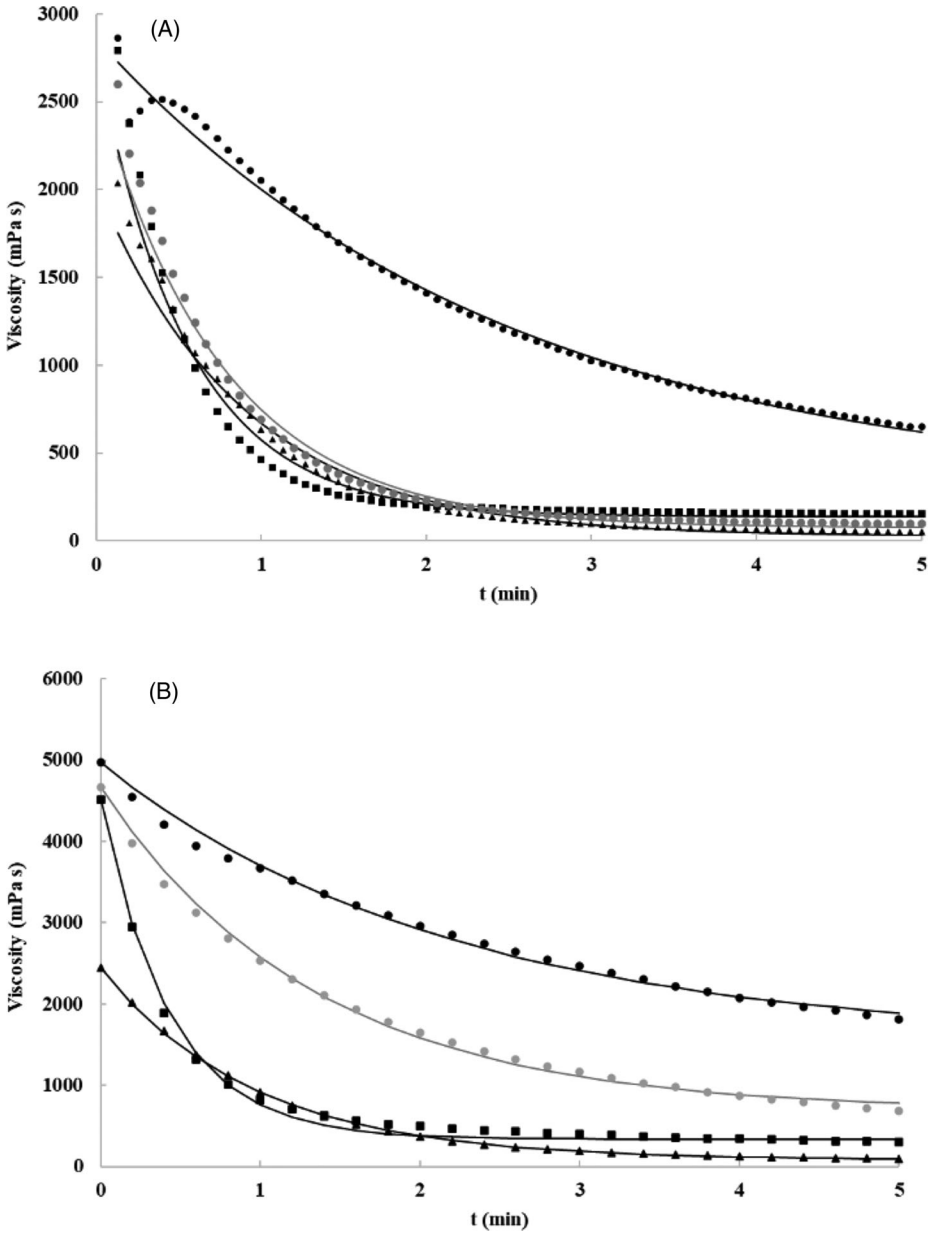

Figure 4. Variation of apparent viscosity during hydrolysis of corn (●), corn pH 5.8 (●), wheat (■), and rice (▲) starchy gels and their modeling by Equation (1) (▬). A) RVA digestograms and B) Rheometer digestograms.

methods (RVA and rheology) were satisfactorily in agreement. Regarding $\mu_{\infty,}$ the lowest value was determined for rice starch (83 mPa s) and the highest with corn (1549 mPa s). Results confirmed the viability of those test to follow enzymatic hydrolysis simulating digestion, being able to discriminate among the type of starches. Conversely, the quantification of glucose release did not show significant differences in their hydrolysis rate.

2.4. Normalized Digestograms

Digestograms were the results of a decrease in viscosity due to the enzymatic hydrolysis of gelatinized starch. To visualize jointly the hydrolysis kinetics of tested starchy gels, **Figure 5** shows the corresponding normalized curves (μN vs dimensionless time, t/t_{final}) of hydrolysis kinetics. Sorba et al.[9] made similar adjustment for studying retrograded gels. Normalized viscosity μN (–) was evaluated considering μ_0 and μ_∞ values by Equation (2), against the results of the biochemical kinetic (C/C_0) in reference to glucose content.

$$\mu_N = \frac{\mu_t - \mu_\infty}{\mu_0 - \mu_\infty} \tag{2}$$

Regardless of the botanical origin of the starch, it can be observed the sharp drop of μN for wheat, intermediate one for rice,

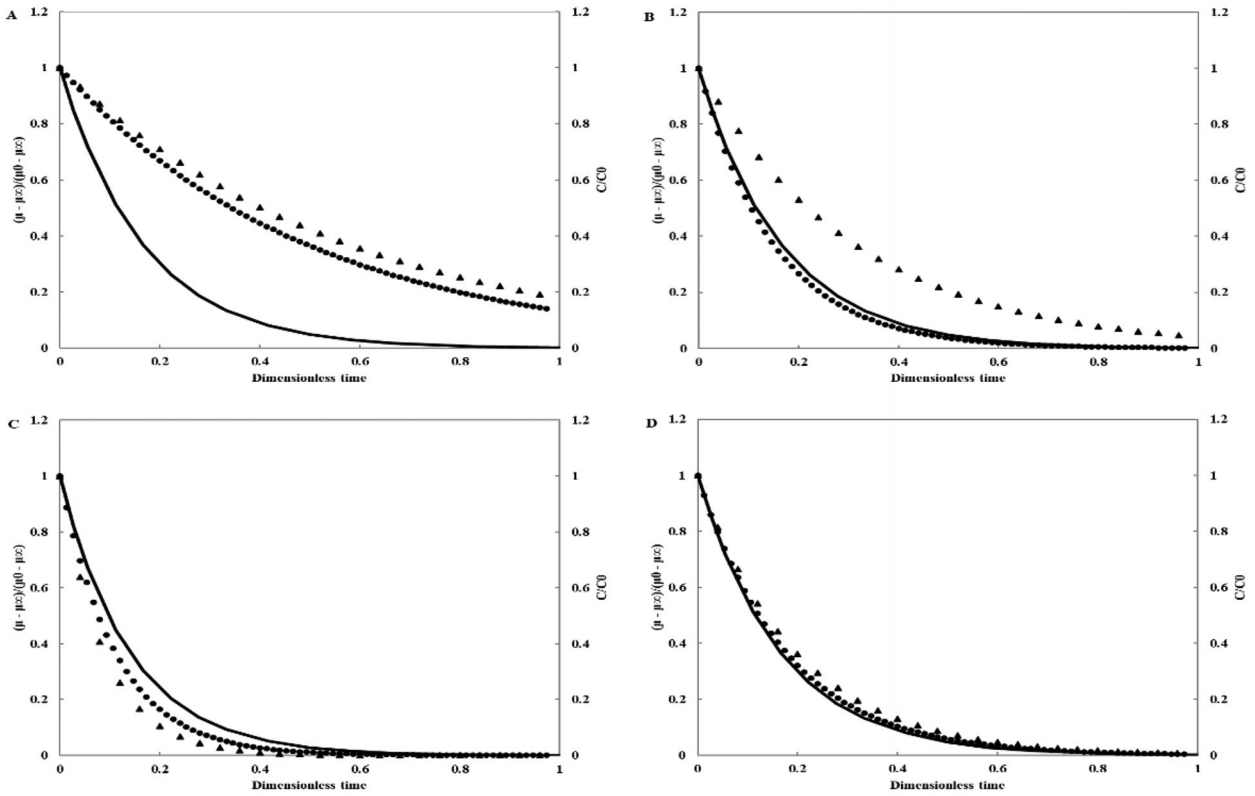

Figure 5. Normalized curve of apparent viscosity using Equation (2) during different hydrolysis: biochemical (▬) RVA (●) and rheometer (▲) methods. Corn A), corn pH 5.8 B), wheat C), and rice D). Biochemical hydrolysis time on the lower X-axis and digestograms time on the upper X-axis.

and moderate drop for corn starch gels (Figure 5). These curves showed the differences in the hydrolysis time of digestible starch in the gels. Then, all curves were asymptotic at long times (all digestible starch was already hydrolyzed). Corn starch was the exception, but it was confirmed that the pH of the sample was a factor that modifies the rheological behavior, mainly in the RVA method. This indicated that the analysis had to be carried out at an optimal pH for the enzymatic activity. In the case of biochemical hydrolysis, the pH of the corn starch gel did not vary the normalized viscosity plots, that was expected since gels pH effect is negligible when diluted into the buffer solution. The models used allowed to know the rate of starch digestion (Table 1), having very good fitting RVA ($R^2 > 0.99$), rheometer ($R^2 > 0.99$), and biochemical kinetics ($R^2 > 0.96$). Differences in the fitting might be attributed to the recording time in each methodology, RVA and rheometer quantifies the viscosity every 4 and 12 s, respectively, whereas aliquots for the biochemical analysis were withdrawn every 5, 15, or 30 min along the enzymatic assay. Most of the starch is digested, at relative high rate, for short period of time when following the apparent viscosity. In both methodologies, wheat gel showed higher hydrolysis rate (k), which indicated that the digestion was faster compared to other starches.

3. Conclusions

Single tests were developed to study the gelatinization performance and the digestion of different starch gels. Viscosity changes of different starches recorded with RVA or rheometer followed by amylase hydrolysis provide digestograms that were used to predict gels digestion by fitting experimental results to a first-order kinetic models. Parameters obtained from the fitting can be used for predicting starch digestion using rapid, simple, and reliable methods. Those can be used to carry out preliminary studies of many samples and identify the rheological behavior with alpha-amylase addition. A preliminary discrimination for predicting starch behavior might be very useful prior to in vitro or in vivo digestions.

4. Experimental Section

Materials: Starches from corn and wheat (EPSA, Valencia, Spain) and rice (Sigma Aldrich, Sigma Chemical, St. Louis, MO, USA) were employed. Moisture content of the starches was 13.08%, 12.60%, and 10.56%, for corn, wheat, and rice, respectively. The enzymes used were VI-B α-amylase from porcine pancreas (EC 3.2.1.1) from Sigma Aldrich (Sigma Chemical, St. Louis, MO, USA) and amyloglucosidase (EC 3.2.1.3) provided by Novozymes (Bagsvaerd, Denmark). Glucose oxidase/peroxidase (GOPOD) kit (Megazyme International Ireland Ltd., Bray, Ireland) was used. All reagents were of analytical grade. Solutions and standards were prepared using deionized water.

Change in Viscosity of Gel and its Hydrolysis Using the Rapid Visco Analyzer: Three grams (14% moisture basis) of starch were placed into the RVA canister and dispersed in 25 mL distilled water. The pH of slurries was determined. Tests were performed in the Rapid Visco Analyzer (RVA 4500; Perten Instruments, Hägersten, Sweden) using the following settings: 50 °C for 1 min, heating from 50 to 95 °C at 10 °C min^{-1}, holding at 95 °C for 2.5 min, cooling down to 37 °C at 10 °C min^{-1}, followed by holding at 37 °C for 36 s for adding the α-amylase solution (900 U mL^{-1}

solution), and then continue recording viscosity at 37 °C for 5 min. Preliminary assays were conducted with corn starch to select the amount of α-amylase (Figure S1, Supporting Information). Different concentrations of α-amylase (56, 90, 169, 225 U) were tested and the enzyme content that induced an intermediate hydrolysis rate was selected (90 U 100 μL^{-1} solution that represented 30 U g^{-1} of starch). Temperature within the slurry/gel was recorded using a Comark N2014 multi-sensor temperature data logger (Comark Instruments, Norwich, Norfolk, UK). Temperature readings were recorded every second. Rotational speed in the first 10 s was 960 rpm and then it was kept at 160 rpm along the test, except when the protocol was stopped (0 rpm) for enzyme addition. Apparent viscosity (mPa s) of starches without adding enzyme was also recorded as reference. RVA analysis was carried out at least duplicate. Pasting parameters extracted from the recorded data included: onset time (min), at which starch viscosity started to increase during heating, peak viscosity (maximum viscosity during heating), peak time (min, at which maximum viscosity is reached), trough viscosity (minimum viscosity when holding at 95 °C), breakdown (difference between maximum and trough viscosity), setback (difference between viscosity at 37 °C and trough viscosity), initial (after adding the enzyme), and final (at the end of the assay) viscosity during the enzymatic hydrolysis.

Rheology of Starch Gels and Enzymatic Hydrolysis Using a Rheometer: The rheological experiments were carried out with a stress-controlled rheometer (MCR 301; Anton Paar Physica, Graz, Austria) using a starch pasting cell (ST24-2D/2V/2V-30) with the following settings: measuring bob radius of 12.00 mm, cup radius of 14.46 mm, and a gap of 2.46 mm. A solvent trap kit was used to minimize water evaporation during tests. A similar protocol, regarding starch concentration (3 g—14% moisture basis—in 25 mL distilled water), times, and temperatures, to the one described above for the RVA, was defined to monitor in the rheometer the gel formation followed by the starch hydrolysis. A pre-shear at 100 rad s^{-1} (960 rpm), 50 °C for 10 s was applied to achieve sample homogenization, followed by a holding time for 1 min at 50 °C and 18 rad s^{-1} (160 rpm). This shear rate was kept for the rest of the assay. A temperature sweep was carried out from 50 to 95 °C at 10 °C min^{-1} to form the gel. High temperature of 95 °C was maintained for 2.5 min. Then, a temperature sweep was made from 95 to 37 °C at 5 °C min^{-1} to achieve the required temperature to make the enzymatic hydrolysis. A rest time of 36 s was needed to introduce the α-amylase (as described in RVA section). Finally, apparent viscosity, μ, at 37 °C for 5 min was monitored to assess the evolution during starch hydrolysis.

Starch Gels Digestion by In Vitro Enzymatic Method: Gels from different starches were prepared in the RVA using Standard 1 method provided by supplier. Starch gels were subjected to hydrolysis digestion following the method reported.[21] Experimental hydrolysis data were used to calculate rapidly digestible starch (RDS) or fraction hydrolyzed during the first 20 min, and the slowly digestible starch (SDS) hydrolyzed within 20 and 120 min.[24] Data were also fitted to a first-order Equation (3) to obtain the kinetic parameters of gels hydrolysis[25]:

$$C = C_{\infty}\ \left(1 - e^{-kt}\right) \tag{3}$$

where C was the concentration (g/100 g gel) of starch hydrolyzed at t time (min), C_{∞} (g/ 100 g gel) was the maximum hydrolysis of starch gels, k (min^{-1}) was the kinetic constant and t was the selected time.

Statistical Data Analysis: The Microsoft Excel Solver was used to model first-order kinetic equations. The digestion results obtained by different methodologies were correlated using Statgraphics Centurion XVII software (Statistical Graphics Corporation, Rockville, MD, USA) by means of analysis of variance (ANOVA) with Fisher's least significant differences test (LSD). Experimental data were expressed as mean ± standard deviation and $p < 0.05$ were considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.S.: conceptualization; data curation; formal analysis; investigation; methodology; roles/writing – original draft; L.M.: investigation; methodology; R.G.: methodology; supervision; data curation; R.M.: formal analysis; writing – review & editing; funding acquisition; C.M.R.: conceptualization; funding acquisition; investigation; supervision; writing – review & editing.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

in vitro digestion, kinetics, modeling, rapid visco analyzers, rheometers

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