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# Legumbres procesadas: propiedades bioactivas y harinas con un perfil nutricional mejorado

**TESIS DOCTORAL**


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El trabajo **"Legumbres procesadas: propiedades bioactivas y harinas con un perfil nutricional mejorado"** ha sido desarrollado por **Milagros Arnal Salinas** bajo su supervisión y reúne las condiciones para optar al título de Doctor en Ciencia, Tecnología y Gestión Alimentaria, por lo que autorizan su presentación.

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**Resumen**

Las legumbres son una de las principales fuentes de proteína vegetal y una alternativa sostenible y asequible a la proteína de origen animal. Además, pueden contener péptidos bioactivos, los cuales pueden liberarse de las proteínas durante la digestión gastrointestinal (DGI). No obstante, su perfil nutricional puede verse comprometido por la presencia de factores antinutricionales (FAN), cuyo efecto puede mitigarse con el procesado. Este puede ayudar a reducir o eliminar dichos FAN, pero también puede afectar a la digestión de las proteínas y, en consecuencia, a la liberación de péptidos. A pesar de sus beneficios, el consumo de legumbres ha disminuido en los últimos años, sin embargo, es fundamental para la salud y el medio ambiente que estas ganen protagonismo en la dieta. Una forma de lograrlo sería a través de su transformación en harinas, lo que permitiría su integración en una amplia variedad de productos. En este contexto, el objetivo de la presente tesis doctoral ha sido evaluar el impacto del procesado y la DGI simulada sobre las propiedades bioactivas de diferentes legumbres y desarrollar harinas de legumbre con un perfil nutricional mejorado.

El primer capítulo de la tesis doctoral se ha centrado en evaluar cómo la cocción convencional, a presión y en microondas, seguida de la DGI simulada, afectan a la actividad antidiabética y al perfil peptídico de la soja, las alubias, los garbanzos y los guisantes. Para ello, se cocieron las legumbres con los distintos tratamientos y se determinó su capacidad de inhibición de las enzimas α-amilasa y α-glucosidasa antes y después de someterlas a una DGI simulada. Se seleccionaron las muestras con mayor actividad inhibidora, se fraccionaron por tamaño molecular o por polaridad, se identificaron los péptidos de las fracciones con mayor actividad mediante espectrometría de masas en tándem y se realizó un análisis *in silico* de las secuencias identificadas. Los resultados mostraron un aumento de la actividad inhibidora de las enzimas α-amilasa y α-glucosidasa tras las DGI simulada, lo que podría atribuirse a la generación de péptidos bioactivos tras la hidrólisis de las proteínas por las enzimas digestivas. La fracción menor a 3 kDa presentó la mayor

actividad inhibidora de la α-amilasa, mientras que la fracción más polar presentó la mayor actividad inhibidora de la α-glucosidasa. Aunque no se observó un patrón definido del efecto de los distintos tratamientos sobre la bioactividad, las legumbres cocidas en microondas mostraron una mayor actividad inhibidora de la α-amilasa, mientras que las legumbres cocidas a presión y de forma convencional mostraron una mayor actividad inhibidora de la α-glucosidasa, antes y después de ser fraccionadas, respectivamente. Según el análisis *in silico*, ninguno de los péptidos identificados había sido descrito previamente como inhibidor de estas enzimas, aunque algunos podrían ser potenciales candidatos por su secuencia y características. En este capítulo se demuestra el potencial de las legumbres como fuente de péptidos antidiabéticos, aunque sería necesario sintetizar las secuencias peptídicas para poder confirmar su actividad biológica.

El segundo capítulo de la tesis se ha centrado en obtener harinas de legumbres enriquecidas en hierro mediante el uso de la tecnología de impregnación a vacío durante la etapa de remojo. Para ello, se seleccionó el haba, por ser la legumbre con mayor porosidad, y se evaluó su cinética de hidratación para comprobar la eficacia del uso de la impregnación a vacío. A continuación, se elaboraron harinas con diferentes variables de procesamiento (enriquecimiento, cocción y/o descascarillado) y se evaluó su impacto sobre distintos FAN, el contenido y la bioaccesibilidad del hierro, las propiedades fisicoquímicas y tecno-funcionales y la estabilidad durante el almacenamiento y la cocción. Los resultados obtenidos indicaron que la impregnación a vacío aceleró el tiempo de remojo, reduciéndolo hasta un 75 %, y permitió la incorporación de hierro a las habas sin alterar la cinética de hidratación. Las harinas enriquecidas presentaron niveles significativamente más altos de contenido y bioaccesibilidad de hierro en comparación con las harinas no enriquecidas. La cocción afectó al contenido en taninos, al contenido en hierro y su bioaccesibilidad, y a las propiedades fisicoquímicas y tecno-funcionales, mientras que el descascarillado afectó al contenido en taninos y hierro, y a la bioaccesibilidad del mineral. La oxidación lipídica y proteica de las harinas enriquecidas se mantuvo estable durante el

almacenamiento. Además, la utilización de dichas harinas en la elaboración de pasta fresca mostró que el contenido de hierro permaneció estable tras la cocción de la pasta y aumentó su bioaccesibilidad. Este capítulo demuestra que la impregnación a vacío es una tecnología efectiva para desarrollar harinas de legumbres enriquecidas en hierro, con aplicaciones potenciales en la formulación de nuevos productos.

Por último, el tercer capítulo de la tesis se ha centrado en obtener harinas de haba hidrolizadas utilizando la impregnación a vacío durante el remojo para incorporar enzimas. Para ello, se evaluó la cinética de hidratación de las habas durante el remojo con las soluciones de las enzimas papaína y bromelina, se obtuvieron las harinas hidrolizadas y se evaluó el efecto del grado de hidrólisis y el descascarillado sobre la digestibilidad proteica y el contenido en FAN. Los resultados obtenidos mostraron que el uso de las enzimas no modificó la cinética de hidratación de las habas y se obtuvieron harinas con un grado de hidrólisis del 9,1 % con papaína y del 8,8 % con bromelina. La hidrólisis aumentó ciertos FAN, como el ácido fítico y los taninos, pero redujo los inhibidores de tripsina. Sin embargo, en general, no se observaron cambios significativos en la digestibilidad proteica. El descascarillado aumentó el contenido en inhibidores de tripsina y ácido fítico, pero redujo los taninos, lo que mejoró la digestibilidad proteica. Este capítulo destaca la importancia de aplicar tecnologías, como la impregnación a vacío, para transformar las legumbres en ingredientes con un mejor perfil nutricional.

En resumen, la presente tesis doctoral demuestra el potencial de las legumbres como fuente de péptidos con actividad antidiabética, y muestra cómo el uso de la impregnación a vacío durante el remojo es una tecnología útil para obtener harinas de haba con un perfil nutricional mejorado.

**Resum**

Els llegums són una de les principals fonts de proteïna vegetal i una alternativa sostenible i assequible a la proteïna d'origen animal. A més, poden contenir pèptids bioactius, els quals poden alliberar-se de les proteïnes durant la digestió gastrointestinal (DGI). No obstant això, el seu perfil nutricional pot veure's compromés per la presència de factors antinutricionals (FAN), l'efecte dels quals pot mitigar-se amb el processament. Aquest pot ajudar a reduir o eliminar aquests FAN, però també pot afectar la digestió de les proteïnes i, en conseqüència, a l'alliberament de pèptids. Malgrat els seus beneficis, el consum de llegums ha disminuït en els últims anys, no obstant això, és fonamental per a la salut i el medi ambient que aquestes guanyen protagonisme en la dieta. Una manera d'aconseguir-ho seria a través de la seua transformació en farines, la qual cosa permetria la seua integració en una àmplia varietat de productes. En aquest context, l'objectiu de la present tesi doctoral ha sigut avaluar l'impacte del processament i la DGI simulada sobre les propietats bioactives de diferents llegums i desenvolupar farines de llegum amb un perfil nutricional millorat.

El primer capítol de la tesi doctoral s'ha centrat en avaluar com la cocció convencional, a pressió i en microones, seguida de la DGI simulada, afecten l'activitat antidiabètica i al perfil peptídic de la soja, els fesols, els cigrons i els pèsols. Per a això, es van coure els llegums amb els diferents tractaments i es va determinar la seua capacitat d'inhibició dels enzims α-amilasa i α-glucosidasa abans i després de sotmetre-les a una DGI simulada. Es van seleccionar les mostres amb major activitat inhibidora, es van fraccionar per grandària molecular o per polaritat, es van identificar els pèptids de les fraccions amb major activitat mitjançant espectrometria de masses en tàndem i es va realitzar una anàlisi *in silico* de les seqüències identificades. Els resultats van mostrar un augment de l'activitat inhibidora dels enzims α-amilasa i α-glucosidasa després de la DGI simulada, la qual cosa podria atribuir-se a la generació de pèptids bioactius després de la hidròlisi de les proteïnes pels enzims digestius. La fracció menor a 3 kDa va presentar la major activitat

inhibidora de la α-amilasa, mentre que la fracció més polar va presentar la major activitat inhibidora de la α-glucosidasa. Encara que no es va observar un patró definit de l'efecte dels diferents tractaments sobre la bioactivitat, els llegums cuits en microones van mostrar una major activitat inhibidora de la α-amilasa, mentre que els llegums cuits a pressió i de manera convencional van mostrar una major activitat inhibidora de la α-glucosidasa, abans i després de ser fraccionades, respectivament. Segons l'anàlisi *in silico*, cap dels pèptids identificats havia sigut descrit prèviament com a inhibidor d'aquests enzims, encara que alguns podrien ser potencials candidats per la seua seqüència i característiques. En aquest capítol es demostra el potencial dels llegums com a font de pèptids antidiabètics, encara que seria necessari sintetitzar les seqüències peptídiques per a poder confirmar la seua activitat biològica.

El segon capítol de la tesi s'ha centrat en obtenir farines de llegums enriquides en ferro mitjançant l'ús de la tecnologia d'impregnació al buit durant l'etapa de remull. Per a això, es va seleccionar la fava, per ser el llegum amb major porositat, i es va avaluar la seua cinètica d'hidratació per a comprovar l'eficàcia de l'ús de la impregnació al buit. A continuació, es van elaborar farines amb diferents variables de processament (enriquiment, cocció i/o eliminació de la casca) i es va avaluar el seu impacte sobre diferents FAN, el contingut i la bioaccessibilitat del ferro, les propietats fisicoquímiques i tecno-funcionals i l'estabilitat durant l'emmagatzematge i la cocció. Els resultats obtinguts van indicar que la impregnació al buit va accelerar el temps de remull, reduint-lo fins a un 75%, i va permetre la incorporació de ferro a les faves sense alterar la cinètica d'hidratació. Les farines enriquides presentaren nivells significativament més alts de contingut i bioaccessibilitat de ferro en comparació amb les farines no enriquides. La cocció va afectar el contingut en tanins, al contingut en ferro i a la seua bioaccessibilitat, i a les propietats fisicoquímiques i tecno-funcionals, mentre que l'eliminació de la casca va afectar el contingut en tanins i ferro, i a la bioaccessibilitat del mineral. L'oxidació lipídica i proteica de les farines enriquides es va mantenir estable durant l'emmagatzematge. A més, la utilització d'aquestes farines en l'elaboració de

pasta fresca va mostrar que el contingut de ferro va romandre estable després de la cocció de la pasta i va augmentar la seua bioaccessibilitat. Aquest capítol demostra que la impregnació al buit és una tecnologia efectiva per a desenvolupar farines de llegums enriquides en ferro, amb aplicacions potencials en la formulació de nous productes.

Finalment, el tercer capítol de la tesi s'ha centrat en obtenir farines de fava hidrolitzades utilitzant la impregnació al buit durant el remull per a incorporar enzims. Per a això, es va avaluar la cinètica d'hidratació de les faves durant el remull amb les solucions dels enzims papaïna i bromelina, es van obtenir les farines hidrolitzades i es va avaluar l'efecte del grau d'hidròlisi i l'eliminació de la casca sobre la digestibilitat proteica i el contingut en FAN. Els resultats obtinguts van mostrar que l'ús dels enzims no va modificar la cinètica d'hidratació de les faves i es van obtenir farines amb un grau d'hidròlisi del 9,1 % amb papaïna i del 8,8 % amb bromelina. La hidròlisi va augmentar uns certs FAN, com l'àcid fític i els tanins, però va reduir els inhibidors de tripsina. No obstant això, en general, no es van observar canvis significatius en la digestibilitat proteica. L'eliminació de la casca va augmentar el contingut en inhibidors de tripsina i àcid fític, però va reduir els tanins, la qual cosa va millorar la digestibilitat proteica. Aquest capítol destaca la importància d'aplicar tecnologies, com la impregnació al buit, per a transformar els llegums en ingredients amb un millor perfil nutricional.

En resum, la present tesi doctoral demostra el potencial dels llegums com a font de pèptids amb activitat antidiabètica, i mostra com l'ús de la impregnació al buit durant el remull és una tecnologia útil per a obtenir farines de fava amb un perfil nutricional millorat.

**Abstract**

Legumes are one of the main sources of plant protein and a sustainable and affordable alternative to animal protein. In addition, they may contain bioactive peptides, which can be released from proteins during gastrointestinal digestion (GID). However, their nutritional profile may be compromised by the presence of anti-nutritional factors (ANF), the effect of which can be mitigated by processing. Processing can help to reduce or eliminate these ANF, but can also affect protein digestion and, consequently, the release of peptides. Despite their benefits, consumption of legumes has declined in recent years, but it is essential for health and the environment that they gain prominence in the diet. One way of achieving this would be through their transformation into flour, which would allow them to be integrated into a wide variety of products. In this context, the aim of this doctoral thesis has been to evaluate the impact of processing and simulated GID on the bioactive properties of different legumes and to develop legume flours with an improved nutritional profile.

The first chapter of the thesis focused on evaluating how conventional, pressure and microwave cooking, followed by simulated GID, affect the antidiabetic activity and peptide profile of soybeans, navy beans, chickpeas, and green peas. For this purpose, legumes were cooked with the different treatments, and their inhibitory capacity of α-amylase and α-glucosidase enzymes was determined before and after undergoing simulated GID. Samples with the highest inhibitory activity were selected and fractionated by molecular size or polarity. Peptides from the fractions with the highest activity were identified by tandem mass spectrometry, and *in silico* analysis of the identified sequences was performed. The results showed an increase in α-amylase and α-glucosidase inhibitory activity after simulated GID, which could be attributed to the generation of bioactive peptides after protein hydrolysis by digestive enzymes. The fraction smaller than 3 kDa showed the highest α-amylase inhibitory activity, while the more polar fraction showed the highest α-glucosidase inhibitory activity. Although no clear pattern of the effect of

the different treatments on bioactivity was observed, microwave-cooked legumes showed higher α-amylase inhibitory activity, while pressure-cooked and conventional-cooked legumes showed higher α-glucosidase inhibitory activity, before and after fractionation, respectively. Based on *in silico* analysis, none of the peptides identified had been previously described as inhibitors of these enzymes, although some of them could be potential candidates due to their sequence and characteristics. This chapter demonstrates the potential of legumes as a source of antidiabetic peptides, although it would be necessary to synthesise the peptide sequences in order to confirm their biological activity.

The second chapter of the thesis focused on obtaining iron-fortified legume flours by using vacuum impregnation technology during the soaking step. For this purpose, the broad bean was selected as the legume with the highest porosity, and its hydration kinetics were evaluated to evaluate the effectiveness of the use of vacuum impregnation. Flours were then produced under different processing variables (fortification, cooking and/or dehulling) and their impact on different ANF, iron content and bioaccessibility, physicochemical and techno-functional properties, and stability during storage and cooking were evaluated. The results obtained indicated that vacuum impregnation accelerated the soaking time, reducing it by up to 75 %, and allowed the incorporation of iron into the beans without altering the hydration kinetics. Fortified flours had significantly higher levels of iron content and bioaccessibility compared to non-fortified flours. Cooking affected tannin content, iron content and bioaccessibility, and physicochemical and techno-functional properties, while dehulling affected tannin and iron content, and mineral bioaccessibility. Lipid and protein oxidation of the fortified flours remained stable during storage. Furthermore, the use of these flours in the production of fresh pasta showed that the iron content remained stable after cooking the pasta and increased its bioaccessibility. This chapter demonstrates that vacuum impregnation is an effective technology for developing iron-fortified legume flours, with potential applications in the formulation of new products.

Finally, the third chapter of the thesis focused on obtaining hydrolysed broad bean flours using vacuum impregnation during soaking to incorporate enzymes. For this purpose, the hydration kinetics of the broad beans during soaking with papain and bromelain enzyme solutions was evaluated, hydrolysed flours were obtained, and the effect of the degree of hydrolysis and dehulling on protein digestibility and ANF content was evaluated. The results obtained showed that the use of enzymes did not modify the hydration kinetics of the broad beans and allowed obtaining flours with a degree of hydrolysis of 9,1 % with papain and 8,8 % with bromelain. Hydrolysis increased certain ANF, such as phytic acid and tannins, but reduced trypsin inhibitors. However, in general, no significant changes in protein digestibility were observed. Dehulling increased the content of trypsin inhibitors and phytic acid, but reduced tannins, which improved protein digestibility. This chapter highlights the importance of applying technologies, such as vacuum impregnation, to transform legumes into ingredients with a better nutritional profile.

In summary, this doctoral thesis demonstrates the potential of legumes as a source of peptides with antidiabetic activity and shows how the use of vacuum impregnation during soaking is a useful technology to obtain broad bean flours with an improved nutritional profile.

# Índice

# Introducción

## 1. CONTEXTUALIZACIÓN

La malnutrición definida como la carencia, el desequilibrio o el exceso en la ingesta de nutrientes de una persona, es un problema de salud mundial. Es un término amplio que engloba tanto a la desnutrición, como a la ingesta insuficiente o excesiva de micronutrientes, y al sobrepeso, la obesidad y las enfermedades no transmisibles relacionadas con la dieta, como la diabetes y las enfermedades cardiovasculares (Organización Mundial de la Salud [OMS], 12/08/24).

En los países en vías de desarrollo, la desnutrición proteico-energética es uno de los problemas más graves de salud pública. Está provocada por el consumo de una dieta basada casi por completo en la ingesta de cereales, los cuales tienen un contenido muy bajo en proteínas, y son deficientes en lisina, triptófano y vitaminas del grupo B (Bessada et al., 2019; Temba et al., 2016). Sin embargo, en los países desarrollados, los problemas nutricionales provienen de una ingesta elevada de grasas saturadas e ingredientes refinados, lo que conlleva a un exceso de calorías y a una ingesta deficiente de micronutrientes y proteínas de calidad (Bessada et al., 2019).

En 2022, unos 149 millones de niños menores de 5 años, unos 190 millones de adolescentes entre 5 y 19 años, y alrededor de unos 390 millones de adultos mayores de 18 años sufrían algún tipo de desnutrición en el mundo. Paralelamente, 37 millones de niños menores de 5 años, unos 390 millones de adolescentes entre 5 y 19 años, y 2500 millones de adultos mayores de 18 años tenían sobrepeso u obesidad (OMS, 12/08/24).

Para solucionar los problemas de malnutrición existentes, la Organización Mundial de la Salud (OMS) y la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) proclamaron el 1 de abril de 2016 el Decenio de las Naciones Unidas de Acción sobre la Nutrición 2016-2025. Una iniciativa que fija un calendario para que se cumplan los compromisos acordados en la Segunda Conferencia Internacional sobre Nutrición (CIN2)

y en la Agenda 2030 para el Desarrollo Sostenible, en cuyos ejes centrales está la eliminación de todas las formas de malnutrición (OMS, 12/08/24).

Para facilitar su implementación, la OMS y la FAO elaboraron una guía de recursos, en donde se menciona la necesidad de que todo el mundo tenga acceso a alimentos suficientes, diversificados, seguros y nutritivos que contribuyan a dietas saludables; se aborda cómo los patrones dietéticos han cambiado y cómo esto ha afectado a la salud global, destacando la necesidad de cambiarlos hacia un mayor consumo de alimentos de origen vegetal y una reducción del consumo de alimentos de origen animal; y donde se destaca la importancia de transformar los sistemas alimentarios para que sean sostenibles y resilientes, proporcionando acceso a alimentos nutritivos durante todo el año (FAO, WHO, 2018).

En este sentido, el fomento del consumo de legumbres puede tener un papel fundamental, y por ello la FAO anunció el 2016 como el Año Internacional de las Legumbres (FAO, 2016). Las legumbres son una fuente de proteína vegetal que, combinada con cereales, aportan una proteína completa y de calidad (Bessada et al., 2019; Venkidasamy et al., 2019). Además, la proteína vegetal es mucho más asequible que la proteína de origen animal (Venkidasamy et al., 2019), lo que facilita que sea accesible en países en vías de desarrollo, y mucho más sostenible ya que las legumbres emiten hasta 250 veces menos gases de efecto invernadero por gramo de proteína, que la carne procedente de rumiantes (Yanni et al., 2024).

## 2. LAS LEGUMBRES

Las legumbres pertenecen a la familia vegetal *Fabaceae*, también llamada *Leguminosae*, y se consideran uno de los cultivos más importantes del mundo, ya que representan aproximadamente el 15 % de la tierra cultivable del planeta (Avilés-Gaxiola et al., 2018; Bessada et al., 2019). Entre las especies que se comercializan en seco destacan las alubias o frijoles, las lentejas, los garbanzos, los guisantes, los altramuces, el frijol bambara, las habas, las vezas o alverjas, los guandúes o gandules y las carillas o frijoles caupíes. Sin

embargo, la soja, las judías verdes o la alfalfa también pertenecen a esta familia, aunque la FAO las incluye en los vegetales y hortalizas (FAO, 2016).

A nivel medioambiental, el cultivo de legumbres es muy importante ya que tienen la capacidad de fijar el nitrógeno atmosférico, lo que permite reducir el uso de fertilizantes sintéticos; contribuyen a reducir las emisiones de gases de efecto invernadero; mejoran la estructura del suelo, favoreciendo la retención de agua y reduciendo la erosión gracias a sus profundas raíces; y tienen la posibilidad de crecer en condiciones desfavorables como la sequía (Azizi et al., 2024; Conti et al., 2021).

### 2.1. Producción y consumo

Según los datos recogidos en la base de datos de la FAO y teniendo en cuenta los granos de soja, en el año 2023 se produjeron más de 465 millones de toneladas de legumbres a nivel mundial, de los cuales más de 94 millones de toneladas fueron de legumbres secas. De estas, más de 11 millones de toneladas fueron producidas en Europa y aproximadamente 367 mil toneladas en España. Tanto a nivel mundial como en Europa, la legumbre con mayor producción fue la soja. Sin embargo, tal y como se puede observar en la Figura 1, la alubia fue la legumbre seca más producida a nivel mundial, mientras que, en Europa y España, el guisante ocupó el primer lugar (FAOSTAT, 2025).

En cuanto a su consumo, según el informe del Ministerio de Agricultura, Pesca y Alimentación (MAPA) sobre el consumo alimentario en España (2023), el mercado de las legumbres cayó un 1,2 % en volumen durante el año 2023, con aproximadamente 153 millones de kilogramos. Sin embargo, aumentó un 6 % en valor, suponiendo 332 millones de euros. El consumo per cápita se situó en 3,26 kg de legumbres por persona y año, lo que representa un 2,3 y 2,4 % menos que el año 2022 y 2019, respectivamente (Ministerio de Agricultura Pesca y Alimentación [MAPA], 2023, p.299). Además, estos datos muestran una gran disminución respecto al consumo que había en los años 60, el cual era de casi 15 kg/persona/año (Enjamio et al., 2016).

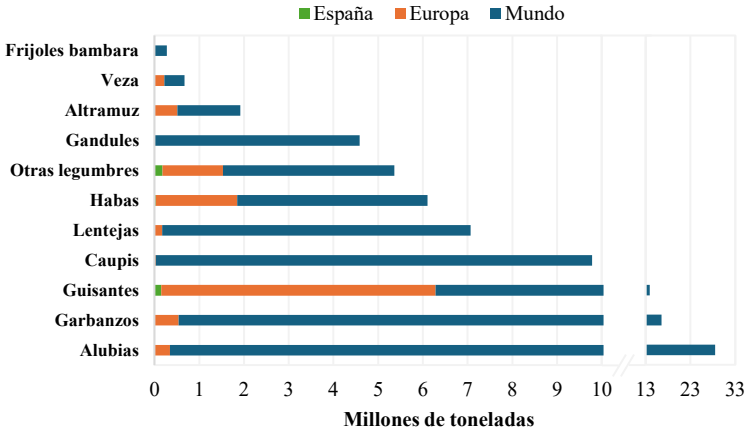


**Figura 1.** Producción de legumbres secas en el año 2023 a nivel mundial, en Europa y en España. Fuente: FAOSTAT, 2025.

La Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) recomienda entre 3 y 4 raciones de unos 60-80 g de legumbre cruda por semana, mientras que la mayoría de la población sólo consume una ración por semana (Enjamio et al., 2016). Esta tendencia puede ser debida a diferentes factores, como las preferencias gustativas, la evolución de las tendencias de la sociedad o la conveniencia. No obstante, el valor nutricional y los beneficios del consumo de legumbres no pueden ser subestimados y la industria alimentaria tiene un papel fundamental. En este sentido, una forma de impulsar e incrementar el consumo de legumbres es transformándolas en harina, ya que esta puede incorporarse a multitud de alimentos.

### 2.2. Perfil nutricional

Las legumbres son ricas en proteínas, hidratos de carbono, fibra, vitaminas, minerales y compuestos bioactivos (Bessada et al., 2019). En la Tabla 1 se resume la composición nutricional de las legumbres secas más comunes. Son una de las principales fuentes de proteína de origen vegetal, ya que, en general, su contenido oscila entre el 15 % y el 40 %. La soja y los altramuces son los que presentan el mayor contenido proteico (36 %), seguidos de las

habas (26 %), las lentejas (25 %), los guisantes y las alubias (22 %) y los garbanzos (19 %) (*BEDCA*). Sin embargo, este contenido en proteínas puede variar según la especie, el genotipo y las condiciones medioambientales (Bessada et al., 2019; Boye et al., 2010).

**Tabla 1.** Información nutricional de los macronutrientes y los principales micronutrientes de las legumbres crudas y secas más comunes (g/100 g).

| | **Alubia** | **Garbanzo** | **Lenteja** | **Guisante** | **Haba** | **Altramuz** | **Soja** |
|---|---|---|---|---|---|---|---|
| Proteína | 22 | 19 | 25 | 22 | 26 | 36 | 36 |
| Hidratos de carbono | 42 | 49 | 49 | 56 | 58 | 40 | 10 |
| Grasa | 1,3 | 6,3 | 1,2 | 2,3 | 1,5 | 9,7 | 18,6 |
| Fibra | 20 | 15 | 10 | 17 | 25 | 18,9 | 16 |
| Vitaminas (mg) | | | | | | | |
| A | 0,001 | 0,02 | 0,01 | 0,042 | 0,005 | 0,006 | 0,002 |
| B1 | 0,5 | 0,45 | 0,62 | 0,7 | 0,5 | 0,64 | 0,61 |
| B2 | 0,17 | 0,14 | 0,39 | 0,2 | 0,26 | 0,22 | 0,27 |
| B3 | 2,4 | 1,7 | 3 | 5,2 | 26 | 2,19 | 7,9 |
| B6 | 0,46 | 0,15 | 0,65 | 0,13 | 0,37 | 0,36 | 0,38 |
| B9 | 0,31 | 0,19 | 0,12 | 0,042 | 0,4 | 0,34 | 370 |
| C | 1,7 | 4,1 | 1,7 | 2 | 1,4 | 4,8 | - |
| Minerales (mg) | | | | | | | |
| Hierro | 5,2 | 6,8 | 6,9 | 5,3 | 5,5 | 4,36 | 9,7 |
| Zinc | 3,7 | 2 | 3,9 | 3,5 | 3,1 | 4,75 | 4,3 |
| Calcio | 139 | 143 | 57 | 72 | 100 | 176 | 240 |
| Magnesio | 135 | 122 | 74 | 123 | 190 | 198 | 250 |
| Fósforo | 470 | 310 | 256 | 330 | 590 | 440 | 660 |
| Potasio | 1718 | 1000 | 463 | 900 | 1090 | 1013 | 1730 |

Fuente: Base de datos BEDCA.

Las proteínas de las legumbres se pueden clasificar según su solubilidad en albúminas (solubles en agua), globulinas (solubles en soluciones salinas), prolaminas (solubles en soluciones ligeramente ácidas o alcalinas) y

glutelinas (solubles en alcohol) (Bouchard et al., 2022; Venkidasamy et al., 2019). En mayor proporción se encuentran las globulinas, de las que destacan la legumina (11S) y la vicilina (7S), así como las albúminas, que engloban principalmente a enzimas, inhibidores de las proteasas, inhibidores de las amilasas y lectinas (Boye et al., 2010).

En general, la calidad nutricional de las proteínas viene determinada por su composición en aminoácidos esenciales y su digestibilidad. En el caso de las legumbres, son generalmente ricas en lisina, leucina, arginina y ácido aspártico, pero son deficientes en metionina, cisteína y triptófano (Bessada et al., 2019; Boye et al., 2010). Por el contrario, los cereales son deficientes en lisina, con lo cual, al combinarlos se obtiene una proteína vegetal completa (Bessada et al., 2019; Venkidasamy et al., 2019). En cuanto a la digestibilidad, la proteína de origen vegetal es generalmente menos digestible que la proteína de origen animal debido a su estructura, ya que predomina la conformación de la hoja β frente a la conformación de hélice α, y a la presencia de factores antinutricionales (FAN) como los inhibidores de las proteasas que inhiben a las enzimas gastrointestinales durante la digestión (Bessada et al., 2019; Venkidasamy et al., 2019).

Dependiendo de la especie, el contenido en hidratos de carbono puede variar entre un 40 y un 75 % en peso seco (Hall et al., 2017), y se componen principalmente por almidón, mayoritariamente resistente y de digestión lenta; polisacáridos como la celulosa, la hemicelulosa y las pectinas (Azarpazhooh y Ahmed, 2022; Venkidasamy et al., 2019); y oligosacáridos como la rafinosa, estaquiosa y verbascosa, los cuales son resistentes a la digestión, y pasan al intestino grueso donde son fermentados por la flora microbiana, provocando la liberación de gases y ácidos grasos de cadena corta (Venkidasamy et al., 2019).

En general, las legumbres contienen un bajo contenido en grasas, mayoritariamente triglicéridos ricos en ácidos grasos monoinsaturados y poliinsaturados. En cuanto a la fibra, las legumbres pueden contener entre un 3 y un 35 % en peso seco, siendo en su mayoría fibra insoluble (Hall et al.,

2017). Esta engloba a los compuestos resistentes a la digestión y absorción en el intestino delgado, con fermentación completa o parcial en el intestino grueso, como los polisacáridos, oligosacáridos, lignina y otras sustancias vegetales asociadas. Por último, en cuanto a las vitaminas y minerales, las legumbres destacan por su contenido en vitaminas del grupo B y ácido fólico, así como en hierro, zinc, calcio, magnesio, fósforo y potasio (Martín-Cabrejas, 2019).

### 2.3. Factores antinutricionales

Las legumbres son un alimento muy completo desde el punto de vista nutricional, aunque su consumo queda muchas veces condicionado por la presencia de FAN, metabolitos secundarios de origen natural asociados a distintos mecanismos de defensa de las plantas (Bessada et al., 2019; Kumar et al., 2022). Estos compuestos pueden ser tóxicos o antinutritivos para los animales y los humanos, ya que afectan a la digestibilidad de las proteínas y a la biodisponibilidad de aminoácidos y minerales (Bessada et al., 2019). Sin embargo, se ha demostrado que pueden tener propiedades beneficiosas si se consumen en cantidades adecuadas (Das et al., 2022).

Tal y como se muestra en la Figura 2, los FAN se pueden clasificar en dos grupos, los FAN de naturaleza proteica y termolábiles y los de naturaleza no proteica y termoestables. Los FAN termolábiles más comunes son los inhibidores enzimáticos (de tripsina, quimotripsina y α-amilasa) y las lectinas, mientras que los FAN termoestables incluyen al ácido fítico, los alcaloides, las saponinas, los oligosacáridos y algunos compuestos fenólicos, entre los que destacan los taninos (Bessada et al., 2019).

Entre los inhibidores enzimáticos, los más comunes son los inhibidores de las proteasas, comúnmente englobados bajo el nombre de inhibidores de tripsina (IT), y los inhibidores de la α-amilasa. En las legumbres, se pueden encontrar dos tipos de IT, los Kunitz con un peso molecular de 20 kDa, y los Bowman-Birk con un peso molecular de 8 kDa (Avilés-Gaxiola et al., 2018; Das et al., 2022; Kumar et al., 2022). Estos son resistentes al pH ácido del

estómago y a la acción de la pepsina (Bessada et al., 2019), y bloquean la actividad de las enzimas pancreáticas, tripsina y quimiotripsina, reduciendo así la digestión y la absorción de las proteínas (Avilés-Gaxiola et al., 2018). Sin embargo, se ha descrito que tienen una potente actividad antiinflamatoria (Bessada et al., 2019; Kumar et al., 2022). Los inhibidores de la α-amilasa interfieren en la actividad de las enzimas α-amilasa salival y pancreática, las cuales son responsables de hidrolizar el almidón en oligosacáridos. Por lo tanto, dificultan y ralentizan la digestión del almidón, lo que es un efecto deseable para pacientes con diabetes (Kumar et al., 2022).

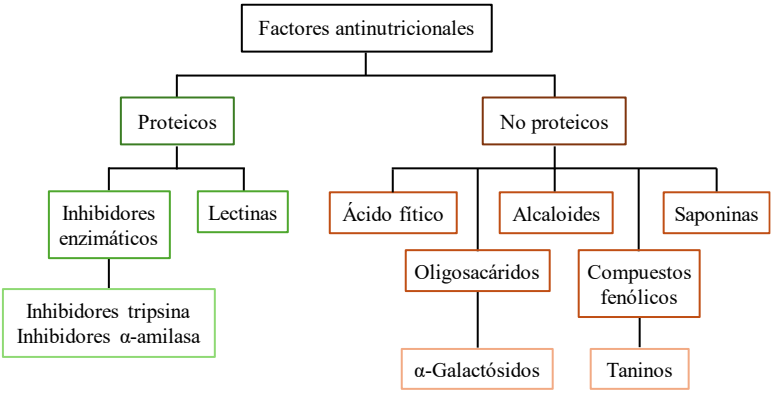


**Figura 2.** Factores antinutricionales más comunes presentes en las legumbres.

Las lectinas, también conocidas como hemaglutininas debido su capacidad para aglutinar eritrocitos, se definen como proteínas o glicoproteínas de naturaleza no inmune capaces de reconocer y unirse de forma reversible y con alta especificidad a carbohidratos simples o complejos (Kumar et al., 2022). Su toxicidad se debe a que son resistentes a las enzimas del tracto gastrointestinal (López-Moreno et al., 2022) y se pueden adherir a las células de la mucosa intestinal interfiriendo en el transporte y la absorción de los nutrientes (Khajali y Rafiei, 2024). Sin embargo, a bajas dosis pueden tener efectos beneficiosos para la salud ya que se ha descrito que tienen actividad antitumoral y antidiabética (López-Moreno et al., 2022).

El ácido fítico, también conocido como inositol fosfato o fitato cuando está en forma de sal, es la principal forma de almacenamiento de fósforo en los tejidos vegetales (Rousseau et al., 2020). En las legumbres secas, el 83 % del inositol fosfato se encuentra como myo-inositol hexafosfato (IP6) (Kumar et al., 2022), el cual es una molécula formada por un anillo de inositol rodeado por 6 grupos fosfato, y localizado principalmente en el cotiledón (Khajali y Rafiei, 2024). Este compuesto existe como ion cargado negativamente en un amplio rango de pH (a partir de pH > 2) (Rousseau et al., 2020), lo que hace que tenga afinidad por iones cargados positivamente, en particular, iones divalentes, proteínas y polisacáridos (Khajali y Rafiei, 2024). En el caso de los minerales como el hierro, el zinc o el calcio, con el pH ácido del estómago, el ácido fítico tiene un efecto quelante y forma complejos solubles con estos. Sin embargo, al aumentar el pH en el intestino, los complejos precipitan, lo que dificulta su absorción y reduce su biodisponibilidad (López-Moreno et al., 2022). Asimismo, los minerales también actúan como cofactores de las enzimas, con lo cual, cuando son quelados pueden disminuir la actividad de las enzimas digestivas. El ácido fítico también forma complejos con las proteínas, lo que disminuye su solubilidad y puede afectar a las enzimas digestivas, reduciendo la digestibilidad de los nutrientes (Bessada et al., 2019). Además, también puede afectar a la digestibilidad del almidón al unirse a este a través de enlaces fosfato (Kumar et al., 2022). Sin embargo, se ha demostrado que el ácido fítico puede tener efectos beneficiosos, ya que puede ejercer actividad antioxidante, neuroprotectora o antitumoral, y puede regular algunos trastornos metabólicos (López-Moreno et al., 2022).

Los alcaloides forman parte de un grupo estructuralmente diverso compuesto por más de 21.000 metabolitos secundarios cíclicos nitrogenados (Osorio y Till, 2022). Dentro del grupo de las legumbres destacan los altramuces por contener cantidades considerables de alcaloides de quinolizidina (Jezierny et al., 2010; Osorio y Till, 2022). Su toxicidad se traduce en alteraciones del sistema nervioso central, de los procesos digestivos, de la reproducción y del sistema inmunitario (Jezierny et al., 2010).

Las saponinas son glucósidos anfipáticos que tienen una parte lipofílica llamada aglicona, que puede ser un esteroide o terpenoide, conjugada con una o más fracciones de oligosacáridos hidrofílicos (pentosa, hexosa o ácido urónico), por lo que presentan propiedades tensioactivas. En la familia de las leguminosas, los glucósidos triterpenoides son los más comunes (Das et al., 2022; Kumar et al., 2022). Su efecto antinutricional se debe a que reaccionan con el colesterol de las membranas de las células de la mucosa del intestino delgado provocando cambios en la permeabilidad celular y hemólisis (Jezierny et al., 2010; Khajali y Rafiei, 2024). Además, pueden inhibir las enzimas digestivas, como la amilasa, la lipasa y la tripsina, lo que afecta a la digestibilidad de los polisacáridos, los lípidos y las proteínas (Khajali y Rafiei, 2024). Sin embargo, son muy importantes en la dieta porque tienen actividad hipocolesterolémica, hipoglucémica, anticancerígena, antinflamatoria, antimutagénica, inmunomuduladora y neuroprotectora (Kumar et al., 2022).

Los galacto-oligosacáridos son oligosacáridos constituidos por una unidad terminal de glucosa y dos o más unidades de galactosa. Dentro de este grupo están los α-galactósidos (Thirunathan y Manickavasagan, 2019), los cuales son oligosacáridos no reductores de bajo peso molecular con enlaces α(1→6) entre las moléculas de α-galactosa, que a su vez se dividen en oligosacáridos de la familia de la rafinosa y en galacto-cilitoles (Das et al., 2022). Las legumbres son el cultivo que contiene mayor cantidad de estos oligosacáridos, siendo la rafinosa, estaquiosa y verbascosa algunos de los más comunes (Das et al., 2022). Su efecto como antinutrientes se debe a que los monogástricos no disponen de la enzima α-galactosidasa en el intestino, con lo cual estos oligosacáridos pasan al intestino grueso sin ser digeridos, y allí son fermentados por las bacterias de la microbiota produciendo ácidos grasos de cadena corta y gases que generan flatulencias y mal estar, pudiendo incluso provocar diarrea si se consumen en grandes cantidades (Das et al., 2022; Kumar et al., 2022). Sin embargo, estos oligosacáridos forman parte de la fibra y tienen sus mismos efectos beneficiosos, como facilitar el tránsito intestinal, aumentar la población de lactobacilos y bifidobacterias, disminuir

las enterobacterias en el intestino, y reducir las posibilidades de enfermedades coronarias (Kumar et al., 2022).

Los compuestos fenólicos son un grupo muy numeroso de metabolitos secundarios presentes en las plantas, compuestos por un anillo aromático al que se unen uno o más grupos hidroxilo, por lo que abarcan desde moléculas simples a polímeros muy complejos. En función del número de grupos hidroxilo fenólicos unidos y de los elementos estructurales que unen los anillos aromáticos, estos se dividen en cuatro subgrupos: ácidos fenólicos, flavonoides, taninos y estibenos. A su vez, los flavonoides se dividen en flavonoles, flavonas, flavanoles, flavanonas, antocianidinas e isoflavonas, mientras que los taninos se dividen en hidrolizables y condensados (no hidrolizables). En las legumbres, los compuestos fenólicos mayoritarios son los ácidos fenólicos, los flavonoides y los taninos condensados (Singh et al., 2017). Dependiendo de la estructura, los compuestos fenólicos pueden tener propiedades quelantes de metales, ya que estos pueden interactuar con los grupos hidroxilo y carboxilo de los compuestos fenólicos y formar complejos. Con lo cual, pueden dificultar la absorción de los minerales, disminuyendo su bioaccesibilidad y biodisponibilidad. Además, pueden formar complejos insolubles con las proteínas y los carbohidratos, reduciendo su digestibilidad (Kumar et al., 2022; Rousseau et al., 2020). Concretamente, los taninos pueden formar complejos con las proteínas a través de interacciones hidrofóbicas o puentes de hidrógeno, y precipitar (Das et al., 2022). En boca forman complejos con las glicoproteínas de la saliva, por ello proporcionan astringencia (Jezierny et al., 2010) y, además, pueden inhibir a las enzimas digestivas, lo que también dificulta la digestibilidad de otros nutrientes (Das et al., 2022). Sin embargo, como bien se conoce, los compuestos fenólicos tienen muchas propiedades beneficiosas para la salud ya que son antioxidantes. Además, se ha demostrado que pueden ayudar a controlar la diabetes tipo 2 y la obesidad, y pueden actuar como agentes antinflamatorios, anticancerígenos y neuroprotectores (Singh et al., 2017).

## 3. DIGESTIBILIDAD, BIODISPONIBILIDAD, BIOACCESIBILIDAD Y PROPIEDADES BIOACTIVAS DE LAS LEGUMBRES

La calidad y eficacia nutricional de un alimento se determina mediante factores como la digestibilidad, biodisponibilidad, bioaccesibilidad y bioactividad. La digestibilidad es la fracción de los componentes de los alimentos que se transforman durante la digestión en materia potencialmente accesible a través de todos los procesos fisicoquímicos que tienen lugar en el lumen del sistema digestivo (Carbonell-Capella et al., 2014; Lucas-Gonzalez et al., 2018). Aunque no tienen una definición consensuada, en los estudios sobre matrices alimentarias y su relación con los efectos nutricionales, la bioaccesibilidad se define como la fracción de un compuesto que se libera de la matriz alimentaria y queda disponible para su absorción (Aguilera, 2019; Cilla et al., 2018; Dima et al., 2020), mientras que la biodisponibilidad se define como a la fracción de un compuesto que se libera, absorbe y llega al torrente sanguíneo (Aguilera, 2019; Lucas-Gonzalez et al. 2018). Asimismo, la bioactividad se refiere a la capacidad de un compuesto para producir un efecto específico en el organismo (Aguilera, 2019; Dima et al., 2020). Para poder estudiar y evaluar cada uno de estos términos son fundamentales los modelos de digestión gastrointestinal.

### 3.1. Modelos de digestión gastrointestinal

La digestión gastrointestinal (DGI) es un proceso complejo, en el que los alimentos ingeridos se transforman en nutrientes mediante alteraciones mecánicas y enzimáticas, para posteriormente ser absorbidos y utilizados por el organismo como fuente de energía para el mantenimiento y el crecimiento celular (Lucas-González et al., 2018). El proceso empieza en la boca con la masticación, acción mecánica que contribuye a descomponer la matriz de los alimentos. A la vez, en la misma fase oral, los alimentos se mezclan con la saliva que contiene α-amilasa, la cual es la enzima responsable de empezar la descomposición de los carbohidratos, y se forma el bolo alimenticio. Seguidamente, al tragar, el bolo llega al estómago y da comienzo la fase gástrica.

En esta etapa, la pepsina comienza la descomposición de las proteínas, generando péptidos grandes u oligopéptidos, y algunos aminoácidos, a la vez que las lipasas inician la descomposición de las grasas. Finalmente, en la fase intestinal, el páncreas libera jugo pancreático con enzimas como proteasas, peptidasas, y lipasa y amilasa pancreáticas. Durante esta fase, continúa la hidrólisis de las proteínas en péptidos pequeños y aminoácidos, mientras las amilasas terminan de descomponer los carbohidratos, y la lipasa pancreática, junto con la colipasa y las sales biliares, completan la digestión de las grasas iniciada en el estómago (Mackie et al., 2020).

Para poder estudiar la transformación de los alimentos en nutrientes se utilizan modelos de digestión que pueden ser *in vivo*, *in vitro* o *in silico*. Para las digestiones *in vivo* se suelen utilizar animales o humanos, y aunque suelen aportar los datos más precisos, son modelos costosos y complejos a nivel técnico, y están limitados por cuestiones éticas. Como alternativa surgen los modelos *in vitro* (Lucas-González et al., 2018). Estos tratan de imitar los procesos fisiológicos que tienen lugar durante la digestión y normalmente incluyen la simulación de las fases oral, gástrica e intestinal, y en ocasiones la fermentación colónica (Minekus et al., 2014). Existen distintos tipos de modelos *in vitro* que pueden ser más o menos complejos. Los modelos más simples son los estáticos donde los parámetros de la digestión (ratio alimento-fluidos digestivos, pH, concentración de enzimas…) son fijos, y se puede simular una sola etapa o todas de forma secuencial (Mackie et al., 2020). Por otro lado, los modelos de digestión semi-dinámicos simulan alguna de las características dinámicas del tracto gastrointestinal, como la modificación del pH del quimo gástrico y/o la liberación controlada de las enzimas (Mulet-Cabero et al., 2020). Por último, los modelos dinámicos tratan de imitar muchas de las variables del tracto digestivo, como la secreción de fluidos digestivos, la concentración variable de las enzimas, los cambios de pH, el tránsito del quimo y la mezcla adecuada en cada una de las etapas debido a los movimientos peristálticos. A su vez, estos modelos pueden ser de un solo compartimento, de dos o de varios, dependiendo del número de partes del sistema digestivo que se simulen. Estos modelos, además de ser técnicamente

mucho más complejos que los estáticos, son costosos, requieren mucho tiempo de preparación y los datos que se obtienen son mucho más difíciles de interpretar (Mackie et al., 2020). De entre los tres tipos de modelos de digestión *in vitro,* los más utilizados son los estáticos (Lucas-González et al., 2018; Minekus et al., 2014), ya que son más baratos y rápidos, requieren poca mano de obra y no tienen limitaciones éticas. Sin embargo, la falta de estandarización de las condiciones fisiológicas dificulta la comparación de los resultados obtenidos (Minekus et al., 2014).

Con motivo de esa falta de armonización y estandarización de las condiciones para simular la DGI *in vitro*, la red internacional INFOGEST publicó el primer protocolo estandarizado en 2014, el cual fue perfeccionado y detallado en 2019 (Brodkorb et al., 2019; Minekus et al., 2014). Este protocolo estático incluye las etapas oral, gástrica e intestinal, y establece una ratio constante de comida y fluidos digestivos en cada fase basada en las condiciones fisiológicas. También especifica la cantidad de electrolitos, el factor de dilución, la actividad de las enzimas y concentración de sales biliares, el pH y el tiempo a utilizar en cada etapa de la digestión tal y como se detalla en la Figura 3. Además, incluye ensayos estandarizados para determinar la actividad enzimática de cada una de las enzimas utilizadas, con el objetivo de armonizar su adición en función de la actividad y no del peso, y proporciona recomendaciones sobre cómo detener las actividades enzimáticas tras la digestión, en función de los análisis posteriores, para mejorar la comparación entre estudios experimentales. El protocolo se ha evaluado y ha sido reproducible y robusto en experimentos entre laboratorios, y los resultados en proteínas lácteas han sido comparables a los resultados *in vivo* (Santos-Sánchez et al., 2024). Así pues, se trata de un protocolo útil y simple para estudiar la degradación de alimentos complejos o sus componentes en el tracto gastrointestinal, así como herramienta previa a estudios de biodisponibilidad en líneas celulares o de fermentación colónica, o a la realización de ensayos *in vivo*.

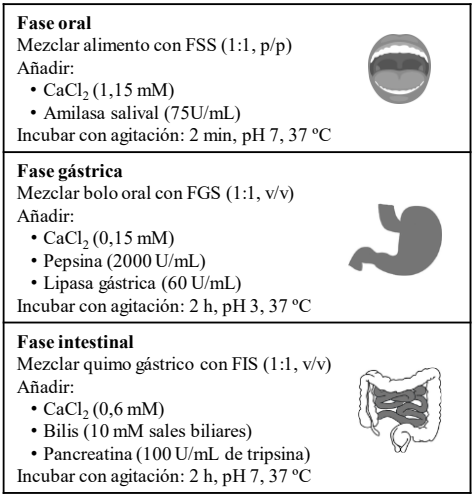


**Figura 3.** Protocolo de la digestión gastrointestinal *in vitro* definido por la red internacional INFOGEST. FSS: fluido salival simulado; FGS: fluido gástrico simulado; FIS: fluido intestinal simulado.

Por otro lado, los modelos de digestión *in silico* utilizan algoritmos informáticos que tratan de reproducir los resultados obtenidos con los modelos de digestión *in vivo* e *in vitro* (Dixit et al., 2024). Estos modelos son muy interesantes porque son rápidos y económicos, sin embargo, todavía están poco desarrollados debido a la complejidad del proceso de digestión (Mackie et al., 2020). En proteómica, por ejemplo, estos modelos computacionales se utilizan para predecir fragmentos proteicos o péptidos que se generarían por acción de proteasas digestivas previamente definidas como pepsina, tripsina y quimotripsina. Para ello, se utilizan herramientas bioinformáticas como PeptideCutter o BIOPEP (Rivero-Pino et al., 2023).

### 3.2. Digestibilidad proteica

Las legumbres son una fuente de proteínas, las cuales son macronutrientes de la dieta que aportan nitrógeno y aminoácidos esenciales. Sin embargo, su utilización biológica depende de su digestibilidad y de la absorción de

aminoácidos, dipéptidos y tripéptidos en el tracto gastrointestinal (Santos-Sánchez et al., 2024). A su vez, la digestibilidad está determinada por la estructura proteica y la accesibilidad de las enzimas, y depende tanto del efecto del procesado como de la matriz alimentaria (Drulyte y Orlien, 2019; Gu et al., 2023).

Para determinar la digestibilidad proteica se pueden utilizar métodos *in vivo* o *in vitro*, dependiendo del modelo utilizado para simular la DGI (Drulyte y Orlien). Tras simular la DGI *in vitro*, los métodos más utilizados son los basados en el cambio de pH, en la precipitación proteica y en la separación por tamaño molecular. Aunque todos ellos presentan limitaciones, el método de precipitación proteica es el más reproducible, ya que se basa en centrifugar la muestra y hacer determinaciones en la fracción soluble e insoluble (Santos-Sánchez et al., 2024).

En general, la digestibilidad proteica de las legumbres es baja. Los valores en legumbres crudas oscilan entre el 40 y el 84 % (King et al., 2024). Sin embargo, los valores de una misma legumbre pueden variar debido al modelo utilizado para simular la DGI, a la metodología empleada para determinar la digestibilidad proteica o, simplemente, a la variedad o las condiciones de crecimiento de la legumbre (Azizi et al., 2024). No obstante, se ha demostrado que la digestibilidad proteica puede mejorar hasta en un 20 % con el procesado de las legumbres (King et al., 2024). Por ello, es de interés seguir investigando en cómo tratar cada una de las legumbres para mejorar su digestibilidad, ya que proporcionan una proteína mucho más asequible (Venkidasamy et al., 2019) y sostenible (Yanni et al., 2024) que la de origen animal.

### 3.3. Bioaccesibilidad y biodisponibilidad

Las legumbres son un alimento de alto valor nutricional debido a su elevado contenido en micronutrientes y compuestos bioactivos. Entre estos destacan los minerales (Martín-Cabrejas, 2019), así como una amplia variedad de péptidos bioactivos (Bouchard et al., 2022; Shea et al., 2024). Sin

embargo, estos compuestos necesitan liberarse de la matriz alimentaria y ser absorbidos para poder ser aprovechados por el organismo. Por ello, resulta fundamental evaluar su bioaccesibilidad y biodisponibilidad.

Para determinar la biodisponibilidad de los minerales y los péptidos se pueden utilizar tanto métodos *in vivo* como *in vitro*. Para la determinación *in vitro* se suele combinar la simulación de la DGI con el estudio de líneas celulares como las Caco-2, que permiten evaluar los mecanismos de transporte a través de la membrana epitelial intestinal. Si sólo se simula la DGI, se estará determinando la fracción bioaccesible y no la biodisponible (Álvarez-Olguín et al., 2022; Cilla et al., 2018).

Entre los distintos minerales, las legumbres son ricas en hierro (Martín-Cabrejas, 2019), el cual es un mineral de gran importancia en la dieta ya que su carencia es una de las principales causas de malnutrición por ingesta insuficiente de micronutrientes en el mundo (Man et al., 2022). Sin embargo, su bioaccesibilidad y biodisponibilidad suelen ser bajas debido a la presencia de FAN, como el ácido fítico y los taninos, que forman complejos insolubles e impiden su absorción (Kumar et al., 2022).

Para determinar el hierro bioaccesible *in vitro*, se utilizan principalmente dos métodos, el de solubilidad y el de dializabilidad. El método de solubilidad se basa en simular la DGI *in vitro* y determinar el hierro presente en el sobrenadante tras centrifugar o filtrar las muestras, mientras que el método de dializabilidad, se basa en lo mismo, pero cuando se simula la fase intestinal de la digestión se introduce una bolsa de diálisis, con lo cual, se determina el hierro presente en el dializado (Bohn et al., 2018; Cilla et al., 2018). Hemalatha et al. (2007ª, 2007b) determinaron el contenido en hierro y su porcentaje bioaccesible en legumbres crudas y procesadas, y observaron que el porcentaje de hierro bioaccesible era bajo, entre un 2 y un 10 %, y que mejoraba ligeramente con el procesado de las legumbres, aunque no en todos los casos. Dada la gran diferencia entre el contenido en hierro y el hierro bioaccesible de las legumbres, será de gran interés evaluar el porcentaje de hierro

bioaccessible y biodisponible cuando se formulen alimentos con legumbres, así como intentar mejorarlo con el procesado.

### 3.4. Bioactividad

Diversos estudios epidemiológicos han relacionado el consumo de legumbres con una menor incidencia de enfermedades cardiovasculares, cáncer colorrectal o diabetes tipo 2 (Bessada et al., 2019; Moreno-Valdespino et al., 2020). Estos beneficios han sido principalmente atribuidos a la presencia de compuestos bioactivos como péptidos y algunos FAN, tal y como se ha comentado anteriormente (Bessada et al., 2019).

Los péptidos bioactivos son pequeños fragmentos proteicos, generalmente de entre 2 y 20 aminoácidos. Estos pueden encontrarse de manera natural en los alimentos o ser generados a través de la hidrólisis de las proteínas (Carbonaro et al., 2015). La hidrólisis puede ocurrir durante el procesado de los alimentos, de forma natural durante la digestión gastrointestinal, o ser inducida mediante el uso de enzimas comerciales o microorganismos en condiciones controladas (Acquah et al., 2022). Los péptidos bioactivos permanecen inactivos mientras forman parte de la proteína de origen, pero se vuelven funcionales una vez que son liberados tras el proceso de hidrólisis (Pérez-Gregorio et al., 2020; Shea et al., 2024).

Se han identificado un gran número de péptidos bioactivos en las legumbres, con diferentes actividades como antihipertensiva, antimicrobiana, antioxidante, anticancerígena, hipocolesterolémica, inmunomoduladora, antidiabética o antiinflamatoria (Bouchard et al., 2022; Shea et al., 2024). No obstante, los péptidos con actividad antidiabética son de los menos estudiados hasta el momento. Falta profundizar en la evaluación de su estabilidad, eficacia y biodisponibilidad, así como en su alergenicidad y seguridad. Además, se necesita un mayor conocimiento de cómo se liberan en el tracto gastrointestinal cuando forman parte de su proteína de origen, lo que dependerá de la matriz alimentaria y las interacciones con otros componentes (Rahmi y Arcot, 2023).

### *3.4.1. Actividad antidiabética*

Según la Organización Mundial de la Salud, más de 220 millones de personas padecen actualmente diabetes *mellitus*, cifra que podría aumentar hasta los 336 millones en 2030 (Li et al., 2022). Esta enfermedad agrupa diversos trastornos metabólicos caracterizados por niveles elevados de glucosa en sangre, siendo los tipos más comunes la diabetes tipo 1 y tipo 2, que representan aproximadamente el 10 % y el 90 % de los casos, respectivamente (Patil et al., 2020). La diabetes tipo 2, la más prevalente, está relacionada con una producción insuficiente de insulina o resistencia a su acción, y suele presentarse en adultos mayores, aunque su incidencia está aumentando en edades más tempranas por factores como la obesidad, el sedentarismo y una dieta poco saludable (Chan-Zapata et al., 2022; Patil et al., 2020). Para su tratamiento, existen fármacos dirigidos a distintos mecanismos fisiológicos, como la inhibición de enzimas digestivas (α-amilasas, α-glucosidasas), la DPP-IV, el cotransportador SGLT2 o como agonistas del receptor GLP-1 (Acquah et al., 2022; Yan et al., 2019). No obstante, se ha descrito que los péptidos derivados de la hidrólisis de las proteínas de algunos alimentos también pueden ejercer esta bioactividad (Kehinde y Sharma, 2020; Patil et al., 2020).

Una de las estrategias más utilizadas para la detección de péptidos bioactivos con actividad antidiabética consiste en evaluar su capacidad de inhibir a las enzimas α-amilasas y α-glucosidasas. Las α-amilasas hidrolizan los carbohidratos complejos, como el almidón, en oligosacáridos y disacáridos, y son liberadas en la boca por las glándulas salivales y en el intestino delgado por el páncreas. Asimismo, las enzimas α-glucosidasas se sitúan en las células del borde en cepillo del intestino delgado e hidrolizan los oligosacáridos y disacáridos en glucosa. Por ello, tal y como se describe en la Figura 4, al inhibir estas enzimas se puede limitar la hidrólisis de los carbohidratos complejos en glucosa y ayudar a controlar la hiperglucemia postprandial (Iwaniak et al., 2018; Kehinde y Sharma, 2020).

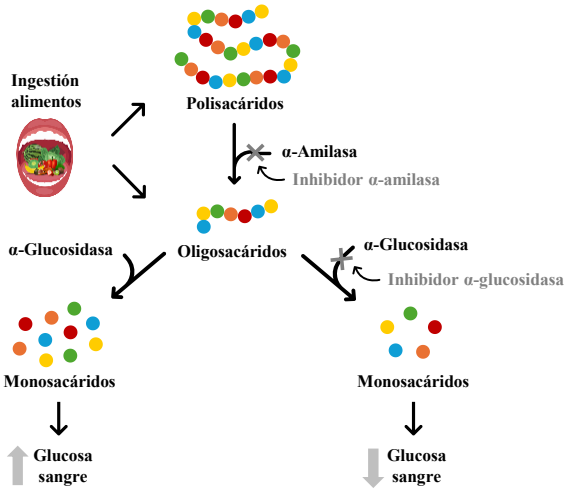


**Figura 4.** Mecanismo de acción de los inhibidores de la α-amilasa y la α-glucosidasa durante la digestión de los carbohidratos.

Distintos estudios señalan que se han obtenido péptidos con actividad inhibidora de la α-amilasa y la α-glucosidasa a partir de legumbres. Aunque la información es limitada (Acquah et al., 2022), el tamaño, la estructura molecular, así como la composición y secuencia de aminoácidos, desempeñan un papel importante en su actividad inhibidora (Li et al., 2022). Algunos estudios apuntan que los péptidos con actividad inhibidora de la α-amilasa presentan prolina en el extremo N- o C-terminal, o en ambos; glicina o fenilalanina en el extremo N-terminal; fenilalanina o leucina en el C-terminal; o dipéptidos que contienen los aminoácidos prolina, cisteína, leucina y glicina (González-Montoya et al., 2018; Ngoh y Gan, 2016). Además, la presencia de aminoácidos hidrofóbicos e hidrofílicos también puede tener un papel importante en dicha actividad (Castañeda-Pérez et al., 2021; Ngoh y Gan, 2018). Asimismo, varios péptidos descritos con actividad inhibidora de la enzima α-glucosidasa se ha detectado que contienen residuos de serina, treonina, tirosina, lisina o arginina en el extremo N-terminal; prolina cerca del extremo C-terminal; metionina o alanina en el extremo C-terminal (Di Stefano et al., 2018; Ibrahim

et al., 2018); valina, isoleucina y prolina en las posiciones 1-4 del extremo N-terminal; arginina y lisina en la extremo C-terminal; o prolina, alanina y serina en las posiciones 2-4 del extremo C-terminal (Mu et al., 2024).

## 4. PROCESADO DE LEGUMBRES Y SUS HARINAS

Las legumbres se comercializan generalmente secas, ya sea enteras o en forma de harina. Para obtener la harina, las legumbres se trituran con o sin piel en molinos de impacto, cuchillas o rodillos hasta obtener partículas muy pequeñas, que se pasan por diferentes tamices para asegurar la homogeneidad del tamaño de partícula (Malcolmson y Han, 2019). En comparación con la harina de trigo, esta harina contiene más proteínas y fibra, y menos hidratos de carbono, lo que la convierte en una buena alternativa para mejorar el perfil nutricional de muchos productos (Edwards et al., 2020; Keskin et al., 2022; Siddiqui et al., 2022). Sin embargo, como se ha comentado previamente, las legumbres contienen importantes cantidades de FAN y su digestibilidad suele ser baja. Además, cuando se añaden como harina cruda, aportan sabores no deseados, conocidos en inglés como "beany flavour" (Simons y Hall, 2018). Por ello, es muy habitual someterlas a distintos tipos de tratamientos antes de molerlas o consumirlas, siendo los más comunes un remojo seguido de un tratamiento térmico como la cocción (Chigwedere et al., 2019a). Sin embargo, otros tratamientos como el descascarillado, la cocción a presión, a vapor o en microondas, la extrusión, la germinación, la fermentación, las altas presiones hidrostáticas o la hidrólisis enzimática, también han demostrado mejorar su calidad nutricional (Bai et al., 2024; Gu et al., 2023).

### 4.1. Remojo

El remojo es un pretratamiento que consiste en hidratar a las semillas, generalmente con agua, y tiene como principales objetivos acortar el tiempo de cocción y reducir el nivel de FAN (Miano y Augusto, 2018ª). Durante el remojo, las legumbres alcanzan un contenido de humedad de equilibrio, que depende del género, la especie y la variedad de la legumbre; de las

características físicas como el tamaño, la dureza y la porosidad; de la composición química; del tipo de remojo, incluyendo la duración del proceso, la temperatura, el pH o la salinidad del medio; y de las condiciones de almacenamiento a las cuales han estado sometidas las legumbres antes del procesado (Chigwedere et al., 2019ª; Miano y Augusto, 2018ª).

Cuando hay un proceso de hidratac¡"n pr'vio, el tiempo de cocción de las legumbres se acorta al activarse las enzimas de la pared celular. Como consecuencia, disminuye el grado de polimerización de algunos polisacáridos pécticos y aumenta la solubilidad (Martínez-Manrique et al., 2011; Miano y Augusto, 2018ª). Además, el agua absorbida facilita la transferencia de calor, lo que mejora la inactivación de los antinutrientes termolábiles y homogeneiza la gelatinización del almidón, la desnaturalización de las proteínas y la solubilización de las pectinas durante la cocción (Miano y Augusto, 2018ª). Asimismo, durante el proceso, la concentración de algunos FAN disminuye debido a la lixiviación al medio de remojo y/o a la activación de algunas enzimas (Kalpanadevi y Mohan, 2013; Rousseau et al., 2020).

Para acelerar el proceso de hidratación de las legumbres se puede utilizar agua a altas temperaturas; un medio de remojo con un pH elevado, agentes quelantes o cationes monovalentes, para facilitar la solubilización de las pectinas; o combinar el proceso con otras tecnologías como los ultrasonidos, las altas presiones hidrostáticas o la impregnación a vacío (Chigwedere et al., 2019ª; Miano y Augusto, 2018ª).

La impregnación a vacío es una técnica que, tal y como se muestra en la Figura 5, consiste en introducir un producto sólido en una solución, reducir la presión del sistema haciendo vacío (despresurización) y, a continuación, restablecer la presión atmosférica. De esta forma se consigue que, durante la despresurización, el aire del interior del producto sea expulsado a través de los poros, y cuando se reestablece la presión, la solución fluya hacia el interior de los poros como consecuencia del gradiente de presión generado (Zanella-Díaz et al., 2014). Así, se potencia la eliminación de los gases, que en ocasiones pueden actuar como factor limitante del proceso de hidratación de las

legumbres (Chigwedere et al., 2019ª), y se reduce el tiempo de remojo. Sin embargo, hasta el momento no existen muchos estudios que evalúen el efecto de la aplicación de la impregnación a vacío en legumbres. Además, bien sea con impregnación a vacío o sin ella, la etapa de remojo consume un tiempo importante del procesado que se puede aprovechar para introducir compuestos hidrosolubles, como por ejemplo el hierro, y enriquecer las legumbres (Miano y Augusto, 2018b), o para incorporar enzimas y aprovechar la etapa para hidrolizar las proteínas.

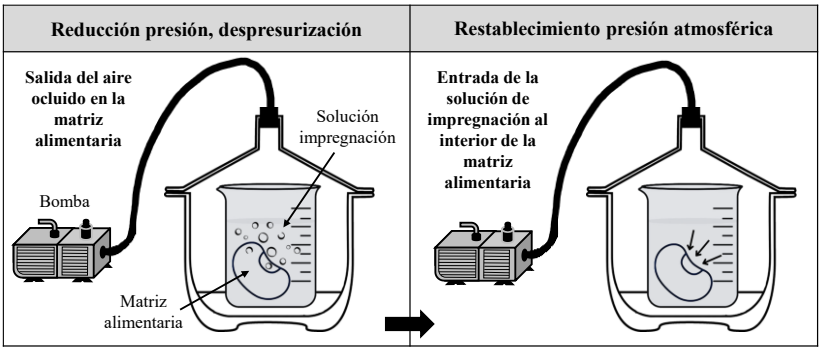


**Figura 5.** Esquema del proceso de impregnación a vacío de una legumbre, como ejemplo de una matriz alimentaria.

### 4.2. Descascarillado

La legumbre está compuesta por diferentes partes: la cubierta, la radícula, el hilio, el lóbulo radicular y el cotiledón. A su vez la cubierta de la semilla está compuesta por cuatro capas, una capa externa de cera que la hace impermeable seguida de una capa compuesta por compuestos hidrofóbicos, que también la hace impermeable al agua (Miano y Augusto, 2018ª). El descascarillado consiste en la eliminación de esta capa de la semilla y, generalmente, se realiza después del remojo de la legumbre. Esta técnica de procesado reduce el tiempo de cocción ya que facilita la entrada de agua. Además, mejora la palatabilidad y el sabor de algunas legumbres y aumenta la digestibilidad de las proteínas debido a la reducción de los niveles de compuestos fenólicos

y taninos que se encuentran principalmente en estas capas (Rousseau et al., 2020; Wang et al., 2009).

### 4.3. Tratamientos térmicos

Los tratamientos térmicos son los métodos de procesado de las legumbres más comunes tanto a nivel industrial como doméstico (Avilés-Gaxiola et al., 2018). Existen diferentes tipos de tratamientos (cocción convencional, a presión, al vapor, en microondas, …) que varían según el perfil de temperatura-tiempo y en el uso de calor seco o húmedo (Rousseau et al., 2020). Sin embargo, todos comparten algunas características. En general, los tratamientos térmicos mejoran la textura, la palatabilidad y el valor nutricional de las legumbres porque promueven la desnaturalización de las proteínas y la gelatinización del almidón mejorando su digestibilidad, la despolimerización y solubilización de las pectinas, y la reducción de los niveles de FAN (Avilés-Gaxiola et al., 2018; Gwala et al., 2020; Khatoon y Prakash, 2004; Teixeira-Guedes et al., 2019). No obstante, los tratamientos térmicos pueden influir negativamente en las características funcionales y nutricionales de las legumbres, ya que pueden reducir la solubilidad de algunos compuestos y dañar a los nutrientes termolábiles (Avilés-Gaxiola et al., 2018).

La cocción convencional es un tratamiento térmico que consiste en poner a las legumbres en una cantidad suficiente de agua y llevarlas lentamente a ebullición, o ponerlas directamente en una olla con agua hirviendo (Drulyte y Orlien, 2019). Independientemente, el objetivo principal del tratamiento es mejorar la palatabilidad de las legumbres consiguiendo una textura adecuada para su consumo. Existe controversia sobre cuál es la razón del ablandamiento de las legumbres durante la cocción. Algunos autores señalan que se debe a la gelatinización del almidón o a la desnaturalización de las proteínas, mientras que otros lo atribuyen a la solubilización de las pectinas. Chigwedere et al. (2019b) demostraron con la cocción de judías que el ablandamiento se debía a la solubilización de las pectinas ya que observaron que los granos seguían duros después de 30 minutos de cocción, cuando ya se había

producido la gelatinización del almidón y la desnaturalización de las proteínas. Además, analizaron la evolución de las pectinas, y observaron que la proporción de pectina poco ligada aumentaba, mientras que la pectina fuertemente ligada disminuía simultáneamente durante la cocción. Sin embargo, no hubo despolimerización de la pectina solubilizada (Chigwedere et al., 2019b).

Las altas temperaturas (superiores a 60 °C), además del ablandamiento de la legumbre, provocan un aumento de las pérdidas de sólidos solubles debido a los cambios de permeabilidad de las células, y una inactivación de los compuestos termolábiles, algunos de los cuales son FAN (Chigwedere et al., 2019ª; Prodanov et al., 2004). Además, también producen la lixiviación de algunos FAN al medio de cocción, siendo mayor en las legumbres que han sido previamente remojadas (Chigwedere et al., 2019b).

El tiempo de cocción es uno de los factores más importantes de este tratamiento y depende tanto del tipo de legumbre, como del pretratamiento realizado (Drulyte y Orlien, 2019). Al igual que en el remojo, es posible acortarlo utilizando agentes quelantes, cationes monovalentes o soluciones con un pH elevado, ya que se facilita la solubilización de las pectinas.

La cocción a presión y el autoclavado son dos tratamientos térmicos de alta intensidad ya que se supera el punto normal de ebullición (100 °C). En la cocción a presión, las legumbres se colocan con una determinada proporción de agua en un recipiente hermético conocido como olla a presión. Este se basa en atrapar el vapor que se produce por la ebullición del agua, lo que provoca que aumente la presión y por ende la temperatura de ebullición del agua hasta aproximadamente los 120 °C. En el caso del autoclavado, generalmente, las legumbres se colocan dentro del autoclave y se someten a vapor saturado a presión a 121 °C. En ambos métodos, se utiliza una presión de entre 1,8 y 2 bares para alcanzar la temperatura (Drulyte y Orlien, 2019). Como en el caso de la cocción convencional, el objetivo principal de estos tratamientos es obtener legumbres con una textura adecuada para su consumo. No obstante, estos tipos de cocción son más rápidos, ya que se alcanza

una temperatura más alta y, por lo tanto, se requiere menos tiempo (Güzel y Sayar, 2012; Thirunathan y Manickavasagan, 2019).

La cocción al vapor consiste en cocinar las legumbres en una atmósfera húmeda sin que entren en contacto con la fuente de calor ni con el líquido productor de vapor, que suele ser agua (Tan et al., 2011). Generalmente, la cocción al vapor tradicional se realiza a presión atmosférica utilizando una olla especializada compuesta por una olla perforada en la que se colocan las legumbres, que encaja con otra olla normal donde se coloca el agua (Xu y Chang, 2008). Sin embargo, también se puede hacer en autoclave o microondas (Tan et al., 2011). El objetivo del tratamiento es el mismo que el de los tratamientos térmicos anteriores, sin embargo, parece ser el mejor método para mantener la calidad nutricional, aunque no está muy estudiado para cocinar legumbres (Fabbri y Crosby, 2016).

Por último, la cocción en microondas es un tratamiento térmico que consiste en introducir las legumbres con una proporción de agua específica, en un microondas durante un periodo de tiempo determinado. Normalmente se utilizan proporciones de legumbre:agua entre 1:2 y 1:15 (p/v) y periodos de tiempo entre 15 y 30 minutos, aunque depende del tipo de legumbre, del pretratamiento y del tipo de microondas utilizado (Khattab y Arntfield, 2009; Ma et al., 2017; Y. Xu et al., 2014; Yadav et al., 2018). El objetivo del tratamiento es el mismo que el del resto de tratamientos térmicos, sin embargo, es más rápido porque el calor es generado por el agua de la propia matriz alimentaria. Las moléculas de agua absorben la energía de las microondas y la convierten en calor debido a la fricción generada por el movimiento de estas (Deng et al., 2015; Drulyte y Orlien, 2019; Embaby, 2010).

### 4.4. Hidrólisis proteica

La hidrólisis proteica es un proceso en el que se rompen los enlaces peptídicos, descomponiendo las proteínas en oligopéptidos, péptidos pequeños y aminoácidos libres. Tras este proceso también quedan expuestos grupos hidrofóbicos, lo cual puede mejorar la digestibilidad de las proteínas. Para

caracterizar el nivel de hidrólisis de la proteína, a menudo se evalúa el grado de hidrólisis, definido como el porcentaje de enlaces peptídicos escindidos en comparación con el total de enlaces peptídicos disponibles para la escisión. Sin embargo, es importante señalar que, el grado de hidrólisis es una simple cuantificación de los enlaces rotos y en ningún caso proporciona información sobre el tipo de péptidos y/o aminoácidos generados (Gouseti et al., 2023).

La hidrólisis puede llevarse a cabo de forma química, con ácidos o bases, o de forma enzimática (Gu et al., 2023). La hidrólisis química tiene la desventaja de que puede generar residuos de aminoácidos no deseados (Gu et al., 2023), mientras que la hidrólisis enzimática permite una hidrólisis controlada y unas condiciones de reacción suaves (Aryee y Boye, 2016).

La hidrólisis proteica con enzimas es una opción interesante para intentar mejorar la baja digestibilidad que presentan las legumbres, como ya se ha comentado previamente. Normalmente, esta hidrólisis se lleva a cabo mezclando harina o aislado de proteína de legumbre con la solución enzimática, se incuba en condiciones controladas durante un periodo de tiempo determinado y se seca (Eckert et al., 2019; Goertzen et al., 2021; Konieczny et al., 2020). El grado de hidrólisis alcanzado dependerá principalmente del tipo de proteína, de la concentración y especificidad de la enzima y las condiciones de reacción utilizadas. Se ha establecido un grado de hidrólisis del 10 % como el umbral entre una hidrólisis limitada o baja y una hidrólisis extensiva, aunque ello no tiene por qué relacionarse con las propiedades nutricionales y funcionales del hidrolizado obtenido (Gouseti et al., 2023). Además, se debe de tener en cuenta que un grado de hidrólisis excesivo puede provocar la agregación y precipitación de las proteínas debido a una mayor exposición de grupos hidrofóbicos (Mookerjee y Tanaka, 2023) así como sabores no deseados debido a la liberación de péptidos hidrofóbicos (Gu et al., 2023). En el caso de las legumbres, se ha descrito que un grado de hidrólisis en torno al 10-15 % es suficiente para mejorar las propiedades tecno-funcionales, nutricionales o sensoriales (Gouseti et al., 2023; Mookerjee y Tanaka, 2023).

Actualmente, las enzimas disponibles en el mercado para la hidrólisis de proteínas pueden ser de origen animal, vegetal o microbiano. Dentro del grupo de las enzimas de origen vegetal, la bromelina y la papaína, extraídas de la piña y la papaya, respectivamente, son de las más utilizadas (Gouseti et al., 2023). Ambas son endopeptidasas, presentan una secuencia de aminoácidos similar (Gurumallesh et al., 2019; Mazorra-Manzano et al., 2018), y han demostrado ser unas buenas candidatas para la obtención de hidrolizados proteicos.

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# Objetivos

El objetivo general de la presente tesis doctoral ha sido evaluar el impacto del procesado y la digestión gastrointestinal simulada sobre las propiedades bioactivas de diferentes legumbres y desarrollar harinas de legumbres con un perfil nutricional mejorado.

Para la consecución del objetivo general se definieron los siguientes objetivos específicos:

- Estudiar el efecto de distintos tratamientos térmicos y la digestión gastrointestinal simulada sobre la actividad antidiabética y el perfil peptídico de diferentes legumbres.
- Desarrollar harinas de legumbres enriquecidas en hierro mediante remojo con impregnación a vacío y evaluar su impacto sobre distintos factores antinutricionales, contenido y bioaccesibilidad del hierro, propiedades fisicoquímicas y tecno-funcionales y estabilidad durante el almacenamiento y la cocción.
- Desarrollar harinas de legumbres hidrolizadas con enzimas mediante remojo con impregnación a vacío y evaluar el efecto de la hidrólisis y el descascarillado sobre la digestibilidad proteica y el contenido en factores antinutricionales.

# Resultados

# Capítulo 1

## Propiedades antidiabéticas de las legumbres

### 1.1. Artículo 1

Arnal, M., Gallego, M., Talens, P., & Mora, L. (2023). Impact of thermal treatments and simulated gastrointestinal digestion on the α-amylase inhibitory activity of different legumes. *Food Chemistry*, *418*, 135884.

### 1.2. Artículo 2

Arnal, M., Gallego, M., Talens, P., & Mora, L. (2024). Peptidomic profile and α-glucosidase inhibitory activity of cooked and gastrointestinal digested legumes. *LWT – Food Science and Technology*, *201*, 116283.

# Impact of thermal treatments and simulated gastrointestinal digestion on the α-amylase inhibitory activity of different legumes

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**Abstract**

Legumes are excellent sources of proteins that can be hydrolysed to generate antidiabetic peptides, which inhibit carbohydrate digestive enzymes. The degree of protein hydrolysis depends on the thermal treatment applied and how it impacts protein denaturation and thus accessibility to enzymes. In this study, α-amylase inhibitory activities of cooked (conventional, pressure, and microwave cooking) and digested (simulated gastrointestinal digestion, GID) green pea, chickpea, and navy beans were evaluated, together with the impact of thermal treatments on peptide profiles after GID. All peptides extracts inhibited α-amylase after cooking and GID, and the peptide fraction <3 kDa was responsible for main activity. In green peas and navy beans, microwave cooking showed the highest impact whereas none thermal treatment highlighted in chickpeas. The peptidomics analysis of the fractions <3 kDa identified a total of 205 peptides, 43 of which were found to be potentially bioactive according to *in silico* analysis. Also quantitative results evidenced differences in the peptide profile between the type of legume and thermal treatment.

*Keywords: diabetes, legume, α-amylase inhibition, in vitro gastrointestinal digestion, peptides.*

## 1. INTRODUCTION

According to the World Health Organization, there are more than 220 million people who live with diabetes mellitus (DM) and prevalence is predicted to increase to 336 million people by 2030 (Li et al., 2022). A strategy to control and prevent it is managing carbohydrate digestibility by inhibiting starch digestive enzymes such as α-amylases as their inhibition can help to control postprandial hyperglycaemia (Iwaniak et al., 2018; Kehinde & Sharma, 2020).

Most common inhibitors are designed as synthetic drugs such as acarbose, miglitol, and voglibose (Li et al., 2022; Rivero-Pino et al., 2020). However, natural compounds present in the diet, such as some polysaccharides, peptides, unsaturated fatty acids, and phytosterols, could also act as inhibitors of carbohydrate hydrolysis (Li et al., 2022). In this regard, legumes are an excellent source of vegetable protein, mainly storage proteins (albumins, globulins and glutelins), whose amino acid profile is rich in lysine, leucine, aspartic acid, and arginine but poor in methionine, cysteine and tryptophan (Bessada et al., 2019). Their consumption is related to a lower incidence of DM (Singhal et al., 2014) and some studies have associated this potential with bioactive peptides. These are protein fragments ranging from 2 to 20 amino acid residues that exert their function once they are released from parent proteins after a hydrolysis process. Nevertheless, the available information about carbohydrate digestive enzyme inhibitory peptides is limited (Acquah et al., 2022). Molecular weight and molecular structure, as well as amino acid composition and sequence, are believed to play an important role in inhibitory activity (Li et al., 2022).

In general, most researches have been focused either on the evaluation of the inhibitory capacity of carbohydrate digestive enzymes of different legumes or on obtaining hydrolysates or peptides from legume proteins (González-Montoya et al., 2018; Mojica et al., 2015; Mojica & De Mejía, 2016; Ngoh & Gan, 2016). However, few studies have evaluated the carbohydrate digestive enzyme inhibitory capacity of processed legumes during

gastrointestinal digestion, which is interesting for different reasons (Ercan & El, 2016; Jakubczyk et al., 2017). On the one hand, the protein will reach a different degree of hydrolysis as it denatures and becomes more accessible to enzymes depending on the thermal treatment performed (Gu et al., 2022; Zhang & Chang, 2019) and, on the other hand, the consumption of legumes could be a natural and affordable way to prevent and control DM.

Therefore, the aim of this work was to study the α-amylase inhibitory activity of green pea, chickpea, and navy bean legumes during simulated gastrointestinal digestion (GID) after three types of household cooking (conventional, pressure, and microwave cooking), as well as to evaluate the impact of these thermal treatments on the peptide profile generated after GID.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Enzymes α-amylase (A3171) and pancreatin (P7545) from porcine pancreas and pepsin from porcine gastric mucosa (P7012), as well as bile extract porcine (B8631), soluble starch (S9765), 3,5-dinitrosalicylic acid (DNS) (D0550), and acarbose (PHR1253), were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Sodium potassium tartrate tetrahydrate was acquired from Scharlau Chemie, S.A. (Sentmenat, Barcelona, Spain). All other reagents and chemicals were of analytical grade.

### 2.2. Preparation of legumes

Green pea (*Pisum sativum*), chickpea (*Cicer Arietinum*), and navy bean (*Phaseolus vulgaris*) seeds were purchased from a supermarket (Lantomanage S.L., Pozuelo de Alarcon, Madrid, Spain). Three independent batches of 1 kg each were used per type of legume. Dry seeds were soaked overnight in distilled water at a legume:water ratio of 1:5 (w/v). After soaking, the water was drained off and the seeds were cooked in distilled water to a suitable texture for consumption, which was considered when the seed could be easily

squeezed with the fingers, using three different types of household cooking (conventional, pressure, and microwave cooking) with a legume:water ratio of 1:10 (w/v). In conventional cooking, green peas were cooked for 90 min, chickpeas for 60 min, and navy beans for 30 min. In pressure cooking, navy beans and chickpeas were cooked for 5 min and green peas for 8 min. Finally, in microwave cooking, chickpeas and navy beans were cooked for 30 min and green peas for 50 min. After cooking, the water was discarded, and the legumes were mashed using a hand blender to simulate the mastication for further *in vitro* gastrointestinal digestion.

### 2.3. *In vitro* gastrointestinal digestion

The *in vitro* gastrointestinal digestion (GID) of cooked legumes was performed according to the standardised INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014). The determination of enzyme activities and the preparation of digestive fluids (simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)) were also carried out as described in the harmonised protocol (Brodkorb et al., 2019; Minekus et al., 2014). In brief, to simulate the oral phase, 1.5 g of mashed legume was diluted (1:1, w/v) with SSF containing α-amylase (75 U/mL) and incubated for 2 min. The reaction was stopped by decreasing the pH at 3 with 1 M HCl. The gastric phase was simulated by diluting the oral bolus (1:1, v/v) with SGF containing pepsin (2000 U/mL) and incubating for 120 min. This phase was stopped by increasing the pH at 7 with 1 M NaOH. Finally, the intestinal phase was carried out by diluting the gastric chyme (1:1, v/v) with SIF containing pancreatin (based on trypsin activity to achieve 100 U/mL) and bile extract (10 mM). After 120 min, the digestion was stopped by heating at 100 °C for 5 min and then the samples were cooled in an ice bath. Digests were centrifuged at 2003 *g*, 4 °C for 10 min, and the supernatants were stored at -20 °C until analysis. The whole digestion protocol was performed at 37 °C under constant gentle mixing using an Intell-MixerTM RM-2 (ELMI Ltd., Riga, Latvia) and an incubator chamber Selecta (JP Selecta, S.A., Barcelona, Spain). The GID was performed in duplicate and separated tubes were used

in each digestion phase (gastric = oral + gastric, intestinal = oral + gastric + intestinal). Samples before digestion were obtained by mixing legumes with distilled water (1:1, w/v), and blank samples were obtained by mixing simulated fluids, enzymes, and bile salts (without sample). Samples were coded as legume + type of cooking + digestion phase, using the legumes code GP (green pea), CP (chickpea) and NB (navy bean), the type of cooking code conventional (C), pressure (P) and microwave (M) cooking and the digestion phase code for before GI digestion (b), gastric (g) and intestinal (i). As an example, pressure-cooked green pea digested up to gastric phase is coded as GPPg.

### 2.4. Measurement of α-amylase inhibitory activity

The α-amylase inhibitory activity was determined following the methodology described by de Souza Rocha et al. (2015) and Ngoh and Gan (2016) with some modifications. Thus, 50 µL of the sample, in triplicate, was mixed with 50 µL of α-amylase (13 U/mL in sodium phosphate buffer 0.02 M, pH 6.9, containing 6 mM NaCl) and incubated at 25 °C for 10 min. Then, 50 µL of 1 % soluble starch (prepared in sodium phosphate buffer 0.02 M, pH 6.9, containing 6 mM NaCl, and boiled for 15 min) was added and the mixture was incubated again at 25 °C for 10 min. The reaction was stopped by adding 100 µL of DNS reagent. Immediately, the mixture was heated in a boiling water bath (100 °C) for 5 min and then cooled in an ice bath. Finally, the reaction mixture was diluted by adding 1 mL of distilled water and the absorbance was measured at 540 nm using a UV–Visible spectrophotometer Helios Zeta (Thermo Fisher Scientific, Loughborough, UK). Acarbose 1 mM was used as positive control whereas phosphate buffer (0.02 M, pH 6.9, containing 6 mM NaCl) was used as negative control. The α-amylase inhibitory activity was calculated as percentage of inhibition using the Equation (1), where $A_{control}$ is the negative control absorbance, $A_{sample}$ is the sample absorbance, and $A_{sample\ blank}$ is the absorbance of the mixture of sample and starch solution without the addition of enzyme.

$$\%\ inhibition = \left[(A_{control} - (A_{sample} - A_{sample\ blank}))/A_{control}\right] \times 100 \quad (1)$$

## 2.5. Fractionation by ultrafiltration

The collected supernatants of gastrointestinal digested legumes were fractionated, in duplicated, using centrifugal ultrafiltration filters (Merck Millipore, Cork, Ireland) with molecular weight cut-offs of 10 and 3 kDa. A total of three fractions (>10, 3-10, and <3 kDa) were obtained and their α-amylase inhibitory activity was measured.

## 2.6. SDS-PAGE electrophoresis

### *2.6.1. Preparation of sample and gel electrophoresis*

The sample was prepared using 4× Laemmli sample buffer with β-mercaptoethanol. A total of 30 µg of sample was diluted 50 times for analysis. Once prepared, sample was denatured at 95 °C during 5 min. The electrophoresis was performed using an 12 % precast gel (Bio-Rad) at 200 V for 30 min. The gel was fixed with 40 % ethanol/10 % acetic acid for 1 h and later stained with colloidal Coomassie (Bio-Rad Laboratories, Madrid, Spain) for 1 h.

### *2.6.2. In-gel digestion*

Gel bands were cut and digested with sequencing grade trypsin enzyme (Promega Corporation, Fitchburg, WI, USA) (100 ng of enzyme to bands 1 and 2, 200 ng to band 3, 300 ng to band 4) as previously described by Shevchenko et al. (1996). The digestion was stopped by adding trifluoroacetic acid (TFA) up to a final concentration of 1 %. A double extraction with acetonitrile (ACN) was done and all peptide solutions were dried in a rotatory evaporator and resuspended with 10 µL (samples 1 and 2), 15 µL (sample 3) and 20 µL (sample 4) of 2 % ACN with 0.1 % TFA. A total of 4 µL of sample were put into each vial for mass spectrometry analysis.

*2.6.3. Mass spectrometry analysis for the protein identification*

The identification of the bands was done using a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) system composed of an Ekspert nanoLC 425 (Eksigent, ABSciex, Framingham, MA, USA) and a mass spectrometer nanoESI qQTOF (6600plus TripleTOF, ABSciex, Framingham, MA, USA). A total of 3 µL of peptide mixture sample was loaded onto a trap column (3 µm C18-CL 120 Ă, 350 µm × 0.5 mm; Eksigent, ABSciex, Framingham, MA, USA) and desalted with 0.1 % TFA at 5 µL/min during 3 min.

The peptides were loaded onto a reversed-phase analytical column (3 µm C18-CL 120 Ă, 0.075 × 150 mm; Eksigent) and equilibrated in 5 % ACN and 0.1 % of formic acid (FA). Elution was carried out with a linear gradient of 7 to 40 % B in A for 20 min. (A: 0.1 % FA; B: ACN, 0.1 % FA) at a flow rate of 300 nL/min. Sample was ionised in an Optiflow 1 µL Nano applying 3.0 kV to the spray emitter at 175 °C. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1400 *m/z* for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired 100–1500 *m/z* for 25 ms in 'high sensitivity' mode. Main conditions established in the instrument were a charge from 2+ to 4+; use of minimum intensity; and a total of 100 counts per second (cps) with a dynamic exclusion in 15 s and up to 100 ions selected for fragmentation after each survey scan.

The data analysis was done using ProteinPilot v5.0. search engine (ABSciex, Framingham, MA, USA). ProteinPilot default parameters were used to generate peak list directly from 6600 plus TripleTOF wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot v 5.0 was used to search in Uniprot_fabaceae database, created from SwissProt (210126), with the following parameters: trypsin specificity, cys-alkylation, taxonomy no restricted, the search effort set to rapid and no FDR analysis. Proteins showing unused score >1.3 were identified with confidence ≥ 95 %.

### 2.7. Mass spectrometry in tandem analysis for the identification of peptides in legume samples after simulated GID

The analysis was done using the LC–MS/MS system described in the previous section. A total of 3 µL of initial peptide mixture (6 µL) was loaded onto a reversed-phase trap column (3 µm C18-CL 120 Ă, 350 µm × 0.5 mm; Eksigent) and desalted with 0.1 % TFA at 5 µL/min during 5 min. The peptides were then loaded onto an analytical column (3 µm C18-CL 120 Ă, 0.075 × 150 mm; Eksigent) equilibrated in 5 % ACN and 0.1 % FA. Elution was carried out using a linear gradient of 15 to 40 % B in A for 20 min at a flow rate of 300 nL/min, where A is 0.1 % FA and B is ACN with 0.1 % FA.

Sample was ionised using an Optiflow 1 µL Nano applying 3.0 kV to the spray emitter at 200 ºC. Analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1400 *m/z* for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired 100–1500 *m/z* for 25 ms in 'high sensitivity' mode. Used switch criteria were: charge between 1+ to 5+; minimum intensity; 100 cps. Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled by analysing 500 ng of K562 trypsin enzyme digestion (ABSciex, Framingham, MA, USA), in these conditions 2550 proteins were identified (FDR <1%) in the 20 min gradient.

The data analysis for the identification of the peptides was done using ProteinPilot v 5.0, as previously described. The Paragon algorithm was used to search the UniProt database with the following parameters: no enzyme specificity, optional iodoacetamide cys-alkylation, fabaceae taxonomy restriction, and the search effort set to throughout.

The data analysis for the label-free quantitative analysis is based on the measurement of the integrated areas of extracted ion chromatograms (XICs) from three replicates to determine the ratios for individual peptides. Peptides

were quantified using PeakView v1.1 software (ABSciex, Framingham, MA, USA).

### 2.8. *In silico* analysis of identified peptides

The Peptide Ranker software was used to predict the potential bioactivity of peptides (http://distilldeep.ucd.ie/PeptideRanker/). A score from 0 to 1 (Peptide Ranker Ratio, PRR) was obtained, where 0 was the minimum bioactivity and 1 was the maximum bioactivity. This prediction was related to the amino acid sequence of the peptide and its specific structural characteristics (Mooney et al. 2012). The peptides with a PRR higher than 0.5 were analysed with the software listed below.

The AllerTOP v. 2.0 software (http://www.ddg-pharmfac.net/AllerTOP/index.html) was used to predict the potential peptide allergenicity, which classified them as allergenic or non-allergenic according to their physicochemical properties (Dimitrov et al., 2014).

The potential toxicity and physicochemical properties of the peptides such as hydrophobicity, steric hindrance, sidebulk, hydropathicity, amphipathicity, hydrophilicity, and charge were predicted using the ToxinPred software (http://crdd.osdd.net/raghava/toxinpred/). Regarding this, amino acid composition of peptides and their position were the main factors for toxicity prediction (Gupta et al., 2013).

The BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) was used to search previously identified antidiabetic sequences and the frequency of the occurrence of these active domains in protein sequence (A). This A value is obtained from the quotient between the number of fragments with given activity in a protein sequence and the number of amino acid residues of protein (Minkiewicz et al., 2019).

Finally, the cell-penetrating probability (CPP) of peptides were predicted using the CPPpred software (http://distilldeep.ucd.ie/CPPpred/). Peptides are

scored from 0 to 1, where 1 is the maximum probability to penetrate the cells and thus crossing the intestinal barrier (Holton et al., 2013).

### 2.9. Statistical analysis

Statistical analysis including one-way analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) tests was performed using Statgraphics Centurion XVII software (Statgraphics Technologies, Inc., The Plains, VA, USA) in order to determine significant differences in α-amylase inhibitory activity of legume samples. Results were expressed as the mean of replicates ± standard deviations, and differences were considered significant at $p < 0.05$.

Data obtained from the relative quantification of peptides from intestinal digested cooked legumes were statistically analysed using MarkerView v1.3 software (AB Sciex, Framingham, MA, USA), performing Principal Component Analysis Discriminant Analysis (PCA-DA).

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of GID on α-amylase inhibitory activity of the different cooked legumes

The α-amylase inhibitory activity of the three legumes subjected to the different cooking treatments (conventional, pressure and microwave cooking) was evaluated before and during *in vitro* GID (after gastric and intestinal phases) (Figure 1). The α-amylase inhibitory activity of all legumes (GP, CP and NB) before digestion and after the gastric phase showed values lower than 12 %, but greatly increased after the intestinal digestion phase. Regarding GP samples (Figure 1A), GPM showed the highest activity with 75 % inhibition, followed by GPC with 58 % inhibition, and GPP with the lowest value (52 % inhibition). In the case of CP samples (Figure 1B), inhibition values were between 58 and 60 % for the different thermal treated legumes (CPC, CPP, CPM), with significant differences between them. In this sense,

CPC showed the highest inhibitory activity value and CPM the lowest. Finally, regarding Figure 1C, NBM reached an inhibition value of 60 % after GID, whereas NBC and NBP samples had similar inhibition rates (around 50 %).

Among the thermal treatments performed, microwave cooking had the greatest impact on the α-amylase inhibitory activity in both GP and NB samples. Non-significant differences between microwave and pressure cooking were observed in CP samples. The increase observed in all thermal treated legumes after GID could be related to the generation of bioactive peptides as a result of protein hydrolysis during digestion, mainly due to the action of intestinal enzymes. In this regard, only a few studies have described the amylase inhibitory activity of cooked and digested legumes or other foods. Ercan and El (2016) studied the α-amylase inhibitory activity of pressure-cooked chickpeas before and after *in vitro* GID, showing that the inhibition activity at 1 mg/mL decreased after GID from 87.8 to 72.6 % and attributing this bioactivity to lectins (Ercan & El, 2016). On the other hand, Nehir El et al. (2015) obtained an increase of α-amylase inhibitory activity after GID of goat milk and kefir, linking this bioactivity to the peptides generated during fermentation and GID. Most studies have evaluated the inhibitory activity of hydrolysates from legume protein isolates. González-Montoya et al. (2018) studied the antidiabetic activity of protein isolate from germinated soybeans, obtaining that the GID sample could inhibit α-amylase ($IC_{50}$= 1.7 mg/mL) but to a less extent than acarbose ($IC_{50}$= 0.16 mg/mL). Castañeda-Pérez et al. (2021) assessed the α-amylase inhibitory activity of lima bean protein isolate hydrolysed with pepsin-pancreatin (100 mg/mL) and Alcalase®-Flavourzyme® (50 mg/mL) enzymes, obtaining inhibitory activities of 18.02 % and 92.58 %, respectively.

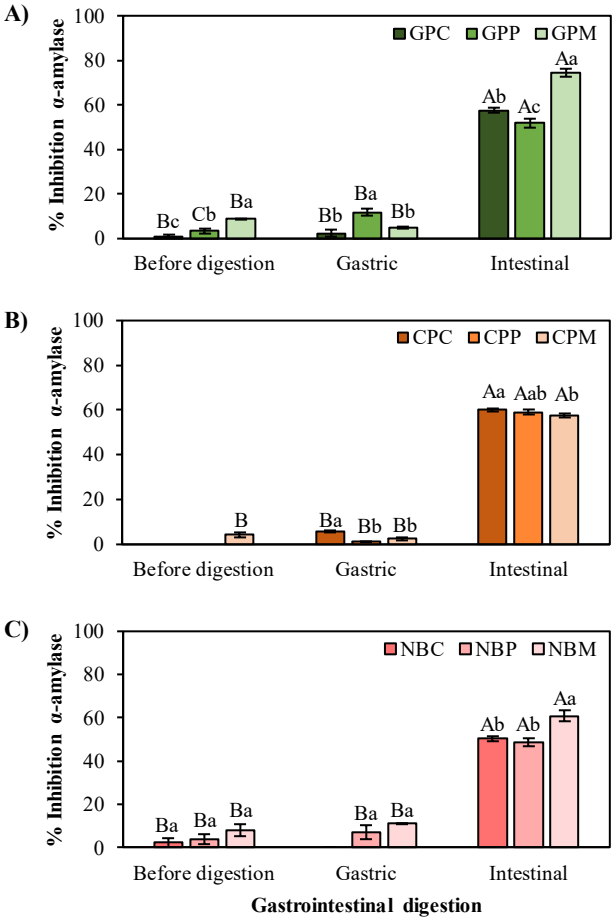


**Figure 1.** α-Amylase inhibition activity of legumes from green pea (A), chickpea (B) and navy bean (C) during gastrointestinal digestion, previously subjected to different types of household cooking (GPC, conventional cooked green pea; GPP, pressure cooked green pea; GPM, microwaved green pea; CPC, conventional cooked chickpea; CPP, pressure cooked chickpea; CPM, microwaved chickpea; NBC, conventional cooked navy bean; NBP, pressure cooked navy bean; NBM, microwaved navy bean). Capital letter indicates significant differences between digestion phases (before, gastric, and intestinal) within the same treatment ($p < 0.05$), whereas lowercase letter indicates significant differences between treated samples (conventional cooking, pressure cooking, and microwave) within the same phase ($p < 0.05$).

In order to determine the influence of the molecular weight of sample chemical compounds on the α-amylase inhibitory activity of thermal-treated legumes, the digested samples with the highest activity (GPCi, GPPi, GPMi, CPCi, CPPi, CPMi, NBCi, NBPi and NBMi) were fractionated by ultrafiltration, and the inhibitory activity of each fraction (<3, 3-10 and >10 kDa) was measured. The results shown in Figure 2, indicated that the highest activity was obtained in those fractions with molecular weight <3 kDa. Moreover, the effect caused by the thermal treatment in these fractions followed the same trend as that observed in the unfractionated samples, although with more marked differences between some treatments. So, in fractions <3 kDa, the inhibitory activity of GP samples was between 42 % and 72 %, with the highest inhibitory activity for GPMi and the lowest for GPPi (Figure 2A). In the case of CP samples, there were not significant differences between CPCi and CPPi samples, showing activities around 58 %, while a slight decrease in the activity was observed for GPMi (52 % inhibition) (Figure 2B). In NB samples, NBMi showed the highest inhibitory activity (54 %), followed by NBCi (49 %), and NBPi (23 %) (Figure 2C). On the other hand, the inhibition percentage of the fractions with a molecular weight of 3-10 kDa and >10 kDa was very low or zero, with the exception of GPMi >10 kDa that showed 47 % inhibition. Similar results were obtained by Ngoh and Gan (2016), Oseguera-Toledo et al. (2015), Valencia-Mejía et al. (2019) and Castañeda-Pérez et al. (2021), who fractionated legume's hydrolysates and obtained the highest inhibitory activity in the smallest molecular size fraction (<1 or <3 kDa). Nevertheless, the fractions with a molecular weight > 10 kDa of some legume hydrolysates also obtained a significant α-amylase inhibitory activity (Castañeda-Pérez et al., 2021; Ngoh & Gan, 2016; Oseguera-Toledo et al., 2015). Likewise, González-Montoya et al. (2018) studied the antidiabetic properties of germinated soybean protein subjected to GID and obtained that the peptide fractions >10 kDa and <5 kDa presented the highest α-amylase inhibitory activities. These results suggested that peptides with different molecular weights could exert α-amylase inhibitory effects.

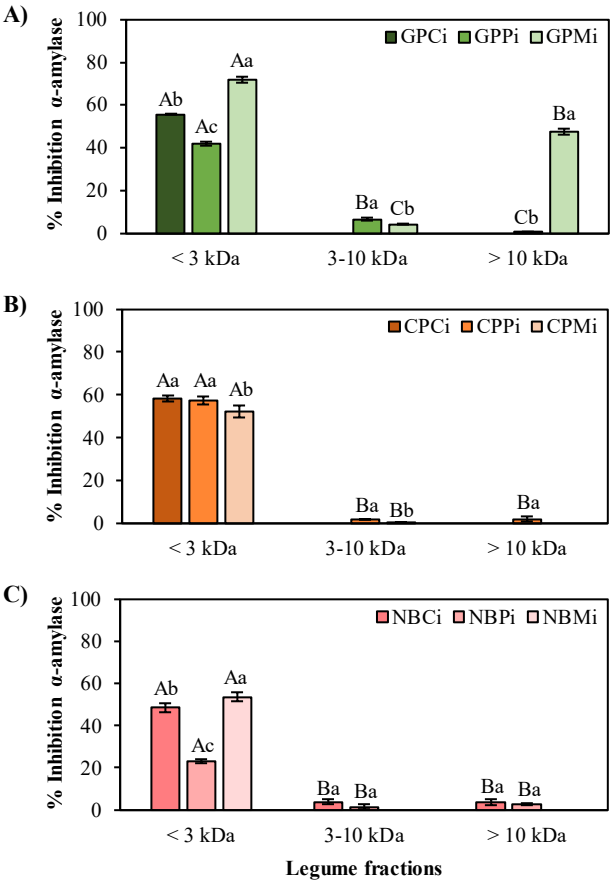


**Figure 2.** α-Amylase inhibition activity of digested legume fractions (<3, 3–10, >10 kDa) from green pea (A), chickpea (B) and navy bean (C), subjected to different types of household cooking (GPCi, intestinal digested conventional cooked green pea; GPPi, intestinal digested pressure cooked green pea; GPMi, intestinal digested microwaved green pea; CPCi, intestinal digested conventional cooked chickpea; CPPi, intestinal digested pressure cooked chickpea; CPMi, intestinal digested microwaved chickpea; NBCi, intestinal digested conventional cooked navy bean; NBPi, intestinal digested pressure cooked navy bean; NBMi, intestinal digested microwaved navy bean).

Capital letter indicates significant differences between fractions (<3, 3–10, >10 kDa) within the same treatment ($p < 0.05$), whereas lowercase letter indicates significant differences between treated samples (conventional cooking, pressure cooking, and microwave) within the same fraction ($p < 0.05$).

### 3.2. SDS-PAGE analysis and protein identification by LC-MS/MS of fraction >10 kDa obtained from gastrointestinal digested microwaved green pea

GPMi was the only fraction with molecular weight >10 kDa with significant α-amylase inhibitory activity (Figure 2A), so its protein profile was evaluated using SDS-PAGE. The results are shown in Supplementary Material Figure S1, in which 4 bands with molecular weight around 50, 37-25, 15 and 10 kDa from band 1 to band 4, respectively, can be distinguished. de Souza Rocha et al. (2015), who studied the effect of germination of common beans and its alcalase hydrolysates to produce and identify antidiabetic peptides, also characterized by SDS-PAGE the polypeptides generated after GID and observed a double band around 17 kDa in the samples where α-amylase peptide inhibitors could have been generated.

The bands were digested and analysed by LC-MS/MS in order to identify their proteins of origin. The accession number, the protein name, legume specie, and coverage percentage are presented in Table 1. In bands 1, 2 and 3, one main protein was identified in each of them, but they were not characterised. The band 4 was identified as a proteinase inhibitor from *Vicia faba*.

**Table 1.** Protein identification of SDS-PAGE bands of microwaved green pea sample after simulated gastrointestinal digestion and ultrafiltration >10 kDa.

| Band | Accession number | Protein name | Specie | % Coverage |
|---|---|---|---|---|
| 1 | tr\|A0A6A5L857\| A0A6A5L857_LUPAL | Uncharacterised | *Lupinus albus* | 93.32 |
| 2 | tr\|A0A2K3KX70\| A0A2K3KX70_TRIPR | Uncharacterised | *Trifolium pratense* | 53.27 |
| 3 | tr\|A0A444WZ61\| A0A444WZ61_ARAHY | Uncharacterised | *Arachis hypogaea* | 52.9 |
| 4 | sp\|P24661\| IBB_VICFA | Proteinase inhibitor | *Vicia faba* | 71.43 |

### 3.3. Peptide identification by LC-MS/MS and *in silico* analysis of intestinal digested legume fractions <3 kDa

The identification of the peptides from the fractions with a molecular weight <3 kDa of intestinal digested cooked legumes (GPCi, GPPi, GPMi, CPCi, CPPi, CPMi, NBCi, NBPi and NBMi), which are the fractions with the highest inhibitory activity, was performed by LC-MS/MS. Table S1 (Supplementary Material) shows the complete list of the 205 identified peptide sequences, in addition to their protein of origin, the observed and calculated molecular masses, and the mass/charge (*m/z*). In brief, a total of 19, 17, and 9 peptides from samples GPCi, GPPi and GPMi, respectively; a total of 27, 11, and 40 peptides from samples CPCi, CPPi and CPMi; and a total of 17, 21, and 44 from samples NBCi, NBPi and NBMi, were identified. These peptides were fragments of different length (from 5 to 62 amino acids) and molecular masses between 540 to 5000 Da, with most of them between 1000 and 2000 Da. Mojica et al., (2015) identified the peptide sequences of protein isolates from raw and precooked beans after pepsin-pancreatin hydrolysis, showing that most of the sequences had molecular weight lower than 700 Da or higher than 2000 Da.

The *in silico* analysis using different databases allowed to obtain the potential bioactivity, cell penetrating probability, allergenicity, toxicity, and physicochemical properties of the identified peptides. After checking in BIOPEP that none of the identified peptides had been reported as bioactive, their potential bioactivity was determined with the PeptideRanker software. About 43 peptides out of the 205 total peptides identified in GPCi (6), GPPi (4), GPMi (3), CPCi (3), CPPi (1), CPMi (2), NBCi (5), NBPi (10) and NBMi (9) obtained a Peptide Ranker Ratio higher than 0.5, which are listed in Table 2.

**Table 2.** *In silico* prediction of the allergenicity, toxicity, cell-penetrating probability (CPP), and physicochemical properties of the identified peptides with Peptide Ranker Ratio (PRR) > 0.5.

| Legume sample* | Peptide sequence | PRR | CPP | Allergenicity prediction | Toxicity prediction | Hydrophobicity | Steric hindrance | Sidebulk | Hydropathicity | Amphipathicity | Hydrophilicity | Charge |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GPCi | GAGGGGFGGGSGGGAGGGIGGGGAGGGFGGGAGGGG | 0.90 | 0.16 | Non-allergen | Non-Toxic | 0.20 | 0.66 | 0.66 | 0.15 | 0 | -0.24 | 0 |
| | NAPLGIGGLPKGF | 0.85 | 0.15 | Non-allergen | Non-Toxic | 0.11 | 0.60 | 0.60 | 0.35 | 0.28 | -0.40 | 1 |
| | CSCNCQGLPGANCVNSDK | 0.84 | 0.11 | Non-allergen | Toxic | -0.18 | 0.63 | 0.63 | -0.31 | 0.27 | -0.02 | 0 |
| | GPSGPVGPSGPVG | 0.83 | 0.08 | Non-allergen | Non-Toxic | 0.08 | 0.56 | 0.56 | -0.12 | 0 | -0.18 | 0 |
| | NGGQSQLVCSGCRSLLMYPVGAT | 0.82 | 0.16 | Non-allergen | Non-Toxic | -0.04 | 0.62 | 0.62 | 0.26 | 0.22 | -0.45 | 1 |
| | VDLLGPLKALSRPIHGKFGNEAL | 0.72 | 0.25 | Allergen | Non-Toxic | -0.05 | 0.58 | 0.58 | 0.13 | 0.54 | -0.03 | 1.5 |
| GPPi | GLGGGGGIGGGGGAGGGIGHGLG | 0.89 | 0.11 | Non-allergen | Non-Toxic | 0.22 | 0.63 | 0.63 | 0.37 | 0.06 | -0.36 | 0.5 |
| | GPSGPVGPSGPVG | 0.83 | 0.08 | Non-allergen | Non-Toxic | 0.08 | 0.56 | 0.56 | -0.12 | 0 | -0.18 | 0 |
| | GTLPCTVLIPGLFFI | 0.70 | 0.11 | Non-allergen | Non-Toxic | 0.31 | 0.59 | 0.59 | 1.82 | 0 | -1.15 | 0 |
| | PGGQPGAP | 0.62 | 0.14 | Allergen | Non-Toxic | -0.02 | 0.54 | 0.54 | -0.96 | 0.16 | -0.04 | 0 |
| GPMi | GAGGPGGAGWGPGPGGPGW | 0.94 | 0.23 | Non-allergen | Non-Toxic | 0.14 | 0.58 | 0.58 | -0.47 | 0 | -0.41 | 0 |
| | SGDVWFPQPAPK | 0.83 | 0.14 | Non-allergen | Non-Toxic | -0.09 | 0.57 | 0.57 | -0.75 | 0.41 | -0.12 | 0 |
| | DIKVKEAAGGILANLAL | 0.58 | 0.24 | Allergen | Non-Toxic | 0.04 | 0.63 | 0.63 | 0.75 | 0.51 | -0.02 | 0 |
| CPCi | AGGGFGGGAGGGAGGGGGI | 0.93 | 0.11 | Non-allergen | Non-Toxic | 0.23 | 0.66 | 0.66 | 0.37 | 0 | -0.31 | 0 |
| | SLASTCMPLSF | 0.71 | 0.09 | Allergen | Non-Toxic | 0.11 | 0.56 | 0.56 | 1.08 | 0 | -0.76 | 0 |
| | SRVLSSFGGQIGALAADADPVSK | 0.69 | 0.21 | Allergen | Non-Toxic | -0.05 | 0.61 | 0.61 | 0.24 | 0.32 | 0.02 | 0 |
| CPPi | DDGGPPGGSGGPA | 0.74 | 0.06 | Non-allergen | Non-Toxic | -0.05 | 0.59 | 0.59 | -1.02 | 0 | 0.45 | -2 |

**Table 2.** Cont.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CPMi | MHPFMGGFT | 0.88 | 0.06 | Non-allergen | Non-Toxic | 0.16 | 0.58 | 0.58 | 0.34 | 0.16 | -0.94 | 0.5 |
| | GAGVGPLGAGGVGH | 0.67 | 0.17 | Allergen | Non-Toxic | 0.20 | 0.58 | 0.58 | 0.59 | 0.10 | -0.45 | 0.5 |
| NBCi | GCDMICLKFFHENWAH | 0.91 | 0.08 | Allergen | Toxic | -0.02 | 0.58 | 0.58 | 0.03 | 0.49 | -0.47 | 0 |
| | GSVLLLGEPLCLGSVFGGILL | 0.83 | 0.14 | Allergen | Non-Toxic | 0.27 | 0.60 | 0.60 | 1.72 | 0.06 | -0.82 | -1 |
| | NVHWDPKPR | 0.71 | 0.31 | Allergen | Non-Toxic | -0.43 | 0.53 | 0.53 | -2.06 | 0.84 | 0.42 | 1.5 |
| | KILGLDHNKFGGSLP | 0.57 | 0.13 | Non-allergen | Non-Toxic | -0.06 | 0.59 | 0.59 | -0.19 | 0.59 | -0.05 | 1.5 |
| | GSGYGSGGGYGSGYGS | 0.50 | 0.04 | Non-allergen | Non-Toxic | 0.03 | 0.65 | 0.65 | -0.67 | 0 | -0.36 | 0 |
| NBPi | GGGAGGGIGGGAGGGGGVGGGVGGGKGGGFGGGGGGGGGGAGGGGSGGAGGGGGGGGGVGGR[1] | 0.99 | 0.29 | Non-allergen | - | - | - | - | - | - | - | - |
| | GGGGGLGGGGGAGGGL | 0.94 | 0.16 | Non-allergen | Non-Toxic | 0.21 | 0.65 | 0.65 | 0.26 | 0 | -0.26 | 0 |
| | GGGGGLGGGGGAGGGI | 0.88 | 0.12 | Non-allergen | Non-Toxic | 0.22 | 0.66 | 0.66 | 0.31 | 0 | -0.26 | 0 |
| | GGGGGIGGGAGGGGGI | 0.88 | 0.10 | Non-allergen | Non-Toxic | 0.24 | 0.67 | 0.67 | 0.35 | 0 | -0.26 | 0 |
| | SELASGCCTLLTCTTKGNASTRS | 0.83 | 0.24 | Allergen | Toxic | -0.15 | 0.58 | 0.58 | -0.02 | 0.32 | -0.04 | 1 |
| | GGGGPLGGGSGVVPLGGGGPL | 0.76 | 0.17 | Non-allergen | Non-Toxic | 0.20 | 0.61 | 0.61 | 0.45 | 0 | -0.39 | 0 |
| | IGLPGLGL | 0.68 | 0.18 | Non-allergen | Non-Toxic | 0.34 | 0.59 | 0.59 | 1.64 | 0 | -0.9 | 0 |
| | RAGRSAENLAGALFAVREIFG | 0.60 | 0.30 | Allergen | Non-Toxic | -0.1 | 0.63 | 0.63 | 0.23 | 0.47 | 0.05 | 1 |
| | IGLPGIGL | 0.60 | 0.10 | Non-allergen | Non-Toxic | 0.37 | 0.61 | 0.61 | 1.72 | 0 | -0.90 | 0 |
| | PIGGPVPPGAFDSTAPAPNA | 0.55 | 0.13 | Allergen | Non-Toxic | 0.06 | 0.55 | 0.55 | -0.03 | 0 | -0.24 | -1 |

**Table 2.** Cont.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NBMi | GLGGGGGSGGGGG-GGL | 0.90 | 0.13 | Non-allergen | Non-Toxic | 0.18 | 0.65 | 0.65 | 0.10 | 0 | -0.21 | 0 |
| | GEGGGGGGGGGGG-GGNG | 0.88 | 0.11 | Non-allergen | Non-Toxic | 0.07 | 0.68 | 0.68 | -0.74 | 0.07 | 0.18 | -1 |
| | SASALGMACGPALA-GILQTDFKI | 0.87 | 0.16 | Non-allergen | Non-Toxic | 0.10 | 0.60 | 0.60 | 0.9 | 0.21 | -0.43 | 0 |
| | SGDVWFPQPAPK | 0.83 | 0.14 | Non-allergen | Non-Toxic | -0.09 | 0.57 | 0.57 | -0.75 | 0.41 | -0.12 | 0 |
| | ASDPQLDRLNSLPW | 0.82 | 0.22 | Non-allergen | Non-Toxic | -0.21 | 0.57 | 0.57 | -0.79 | 0.26 | 0.05 | -1 |
| | DSLGLPDFGYDGPL | 0.76 | 0.07 | Non-allergen | Non-Toxic | 0.01 | 0.61 | 0.61 | -0.2 | 0 | -0.06 | -3 |
| | GPVFNPQPPKPPV | 0.72 | 0.19 | Non-allergen | Non-Toxic | -0.08 | 0.54 | 0.54 | -0.75 | 0.38 | -0.16 | 1 |
| | NVHWDPKPR | 0.71 | 0.31 | Allergen | Non-Toxic | -0.43 | 0.53 | 0.53 | -2.06 | 0.84 | 0.42 | 1.5 |
| | NAGPADGL | 0.54 | 0.12 | Allergen | Non-Toxic | -0.01 | 0.60 | 0.60 | -0.25 | 0 | 0.05 | -1 |

*Legume samples are: GPCi, intestinal digested conventional cooked green pea; GPPi, intestinal digested pressure cooked green pea; GPMi, intestinal digested microwaved green pea; CPCi, intestinal digested conventional cooked chickpea; CPPi, intestinal digested pressure cooked chickpea; CPMi, intestinal digested microwaved chickpea; NBCi, intestinal digested conventional cooked navy bean; NBPi, intestinal digested pressure cooked navy bean; NBMi, intestinal digested microwaved navy bean.

[1]ToxinPred does not provide information for peptides > 50 amino acid residues.

According to AllerTop and ToxinPred, 27 of these 43 peptides were predicted to be neither allergen nor toxic. In addition, each of these peptides was analysed with the BIOPEP database to find active domains previously reported. Table 3 presents the list of peptides with each of their bioactive sequences and their respective biological activities, and the frequency of occurrence in protein sequence (A). As can be seen by the number of bioactive sequences and the A value, ACE-I inhibition and DPP-IV inhibition were the main biological activities predicted for these sequences. Moreover, some sequences were reported as α-glucosidase inhibitory or glucose uptake which, together with DPP-IV inhibition, are related to antidiabetic activity. It seems that molecular weight and structure as well as amino acid composition and sequences of the peptides could influence in the mechanism and inhibitory activity of α-amylase (Li et al., 2022). Ngoh and Gan (2016) proposed that the residues Leu, Pro, Gly and Phe had the potential to inhibit α-amylase. Moreover, the position of these amino acids, Pro at the N- or C- terminal or both terminals of the peptide sequence, Gly or Phe at N-terminal and Phe or Leu at C-terminal, as well as the presence of dipeptides such as Pro-Pro, Cys-Cys, Leu-Leu and Gly-Gly could be related to the inhibitory activity in pinto bean protein isolates (González-Montoya et al., 2018; Ngoh & Gan, 2016). Regarding the peptides identified in this study, 19 of the 43 peptides identified with a PRR > 0.5 showed Gly or Pro at the N-terminal, 14 of them showed Pro, Phe or Leu at the C-terminal, and 31 of them showed the dipeptides Pro-Pro, Cys-Cys, Leu-Leu or Gly-Gly in their sequences. Likewise, Ngoh et al. (2016) reported that the active binding sites of pinto bean bioactive peptides were found to be His, Pro, and Met, which were in accordance with the results obtained in wheat peptides. Besides that, Ngoh and Gan (2018) studied the interactions between pinto bean peptides and human salivary α-amylase, and *in silico* results showed that hydrophilic (Cys, Met, His, and Ser) and hydrophobic (Ala, Leu, Phe, Val, Pro, and Gly) amino acids could play a crucial role in inhibiting α-amylase activity because they interact with its active site through hydrophobic and hydrogen bonds. In addition, they also proposed that hydrophobic amino acids at the N-terminal played a

key role in inhibitory activity. Castañeda-Pérez et al. (2021) analysed the amino acid profile of pepsin-pancreatin and Alcalase®-Flavourzyme® hydrolysate from *P. lunatus* protein isolate and their fractions, obtaining that the fractions with higher α-amylase inhibitory activity also had high levels of hydrophobic (Leu, Phe, and Ala) and hydrophilic (Lys, Asp, and Glu) amino acid residues. In this study, the peptides identified also presented a high proportion of hydrophobic (Gly, Pro, Leu, and Ala) and hydrophilic (Ser) amino acids. Regarding the inhibitory mechanism, it has been proposed that amino acids interact with α-amylase active site and change the position of the substrate by displacing it from the catalytic domain (González-Montoya et al., 2018; Ngoh et al., 2016).

To conclude the *in silico* analysis of the peptides, their cell penetrating probability (CPP) was determined (Table 2). This is interesting because although peptides with a-amylase inhibitory activity do not need to cross the intestinal barrier to exert their function, most of them have bioactive sequences with multiple functions that do need it. The probabilities obtained were between 0 and 0.3, with the most part being between 0.1 and 0.2, indicating that peptides have low probability to penetrate cells.

**Table 3.** Potential biological activity according to BIOPEP database of the identified peptides with Peptide Ranker Ratio > 0.5.

| Legume sample* | Peptide Sequence | Bioactive sequence | Biological activity | A** |
|---|---|---|---|---|
| GPCi | GAGGGGFGGGS-GGGAGGGIGGG-GAGGGFGGGA-GGGG | AG, FG, FGG, GA, GF, GG, GI, GS, IG, SG | ACE | 1.0278 |
| | | GF | DPP-III | 0.0556 |
| | | AG, GA, GF, GG, GI | DPP-IV | 0.8333 |
| | NAPLGIGGLPKGF | AP, GF, GG, GI, GL, GLP, IG, KG, LG, LGI, LP, PL, PLG | ACE | 1.0000 |
| | | GF | DPP-III | 0.0769 |
| | | AP, GF, GG, GI, GL, KG, LP, NA, PK, PL | DPP-IV | 0.7692 |
| | | PLG | OP | 0.0769 |
| | CSCNCQGLPGAN-CVNSDK | GA, GL, GLP, LP, LPG, PG, QG | ACE | 0.3889 |
| | | PG | AM, AT, RE | 0.0556 |
| | | GA, GL, LP, PG, QG, VN | DPP-IV | 0.3333 |
| | GPSGPVGPSGPVG | GP, GPV, SG, SGP, VG, VGP | ACE | 1.0000 |
| | | GP | AM, AT, RE | 0.3077 |
| | | GP, GPV, PS, PV, VG | DPP-IV | 1.0769 |
| | NGGQSQLVCSGC-RSLLMYPVGAT | GA, GG, GQ, MY, NG, SG, VG, YP | ACE | 0.3478 |
| | | YP | GLUI | 0.0435 |
| | | MY | AO | 0.0435 |
| | | AT, GA, GG, LL, LM, LV, MY, NG, PV, QL, QS, SL, VG, YP | DPP-IV | 0.6087 |
| | | GQ | NE | 0.0435 |
| | | SL | RE | 0.0435 |
| | | LL, LV | ST | 0.0870 |
| | VDLLGPLKALSRP-IHGKFGNEAL | EA, FG, GK, GP, GPL, HG, KA, KF, LG, LGP, LKA, PL, RP | ACE | 0.5652 |
| | | EA | GLUI | 0.0435 |
| | | GP | AM, AT, RE | 0.0435 |
| | | LK | AO | 0.0435 |
| | | KF | 35PD, REN | 0.0435 |
| | | IH, KA | DPP-III | 0.0870 |
| | | AL, GP, IH, KA, KF, LL, NE, PI, PL, RP, VD | DPP-IV | 0.5217 |
| | | LL | ST | 0.0435 |
| GPPi | GLGGGGGIGGGG-GAGGGIGHGLG | AG, GA, GG, GH, GHG, GI, GL, HG, IG, LG | ACE | 1.0000 |
| | | AG, GA, GG, GH, GI, GL | DPP-IV | 0.7391 |
| | GPSGPVGPSGPVG | GP, GPV, SG, SGP, VG, VGP | ACE | 1.0000 |
| | | GP | AM, AT, RE | 0.3077 |
| | | GP, GPV, PS, PV, VG | DPP-IV | 1.0769 |
| | GTLPCTVLIPGLFFI | FF, GL, GT, IP, LF, LP, PG, PGL | ACE | 0.5333 |
| | | PG | AM, AT | 0.0667 |
| | | FF, GL, IP, LI, LP, PG, TL, TV, VL | DPP-IV | 0.6000 |
| | | GLF | IM | 0.0667 |
| | | PG, GLF | RE | 0.1333 |
| | | LI, VL | ST | 0.1333 |
| | PGGQPGAP | AP, GA, GG, GQ, GQP, PG, QP | ACE | 1.0000 |
| | | PG | AM, AT, RE | 0.2500 |
| | | AP, GA, GG, PG, QP | DPP-IV | 0.7500 |
| | | GQ | NE | 0.1250 |

**Table 3.** Cont.

| | | | | |
|---|---|---|---|---|
| GPMi | GAGGPGGAGWG-PGPGGPGW | AG, GA, GG, GP, GW, PG, WG | ACE | 0.9474 |
| | | GP, GPGG, PG, PGP | AM, AT, RE | 0.5789 |
| | | WG | AO | 0.0526 |
| | | PGP | CHE, INH | 0.0526 |
| | | AG, GA, GG, GP, GW, PG, WG | DPP-IV | 0.9474 |
| | SGDVWFPQPAPK | AP, FP, GD, PAP, PQ, QP, SG, VW | ACE | 0.6667 |
| | | VW | GLUI, AO | 0.0833 |
| | | AP, FP, PA, PK, PQ, QP, VW, WF | DPP-IV | 0.6667 |
| | DIKVKEAAGGILA-NLAL | AA, AG, EA; GG, GI, IL, KE, LA, VK | ACE | 0.5882 |
| | | LA | APR, DPP-III | 0.1176 |
| | | LAN | AO | 0.0588 |
| | | EA | GLUI | 0.0588 |
| | | AA, AG, AL, GG, GI, IL, KE, KV, LA, NL, VK | DPP-IV | 0.7059 |
| | | AA | HYP | 0.0588 |
| | | IL | ST | 0.0588 |
| CPCi | AGGGFGGGAGG-GAGGGGGI | AG, FG, FGG, GA, GF, GG, GI | ACE | 1.0000 |
| | | AG | DPP-III | 0.0526 |
| | | AG, GA, GF, GG, GI | DPP-IV | 0.8947 |
| | SLASTCMPLSF | LA, PL, SF, ST | ACE | 0.3636 |
| | | LA | APR, DPP-III | 0.0909 |
| | | AS, LA, MP, PL, SF, SL | DPP-IV | 0.5455 |
| | | SL | RE | 0.0909 |
| | | SF | REN | 0.0909 |
| | SRVLSSFGGQIGA-LAADADPVSK | AA, DA, FG, FGG, GA, GG, GQ, IG, LA, LAA, SF | ACE | 0.4783 |
| | | LA | APR | 0.0435 |
| | | AD | GLUI | 0.0870 |
| | | DA, LA, RV | DPP-III | 0.1304 |
| | | AA, AD, AL, DP, GA, GG, LA, PV, QI, SF, SK, VL, VS | DPP-IV | 0.6087 |
| | | AA | HYP | 0.0435 |
| | | GQ | NE | 0.0435 |
| | | SF | REN | 0.0435 |
| | | VL | ST | 0.0435 |
| CPPi | DDGGPPGGSGGPA | DG, GG, GP, GPA, GPP, GS, PG, PP, SG | ACE | 0.9231 |
| | | PP | GLUI | 0.0769 |
| | | PP | AM | 0.2308 |
| | | GP, PG | AO | 0.0769 |
| | | GP, PG | DPP-IV | 0.7692 |
| | | GPP | AT | 0.2308 |
| | | PG | RE | 0.2308 |
| CPMi | MHPFMGGFT | GF, GG, HP, MG | ACE | 0.4444 |
| | | FM, GF, HP, PF | DPP-III | 0.4444 |
| | | GF, GG, HP, MG, MH, PF | DPP-IV | 0.6667 |
| | | MH | NE | 0.1111 |
| | | FT | REN | 0.1111 |
| | GAGVGPLGAGGV-GH | AG, GA, GG, GH, GP, GPL, GV, LG, PL, PLG, VG, VGP | ACE | 1.1429 |
| | | GP | AM, AT, RE | 0.0714 |
| | | AG, GA, GG, GH, GP, GV, PL, VG | DPP-IV | 0.8571 |

**Table 3.** Cont.

| | | | | |
|---|---|---|---|---|
| CPMi | GAGVGPLGAGGV-GH | GGV | HMGI | 0.0714 |
| | | PLG | OP | 0.0714 |
| NBCi | KILGLDHNKFGG-SLP | FG, FGG, GG, GL, GS, IL, KFLG, LP, NK | ACE | 0.6667 |
| | | KF | 35PD, REN | 0.0667 |
| | | GG, GL, IL, KF, KI, LP, SL | DPP-IV | 0.4667 |
| | | SL | RE | 0.0667 |
| | | IL | ST | 0.0667 |
| | GSGYGSGGGYGS-GYGS | GG, GGY, GS, GY, SG, YG | ACE | 1.0000 |
| | | YG | DPP-III, IM | 0.1875 |
| | | GG, GY, YG | DPP-IV | 0.5000 |
| | GCDMICLKFFHE-NWAH | AH, DM, FF, KF, WA | ACE | 0.3125 |
| | | WA | APR | 0.0625 |
| | | AH, LK | AO | 0.1250 |
| | | KF | 35PD, REN | 0.0625 |
| | | AH, FF, HE, KF, MI, NW, WA | DPP-IV | 0.4375 |
| | GSVLLLGEPLCLG-SVFGGILL | FG, FGG, GE, GEP, GG, GI, GS, IL, LG, PL, VF | ACE | 0.6190 |
| | | SVL | AO | 0.0476 |
| | | GE | DPP-III | 0.0476 |
| | | EP, GE, GG, GI, IL, LL, PL, SV, VF, VL | DPP-IV | 0.6190 |
| | | IL, LL, LLL, VL | ST | 0.2857 |
| | NVHWDPKPR | KP, PR, VHW | ACE | 0.3333 |
| | | KP, PR, VHW | AO | 0.1111 |
| | | PR | DPP-III | 0.1111 |
| | | DP, HW, KP, NV, PK, VH, WD | DPP-IV | 0.7778 |
| NBPi | GGGAGGGIGGGA-GGGGGVGGGVG-GGKGGGFGGGG-GGGGGGAGGGG-SGGAGGGGGGG-GGVGGR | AG, FG, FGG, GA, GF, GG, GI, GK, GR, GS, GV, IG, KG, SG, VG | ACE | 1.0000 |
| | | FG | DPP-III | 0.0161 |
| | | AG, GA, GF, GG, GI, GV, KG, VG | DPP-IV | 0.8871 |
| | | GGV | HMGI | 0.0484 |
| | GGGGGLGGGGG-AGGGL | AG, GA, GG, GL, LG | ACE | 0.9375 |
| | | AG, GA, GG, GL | DPP-IV | 0.8750 |
| | GGGGGLGGGGG-AGGGI | AG, GA, GG, GI, GL, LG | ACE | 0.9375 |
| | | AG, GA, GG, GI, GL | DPP-IV | 0.8750 |
| | GGGGGIGGGAG-GGGGI | AG, GA, GG, GI, IG | ACE | 0.9375 |
| | | AG, GA, GG, GI | DPP-IV | 0.8750 |
| | SELASGCCTLLTC-TTKGNASTRS | KG, LA, SG, ST | ACE | 0.1739 |
| | | LA | APR, DPP-III | 0.0435 |
| | | EL, LTC | AO | 0.0870 |
| | | AS, KG, LA, LL, LT, NA, TK, TL, TR, TT | DPP-IV | 0.4783 |
| | | LL, SE | ST | 0.0870 |
| | GGGGPLGGGSGV-VPLGGGGPL | GG, GP, GPL, GS, GV, LG, PL, PLG, SG, VP | ACE | 1.0952 |
| | | GP, VPL | AM | 0.1429 |
| | | GP | AT, RE | 0.0952 |
| | | GG, GP, GV, PL, VP, VPL, VV | DPP-IV | 0.8095 |
| | | PLG | OP | 0.0952 |
| | | VPL | ST | 0.0476 |
| | IGLPGLGL | GL, GLP, IG, LG, LP, LPG, PG, PGL | ACE | 1.2500 |
| | | PG | AM, AT, RE | 0.1250 |
| | | GL, LP, PG | DPP-IV | 0.7500 |

**Table 3.** Cont.

| | | | | |
|---|---|---|---|---|
| NBPi | RAGRSAENLAGA-LFAVREIFG | AG, AV, EI, FG, GA, GR, IF, IFG, LA, LF, RA, VR | ACE | 0.6190 |
| | | LA, RA | APR | 0.0952 |
| | | AGA | 35PD | 0.0476 |
| | | FA, LA | DPP-III | 0.0952 |
| | | AE, AG, AL, AV, EI, FA, GA, LA, NL, RA, VR | DPP-IV | 0.5714 |
| | IGLPGIGL | GI, GL, GLP, IG, LP, LPG, PG | ACE | 1.1250 |
| | | PG | AM, AT, RE | 0.1250 |
| | | GI, GL, LP, PG | DPP-IV | 0.8750 |
| | PIGGPVPPGAFDS-TAPAPNA | AF, AP, GA, GG, GP, GPV, IG, PAP, PG, PP, ST, TAP, VP, VPP | ACE | 0.7500 |
| | | PP | GLUI | 0.0500 |
| | | VPP | AI | 0.0500 |
| | | GP, PG | AM, AT, RE | 0.1000 |
| | | AF, AP, GA, GG, GP, GPV, NA, PA, PG, PI, PN, PP, PPG, PV, TA, VP | DPP-IV | 0.8500 |
| NBMi | GLGGGGGSGGGG-GGGL | GG, GL, GS, LG, SG | ACE | 0.9375 |
| | | GG, GL | DPP-IV | 0.7500 |
| | GEGGGGGGGGG-GGGGGNG | EG, GE, GG, NG | ACE | 0.8889 |
| | | GE | DPP-III | 0.0556 |
| | | EG, GE, GG, NG | DPP-IV | 0.8889 |
| | SASALGMACGPA-LAGILQTDFKI | AG, DF, GI, GM, GP, GPA, IL, LA, LG, LQ | ACE | 0.4348 |
| | | LA | APR, DPP-III | 0.0435 |
| | | GP | AM, AT, RE | 0.0435 |
| | | AG, AL, AS, GI, GP, GPA, IL, KI, LA, MA, PA, QT, TD | DPP-IV | 0.6087 |
| | | IL | ST | 0.0435 |
| | SGDVWFPQPAPK | AP, FP, GD, PAP, PQ, QP, SG, VW | ACE | 0.6667 |
| | | VW | GLUI, AO | 0.0833 |
| | | AP, FP, PA, PK, PQ, QP, VW, WF | DPP-IV | 0.6667 |
| | ASDPQLDRLNSL-PW | DR, LN, LP, PQ, RL | ACE | 0.3571 |
| | | PW | AO | 0.0714 |
| | | AS, DP, DR, LN, LP, PQ, PW, QL, RL, SL | DPP-IV | 0.7143 |
| | | SL | RE | 0.0714 |
| | DSLGLPDFGYDGPL | DF, DFG, DG, FG, GL, GLP, GP, GPL, GY, LG, LP, PL | ACE | 0.8571 |
| | | GP | AM, AT | 0.0714 |
| | | GL, GP, GY, LP, PL, SL, YD | DPP-IV | 0.5000 |
| | | GP, SL | RE | 0.1429 |
| | GPVFNPQPPKPPV | GP, GPV, KP, PP, PPK, PQ, QP, VF | ACE | 0.6923 |
| | | PP | GLUI | 0.1538 |
| | | GP, KPPV | AM | 0.1538 |
| | | KP | AO | 0.0769 |
| | | GP, PPK | AT | 0.1538 |
| | | FN, GP, GPV, KP, NP, PK, PP, PQ, PV, QP, VF | DPP-IV | 1.0000 |
| | | GP | RE | 0.0769 |
| | NVHWDPKPR | PK, PR, VHW | ACE | 0.3333 |
| | | KP | AO | 0.1111 |
| | | PR | DPP-III | 0.1111 |
| | | DP, HW, KP, NV, PK, VH, WD | DPP-IV | 0.7778 |

**Table 3.** Cont.

| | | | | |
|---|---|---|---|---|
| NBMi | NAGPADGL | AG, AGP, DG, DGL, GL, GP, GPA | ACE | 0.8750 |
| | | AD | GLUI | 0.1250 |
| | | GP | AM, AT, RE | 0.1250 |
| | | AD, AG, GL, GP, GPA, NA, PA | DPP-IV | 0.8750 |

*Legume samples are: GPCi, intestinal digested conventional cooked green pea; GPPi, intestinal digested pressure cooked green pea; GPMi, intestinal digested microwaved green pea; CPCi, intestinal digested conventional cooked chickpea; CPPi, intestinal digested pressure cooked chickpea; CPMi, intestinal digested microwaved chickpea; NBCi, intestinal digested conventional cooked navy bean; NBPi, intestinal digested pressure cooked navy bean; NBMi, intestinal digested microwaved navy bean.
**A: frequency of the occurrence of the active domain in protein sequence.
AM: antiamnestic; ACE: ACE inhibitor; AT: antithrombotic; AO: antioxidative; AI: anti-inflammatory; APR: activating ubiquitin-mediated proteolysis; CHE: chemotactic; DPP-III: dipeptidyl peptidase III inhibitor; DPP-IV: dipeptidyl peptidase IV inhibitor; GLUI: alpha-glucosidase inhibitor; GU: glucose uptake; HYP: hypotensive; HMGI: HMG-CoA reductase inhibitor; IM: immunomodulating; INH: inhibitor; NE: neuropeptide; OP: opioid; RE: regulating; REN: renin inhibitor; ST: stimulating; 35PD: CaMPDE inhibitor.

### 3.4. Peptide quantification of gastrointestinal digested legume fractions <3 kDa using a label-free MS methodology

A relative quantification of the peptides identified in the gastrointestinal digested legume fractions <3kDa using a label-free methodology was performed to assess possible differences according to the type of legume and applied thermal treatment. For this purpose, a PCA-DA on peptide profiles was performed and the obtained score plots are shown in Figure 3. According to the type of legume (Figure 3A), the discriminant component 1 explained 52.8 % of the variability in the dataset whereas the discriminant component 2 explained 47.2 % of the variance within the dataset for these two discriminant components. So, three groups of samples were statistically differentiated in the plot, corresponding to the three types of legumes (GP, CP, and NB). The observed differences could be due to the fact that although most legumes contain the same storage proteins, mostly legumin and vicilin, the ratio of these are different according to the legume specie (Gu et al., 2022). Also, it is possible that physicochemical differences between legumes can make them respond differently to different treatments and to GID. However, regarding the type of thermal treatment (Figure 3B), the discriminant component 1 was

responsible for 59.9 % of the variance within the dataset, and the discriminant component 2 was responsible for 40.1 % within the dataset. So, two different groups were statistically differentiated in the plot. One cluster included the conventional cooked and pressure cooked samples, whereas the other cluster included the microwaved samples with the exception of GPM sample, whose peptide profile was statistically different from the other microwaved samples and clustered better with conventional cooked and pressure cooked samples group. This fact could be due to differences in the effect of the microwave treatment on legumes, which could affect the later action of GI enzymes by obtaining a different profile of peptides.

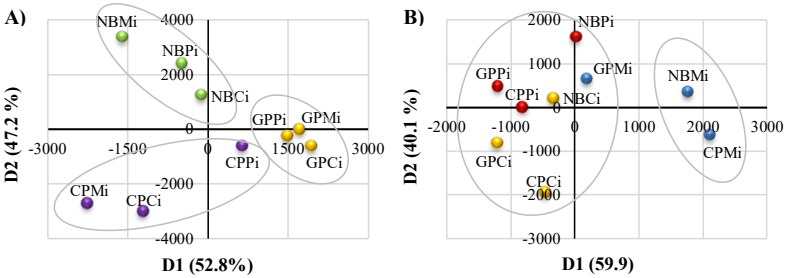


**Figure 3.** Principal Component Analysis Discriminant Analysis (PCA-DA) of samples: GPCi, intestinal digested conventional cooked green pea; GPPi, intestinal digested pressure cooked green pea; GPMi, intestinal digested microwaved green pea; CPCi, intestinal digested conventional cooked chickpea; CPPi, intestinal digested pressure cooked chickpea; CPMi, intestinal digested microwaved chickpea; NBCi, intestinal digested conventional cooked navy bean; NBPi, intestinal digested pressure cooked navy bean; NBMi, intestinal digested microwaved navy bean. A) Samples have been grouped according to the type of legume. B) Samples have been grouped according to the type of treatment.

## 4. CONCLUSIONS

In this study, it was established that GP, CP, and NB samples exerted α-amylase inhibitory activity after different types of household cooking (conventional, pressure, and microwave cooking) and GID, being the peptide fraction < 3 kDa main responsible for this bioactivity. In GP and NB, the microwave cooking showed the highest impact on the inhibition of the

enzyme whereas in CP similar values were obtained for the three types of household cooking. A total of 205 peptides were identified by LC-MS/MS, 43 of which were found to be potential bioactive peptides using *in silico* tools. The quantitative analysis of the peptide profiles showed significant differences between the type of legume and type of cooking treatment. So, the results obtained in this study proved the antidiabetic potential of legumes, which increased after GID probably due to the generation of bioactive peptides after the hydrolysis of proteins. Further studies are needed in order to identify the α-amylase inhibitory peptides responsible for the observed bioactivity as well as to define the characteristics that confer it.

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**Author contribution**

Mila Arnal: Conceptualization, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. Marta Gallego: Conceptualization, Supervision, Writing – Review & Editing. Pau Talens: Conceptualization, Supervision, Resources, Writing – Review & Editing, Project administration, Funding acquisition. Leticia Mora: Conceptualization, Formal analysis, Investigation, Supervision, Resources, Writing – Review & Editing.

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## Supplementary data

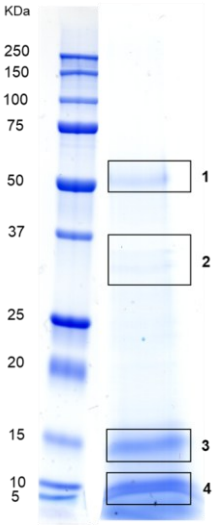


**Figure S1.** Gel electrophoresis separation of microwaved green pea sample after simulated gastrointestinal digestion and ultrafiltration > 10 kDa.

**Table S1.** Peptide sequences identified by LC-MS/MS in the legume intestinal fractions <3 kDa.

| Legume sample[a] | Peptide sequence | Parental protein[b] | Observed MW (Da)[c] | Observed *m/z* | Calculated MW (Da)[d] |
|---|---|---|---|---|---|
| GPCi | EGSLLLPNYNSR | Vicilin 47k OS | 1361.70 | 681.86 | 1361.69 |
| | NQQLQDLDIFVNSV | Vicilin 47k OS | 1631.81 | 816.91 | 1631.82 |
| | GLLGSVDSPNVSVNAP | Serine/threonine-protein kinase Nek4 (Fragment) OS | 1524.74 | 763.38 | 1524.78 |
| | CSCNCQGLPGANCVNSDK | COBRA-like protein 6 OS | 1811.89 | 604.97 | 1811.71 |
| | SIIALPSV | REVERSED SBP-type domain-containing protein OS; REVERSED Putative transcription factor SBP family OS | 798.46 | 400.24 | 798.49 |
| | ASAQTIGSHSVAG | PMD domain-containing protein OS | 1178.46 | 590.24 | 1184.58 |
| | NGGQSQLVCSGCRSLLMYPVGAT | Uncharacterized protein OS | 2356.95 | 590.24 | 2357.08 |
| | NAPLGIGGLPKGF | Uncharacterized protein OS | 1239.59 | 620.80 | 1239.70 |
| | DKGHSGSVGPIIIIR | Serrate RNA effector molecule OS | 1547.77 | 516.93 | 1547.88 |
| | SSIRTVLALAATLDLEVEQ | Integrase catalytic domain-containing protein OS | 2028.11 | 677.04 | 2028.11 |
| | LGTGSGRTIGLRA | M20_dimer domain-containing protein OS | 1257.58 | 629.80 | 1257.72 |
| | SVSLQTLSSLKGLSKPFEGGI | REVERSED probable leucine-rich repeat receptor-like protein kinase At1g35710 OS | 2147.10 | 1074.56 | 2147.18 |
| | QYDDLLDIDGPQ | BAH domain-containing protein OS; BAH domain-containing protein OS | 1390.68 | 696.35 | 1390.63 |
| | GAGGGGFGGGSGGGAGGGIGGG-GAGGGFGGGAGGGG | glycine-rich cell wall structural protein OS | 2392.73 | 1197.37 | 2393.01 |
| | GPSGPVGPSGPVG | Uncharacterized protein OS | 1063.54 | 532.78 | 1063.53 |
| | VDLLGPLKALSRPIHGKFGNEAL | REVERSED Protein kinase domain-containing protein OS | 2445.08 | 612.28 | 2444.39 |
| | GMISQIRVARPPAEGR | Uncharacterized protein OS | 1736.84 | 869.43 | 1736.95 |
| | SVGQGGGVSPCGCADA | REVERSED DUF4283 domain-containing protein (Fragment) OS | 1363.61 | 682.81 | 1363.55 |
| | DGNDDEVAHVVAL | Protein ALWAYS EARLY 3 (Fragment) OS | 1352.65 | 677.33 | 1352.62 |
| GPPi | TETWNPNNPEL | Minor legumin (Fragment) OS | 1313.58 | 657.80 | 1313.59 |
| | GTLPCTVLIPGLFFI | MFS domain-containing protein OS | 1589.80 | 795.91 | 1589.89 |
| | GLGGGGGIGGGGAGGGIGHGLG | REVERSED m-rich cell wall structural protein-like OS | 1647.78 | 824.90 | 1647.81 |
| | VAEHPQ | DNA-directed RNA polymerase subunit OS; SAP-like protein BP-73 OS | 679.33 | 340.67 | 679.33 |
| | PGVGVAAPQIG | Peptide deformylase OS | 964.47 | 483.24 | 964.53 |
| | GSVVDAH | Putative pentatricopeptide repeat-containing protein OS | 683.36 | 342.69 | 683.32 |
| | KNDTGSNAGCSGV | Nucleic acid binding protein OS | 1208.33 | 605.17 | 1208.51 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| GPPi | SFNTDYEEIEK | Vicilin OS | 1373.60 | 687.81 | 1373.60 |
| | KILCSPFDQPELPEG | Proline--tRNA ligase OS | 1671.90 | 836.96 | 1671.82 |
| | PTIDCISTPL | REVERSED Uncharacterized protein OS; REVERSED NB-ARC domain-containing protein OS | 1058.57 | 530.29 | 1058.53 |
| | GSVGSGVGAGYGSGYGQ | REVERSED Uncharacterized protein OS | 1458.47 | 730.24 | 1458.64 |
| | TGFKGPQAVGQAIVNN | Arginyl-tRNA synthetase (Fragment) OS | 1599.72 | 800.87 | 1599.84 |
| | SSASTVNQHGVGGE | REVERSED HECT-type E3 ubiquitin transferase OS | 1328.53 | 665.27 | 1328.60 |
| | GPSGPVGPSGPVG | Uncharacterized protein OS | 1063.54 | 532.78 | 1063.53 |
| | PGGQPGAP | Uncharacterized protein OS; ER membrane protein complex subunit 10 OS | 679.36 | 340.69 | 679.33 |
| | EGSLLLPNYNSR | Allergen Len c 1.0101 (Fragment) OS; Allergen Len c 1.0102 (Fragment) OS; Vicilin (Fragment) OS; Vicilin 47k OS; Beta-lathyrin 1 (Fragment) OS; Vicilin, 14 kDa component OS | 1361.70 | 681.86 | 1361.69 |
| | VDEWLYSGGPY | Uncharacterized protein (Fragment) OS | 1284.54 | 643.28 | 1284.57 |
| GPMi | DIKVKEAAGGILANLAL | Uncharacterized protein OS; uncharacterized protein LOC106777739 isoform X1 OS; uncharacterized protein LOC106777739 isoform X3 OS; Uncharacterized protein (Fragment) OS; uncharacterized protein LOC106777739 isoform X2 OS; U-box domain-containing protein 12 OS | 1696.12 | 849.07 | 1695.98 |
| | VNKLMVPLLPV | Putative leucine-rich repeat domain, L domain-containing protein OS | 1237.74 | 1238.75 | 1237.75 |
| | GAGGPGGAGWGPGPGGPGW | Uncharacterized protein (Fragment) OS | 1547.77 | 774.89 | 1547.69 |
| | LSPGDVVIIPAGHPV | Convicilin (Fragment) OS; Convicilin OS; Cvc protein (Fragment) OS; Putative rmlC-like jelly roll protein OS | 1469.82 | 735.92 | 1469.82 |
| | SYNDNLMLSY | Transcription factor MYB36 (Fragment) OS | 1218.35 | 610.18 | 1218.52 |
| | GSGYGSGYGYGEGSGYGS | Uncharacterized protein OS | 1660.52 | 831.27 | 1660.63 |
| | GAEGNSDEPR | Protein kinase domain-containing protein OS; Brassinosteroid insensitive 1-associated receptor kinase 1 OS | 1030.30 | 516.16 | 1030.43 |
| | APISAENDVAPG | Uncharacterized protein OS | 1139.39 | 570.70 | 1139.55 |
| | SGDVWFPQPAPK | Formate dehydrogenase, mitochondrial OS; Formate dehydrogenase OS; 2-Hacid_dh_C domain-containing protein; Formate dehydrogenase (Fragment) OS | 1327.66 | 664.84 | 1327.66 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| CPCi | GNSVFDGELEAGR | legumin-like OS | 1349.63 | 675.82 | 1349.62 |
| | LHQNIGSSSSPDIY | legumin-like OS | 1516.72 | 759.37 | 1516.72 |
| | SEGGLIETWNPSNK | legumin-like OS | 1530.74 | 766.38 | 1530.73 |
| | SLASTCMPLSF | ATP-dependent Clp protease proteolytic subunit OS | 1187.46 | 594.74 | 1187.52 |
| | GNVPGIDGEEIAPF | Starch synthase, chloroplastic/amyloplastic OS | 1413.69 | 707.85 | 1413.68 |
| | LSSGDVVVIPASHPF | vicilin-like OS | 1523.80 | 762.91 | 1523.80 |
| | AAASSAEAQLSSPK | J domain-containing protein OS; Platelet binding protein GspB-like OS | 1316.57 | 659.29 | 1316.66 |
| | IILGPK | Lectin OS; albumin-2-like OS; Albumin-2 OS | 639.44 | 320.73 | 639.43 |
| | SVEINEGSLLLPHFNSR | vicilin-like OS | 1910.99 | 638.00 | 1910.98 |
| | TETWNPNHPELQ | legumin J-like OS | 1464.67 | 733.34 | 1464.66 |
| | MGYDVGLGPPQ | Uncharacterized protein OS | 1132.53 | 567.27 | 1132.52 |
| | ILEASFNSDYEEIER | vicilin-like OS | 1813.84 | 907.93 | 1813.84 |
| | DFLEDALNVNR | legumin-like OS | 1305.60 | 436.21 | 1304.64 |
| | LHQNIDSSSSPDIY | legumin A-like OS | 1574.72 | 788.37 | 1574.72 |
| | LHQNIGSSSSPDIYNPQ | legumin-like OS | 1855.88 | 928.95 | 1855.87 |
| | EASFNSDYEEIER | vicilin-like OS | 1587.68 | 794.85 | 1587.67 |
| | VVGECARANVAVVPQG | FAD-binding oxidoreductase OS | 1568.75 | 785.38 | 1568.80 |
| | GPIESGALSGPLDDAVSSSGGVPF-SAP | protein phosphatase 2C 29-like OS | 2470.23 | 824.42 | 2470.19 |
| | SRVLSSFGGQIGALAADADPVSK | REVERSED uncharacterized protein LOC113867461 OS | 2246.02 | 749.68 | 2246.15 |
| | LHQNIGSSSSPDIYNPQAG | legumin-like OS | 1983.94 | 992.98 | 1983.93 |
| | FTILLK | CST complex subunit CTC1 OS; NAD(P)H-quinone oxidoreductase chain 4 OS; ABC transporter G family member 9-like OS; B3 domain-containing protein At5g24050 family OS; REVERSED VOC domain-containing protein OS; REVERSED Sister-chromatide cohesion protein OS; REVERSED NAD(P)H-quinone oxidoreductase chain 4, chloroplastic OS; REVERSED Uncharacterized protein OS; REVERSED Phosphatidylinositide phosphatase SAC1-like protein OS; REVERSED Reticulon-like protein OS; REVERSED Uncharacterized protein OS | 733.48 | 367.75 | 733.47 |
| | SFNSDYEEIER | vicilin-like OS | 1387.59 | 694.80 | 1387.59 |
| | DIDEIGNLWYHH | REVERSED Putative ETHYLENE INSENSITIVE 3-like 4 protein OS | 1510.59 | 756.30 | 1510.68 |
| | SIITPPEKEPR | legumin-like OS | 1265.70 | 422.91 | 1265.70 |
| | AGGGFGGGAGGGAGGGGGI | REVERSED glycine-rich cell wall structural protein-like OS | 1289.55 | 645.78 | 1289.57 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| CPCi | GLHIPSFSPSPQ | legumin J-like OS | 1265.65 | 633.83 | 1265.64 |
| | VNYPIISDPK | Peroxiredoxin OS | 1144.62 | 573.32 | 1144.61 |
| CPPi | TETWNPNHPELQ | Uncharacterized protein OS | 1464.66 | 733.34 | 1464.66 |
| | GLHLPSFSPSPQ | Uncharacterized protein OS | 1266.62 | 634.32 | 1265.64 |
| | PAPVPQSSSTFV | Uncharacterized protein OS | 1212.31 | 607.16 | 1215.61 |
| | ESALSMGIHLHTKEKKITRDLL | Uncharacterized protein OS | 2536.83 | 635.21 | 2535.38 |
| | LGTGSGRTIGLRA | M20_dimer domain-containing protein OS | 1257.59 | 629.80 | 1257.72 |
| | WLALGLAQRARGHH | Uncharacterized protein OS | 1584.76 | 793.39 | 1584.88 |
| | DDGGPPGGSGGPA | Uncharacterized protein OS | 1038.43 | 520.22 | 1039.42 |
| | EREGSDALVDPLVNYWGLSLFPS-PSLP | Centromere protein C (Fragment) OS | 2977.21 | 745.31 | 2977.47 |
| | VQMIGKGAVREN | Uncharacterized protein OS | 1300.61 | 651.31 | 1300.69 |
| | AAVGGGGGIGPS | Eukaryotic translation initiation factor 3 subunit C OS | 894.35 | 448.18 | 898.45 |
| | ISEMNTDP | REVERSED Uncharacterized protein OS | 904.41 | 453.21 | 905.38 |
| CPMi | LHQNIGSSSSPDIYNPQAGR | legumin-like OS | 2140.04 | 714.35 | 2140.03 |
| | ILEASFNSDYEEIER | vicilin-like OS | 1813.85 | 907.93 | 1813.84 |
| | LHQNIDSSSSPDIYIPQAGR | legumin A-like OS | 2194.14 | 732.39 | 2197.08 |
| | MHPFMGGFT | uncharacterized protein LOC101511319 isoform X2 OS; uncharacterized protein LOC101511319 isoform X3 OS; uncharacterized protein LOC101511319 isoform X1 OS | 1023.40 | 512.71 | 1023.43 |
| | SFNSDYEEIER | vicilin-like OS | 1386.62 | 694.32 | 1387.59 |
| | LHQNIGSSSSPDIYNPQ | legumin-like OS | 1855.87 | 928.94 | 1855.87 |
| | LHQNIGSSSSPDIYNPQAG | legumin-like OS | 1983.93 | 992.97 | 1983.93 |
| | IGALFEEPQ | Glucose-6-phosphate dehydrogenase (NADP(+)) OS; Glucose-6-phosphate 1-dehydrogenase OS | 1002.43 | 502.22 | 1002.50 |
| | SGYGSGYGYGEGSGYGSG | Uncharacterized protein OS | 1660.67 | 831.34 | 1660.63 |
| | SQGNEPEAGS | Chitin elicitor receptor kinase 1 OS; lysM domain receptor-like kinase 4 OS; Uncharacterized protein OS | 974.38 | 975.39 | 974.39 |
| | NDHDMVHYYGGDMF | Ethylene-responsive transcription factor ERF114 family OS | 1700.54 | 851.28 | 1700.62 |
| | LAGNQEQEFLQYQQQEGR | legumin A-like OS | 2165.02 | 722.68 | 2165.01 |
| | ATVILVVNEGKGEVELV | vicilin-like OS | 1768.00 | 885.01 | 1768.00 |
| | LSSGDVVVIPASHPF | vicilin-like OS | 1523.80 | 762.91 | 1523.80 |
| | SLIDTSSFQNQLDQMPR | legumin-like OS | 1981.07 | 991.54 | 1978.94 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| | DGVMDSPS | Uncharacterized protein OS | 806.31 | 404.16 | 806.31 |
| | GDSSGAAVNKDDPSICSR | REVERSED Protein tipD-like (Fragment) OS; REVERSED Protein tipD-like OS; REVERSED Nucleotide-binding protein OS | 1777.79 | 593.60 | 1777.79 |
| | DTSSFQNQLDQMPR | legumin-like OS | 1665.75 | 833.88 | 1665.74 |
| | LHQNIGSSSSPDI | legumin-like OS | 1353.65 | 677.83 | 1353.65 |
| | LSDNSPISANIKGQ | REVERSED DNA excision repair protein ERCC-6 OS | 1443.75 | 722.88 | 1443.72 |
| | GDTIKLPAGTI | vicilin-like OS | 1084.61 | 543.31 | 1084.61 |
| | TETWNPNHPELQ | legumin J-like OS | 1465.64 | 733.83 | 1465.65 |
| | SEKGKSVQADSAGETDAT | REVERSED Uncharacterized protein (Fragment) OS | 1779.83 | 594.28 | 1779.81 |
| | VVGGMVAGA | REVERSED ADP/ATP translocase OS | 759.38 | 760.38 | 759.39 |
| | EHAMNPTPQ | NAD(P)-binding rossmann-fold protein OS; NADPH-dependent aldehyde reductase 1, chloroplastic-like OS; NADPH-dependent aldehyde reductase 1, chloroplastic (Fragment) OS; Glucose and ribitol dehydrogenase-like protein OS | 1023.45 | 512.73 | 1023.44 |
| | TETWNPNHPE | legumin J-like OS | 1223.52 | 612.77 | 1223.52 |
| CPMi | ASAGLSTAPLVHPDAFLEEGQNE-SNL | REVERSED Subtilisin-like protease SBT1.1 (Fragment) OS; REVERSED Subtilisin-like protease OS | 2666.28 | 889.77 | 2666.28 |
| | GNSVFDGELEAGR | legumin-like OS | 1349.63 | 675.82 | 1349.62 |
| | GGSGGGGGVT | WD repeat-containing protein 44 isoform X1 OS | 704.31 | 353.16 | 704.31 |
| | VHQRVVDQHQ | Lactamase_B domain-containing protein OS | 1244.54 | 623.28 | 1244.64 |
| | TNAPQEIFIQQGNGY | legumin-like OS | 1678.79 | 840.40 | 1678.80 |
| | SIITPPEKEPR | legumin-like OS | 1265.70 | 422.91 | 1265.70 |
| | HNENSGSEGTYDHSFCDK | uncharacterized protein LOC106765885 OS | 2025.69 | 1013.85 | 2025.78 |
| | LGGNPEVEFPETQ | legumin J-like OS | 1415.66 | 708.84 | 1415.66 |
| | VNYPIISDPK | Peroxiredoxin OS; Thioredoxin-dependent peroxiredoxin OS; 1-Cys peroxiredoxin OS | 1144.61 | 573.31 | 1144.61 |
| | DAVKPGDPPENSPGLGSGE | REVERSED RVP_2 domain-containing protein OS | 1821.88 | 911.95 | 1821.84 |
| | GAGVGPLGAGGVGH | REVERSED Uncharacterized protein OS | 1104.57 | 553.29 | 1104.57 |
| | LSCVGTPQ | Peptide transporter PTR2 (Fragment) OS | 803.40 | 402.71 | 803.38 |
| | LKGAPKGAPK | 50S ribosomal protein L7/L12 OS | 965.46 | 483.74 | 965.60 |
| | GDTIKLPAGTIAY | vicilin-like OS | 1318.71 | 660.36 | 1318.71 |
| | GSVLLLGEPLCLGSVFGGILL | WAT1-related protein OS | 2056.42 | 1029.22 | 2056.16 |
| NBCi | SGYGGNMAGSGGYGS | Uncharacterized protein OS; zinc finger Ran-binding domain-containing protein 2-like OS; Putative Zinc finger, RanBP2-type OS | 1336.41 | 669.21 | 1336.50 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| NBCi | KILGLDHNKFGGSLP | Putative LRR receptor-like serine/threonine-protein kinase OS | 1595.12 | 798.57 | 1594.88 |
| | GSGYGSGEGYGYGSGYGS | REVERSED Uncharacterized protein OS | 1660.52 | 831.27 | 1660.63 |
| | VAEHPK | Dihydroflavonol 4-reductase (Fragment) OS; ATP-dependent helicase BRM isoform X2 OS; ATP-dependent helicase BRM isoform X1 OS; Cinnamyl alcohol dehydrogenase-like protein (Fragment) OS; Uncharacterized protein (Fragment) OS; REVERSED PPR containing plant-like protein OS; REVERSED Putative tetratricopeptide-like helical domain-containing protein OS; REVERSED Uncharacterized protein (Fragment) OS | 679.33 | 340.67 | 679.37 |
| | ILKIGVPVGVKK | Tudor domain-containing protein OS | 1249.90 | 1250.91 | 1249.85 |
| | SLSLTFADRNAC | Hexosyltransferase OS | 1296.41 | 649.21 | 1296.61 |
| | VLVPIEPPHHQPL | Uncharacterized protein (Fragment) OS | 1474.83 | 492.62 | 1474.83 |
| | GSGYGSGGGYGSGYGS | putative glycine-rich cell wall structural protein 1 OS | 1368.46 | 685.24 | 1368.52 |
| | YEAGVLPGPGYE | Uncharacterized protein OS | 1250.58 | 626.30 | 1250.58 |
| | GCDMICLKFFHENWAH | REVERSED Putative sulfate transporter (Fragment) OS | 1965.74 | 656.25 | 1965.83 |
| | VTRGSNSPLRSG | Uncharacterized protein (Fragment) OS | 1229.78 | 615.90 | 1229.65 |
| | GSVPDEDII | F-box/RNI/FBD-like domain protein OS | 943.47 | 472.74 | 943.45 |
| | TSEEASST | calmodulin-binding transcription activator 4 isoform X2 OS; calmodulin-binding transcription activator 4 isoform X1 OS; calmodulin-binding transcription activator 4 isoform X3 OS | 810.28 | 406.14 | 810.32 |
| | NVHWDPKPR | Lectin_legB domain-containing protein OS; Erythroagglutinin OS; Erythroagglutinating phytohemagglutinin OS; Phytohemagglutinin OS; PHA-E protein OS | 1147.59 | 383.54 | 1147.59 |
| | GVVVVMVVVVVVLVVVVG | loricrin-like OS | 1763.26 | 882.64 | 1763.14 |
| | SGYGSGYGYGEGSGYGSG | Uncharacterized protein OS | 1660.52 | 831.27 | 1660.63 |
| NBPi | NIDVPNNSGPADGLA | Lectin_legB domain-containing protein OS; Erythroagglutinin OS; Erythroagglutinating phytohemagglutinin OS; Phytohemagglutinin OS; Lectin OS; PHA-E protein OS | 1452.67 | 727.34 | 1452.68 |
| | GVIPR | SDR family oxidoreductase OS; Kinesin motor domain-containing protein OS; BRO1 domain-containing protein OS | 540.34 | 271.18 | 540.34 |
| | GGGGGIGGGAGGGGGI | Uncharacterized protein OS | 1056.50 | 529.26 | 1056.49 |
| | GGGGGLGGGGGAGGGL | Uncharacterized protein OS | 1056.50 | 529.26 | 1056.49 |
| | GGGGGLGGGGGAGGGI | REVERSED Uncharacterized protein OS | 1056.50 | 529.26 | 1056.49 |
| | GVLPR | pollen-specific leucine-rich repeat extensin-like protein 3 OS; Uncharacterized protein OS | 540.34 | 271.18 | 540.34 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| NBPi | YEAGVLPGPGYE | Uncharacterized protein OS | 1250.58 | 1251.58 | 1250.58 |
| | IGLPGIGL | Uncharacterized protein OS | 738.39 | 370.20 | 738.46 |
| | IGLPGLGL | Uncharacterized protein OS | 738.39 | 370.20 | 738.46 |
| | TFNIQVPNNAGPA | Lectin_legB domain-containing protein OS; Phytohemagglutinin OS; Leucoagglutinating phytohemagglutinin OS | 1341.66 | 671.84 | 1341.67 |
| | GGGAGGGIGGGAGGGGGVGGGVGGGKGGGFGGGGGGGGGGGAGGGGSGGAGGGGGGGGGVGGR | REVERSED Uncharacterized protein OS | 4081.73 | 1361.58 | 4081.82 |
| | IGAVAESKCNDDSAVAVK | REVERSED Uncharacterized protein OS; REVERSED Putative nucleobase-ascorbate transporter 10 OS | 1775.84 | 592.95 | 1775.87 |
| | PIGGPVPPGAFDSTAPAPNA | Transmembrane protein, putative OS | 1831.84 | 916.93 | 1831.91 |
| | SGNVHVPQD | REVERSED PIPK domain-containing protein OS; REVERSED 1-phosphatidylinositol-3-phosphate 5-kinase OS | 951.40 | 476.71 | 951.44 |
| | GGGGPLGGGSGVVPLGGGGPL | REVERSED Uncharacterized protein OS | 1617.80 | 809.91 | 1617.85 |
| | CLGKKNSLGEEIRGLDIH | Protein kinase domain-containing protein OS | 1981.02 | 991.52 | 1981.04 |
| | SELASGCCTLLTCTTKGNASTRS | Uncharacterized protein OS | 2303.14 | 1152.58 | 2303.06 |
| | IQGIERGDALPGLRSYSDIA | 24-methylenesterol C-methyltransferase 2-like OS; Methyltransferase OS; Methyltransferase (Fragment) OS | 2130.10 | 711.04 | 2130.11 |
| | TREYFKEGMFQDVNIANKQLR | Protein SCAI OS; Nuclear pore complex protein OS; Uncharacterized protein OS | 2586.26 | 863.09 | 2586.30 |
| | RAGRSAENLAGALFAVREIFG | Mannitol dehydrogenase family protein OS | 2204.06 | 735.69 | 2204.18 |
| | DPVVVFNET | REVERSED Uncharacterized protein OS; REVERSED 9-cis-epoxycarotenoid dioxygenase OS; REVERSED Nine-cis-epoxycarotenoid dioxygenase2 OS; REVERSED 9-cis-epoxycarotenoid dioxygenase NCED1, chloroplastic OS; REVERSED Putative 9-cis-epoxycarotenoid dioxygenase (Fragment) OS; REVERSED 9-cis-epoxycarotenoid dioxygenase chloroplastic-like OS | 1018.38 | 510.20 | 1018.50 |
| NBMi | QVEEEGSNVLGGFGK | Uncharacterized protein OS; Legumin OS | 1531.71 | 766.86 | 1531.72 |
| | FLTSDNPIFSDHQ | Alpha-phaseolin OS; Cupin type-1 domain-containing protein (Fragment) OS; Phaseolin OS | 1519.68 | 760.85 | 1519.69 |
| | ASDPQLDRLNSLPW | Uncharacterized protein OS | 1610.79 | 806.40 | 1610.81 |
| | VVGECARANVAVVPQG | FAD-binding oxidoreductase OS | 1568.73 | 785.37 | 1568.80 |
| | LAVANYPQWVANIVSVP | G-patch domain-containing protein (Fragment) OS | 1839.88 | 614.30 | 1839.99 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| | GPLGGGSGVVPLGGGGPLGGGS-GVGPLGGGGP | REVERSED Uncharacterized protein OS; REVERSED Uncharacterized protein OS | 2453.21 | 818.75 | 2453.27 |
| | GEGGGGGGGGGGGGGGNG | Uncharacterized protein OS | 1173.51 | 587.76 | 1173.44 |
| | IQVDNLKVPN | Uncharacterized protein OS; cytokinin hydroxylase OS | 1138.48 | 570.25 | 1138.63 |
| | VGDNTADTGTGPR | Uncharacterized protein OS | 1259.57 | 630.79 | 1259.57 |
| | QVEEEGSNVLGGFGKR | Uncharacterized protein OS; Legumin OS | 1704.84 | 569.29 | 1704.84 |
| | GNPEALYRQGVPLRNLKSTVIDE-PPW | Component of oligomeric Golgi complex 7 OS | 2949.53 | 984.18 | 2949.53 |
| | TSNDYNQLDQSPR | Uncharacterized protein OS; Legumin OS | 1536.65 | 769.33 | 1536.68 |
| | YEAGVLPGPGYE | Uncharacterized protein OS | 1250.58 | 626.30 | 1250.58 |
| | SGDVWFPQPAPK | Formate dehydrogenase, mitochondrial OS; Formate dehydrogenase, mitochondrial OS; Formate dehydrogenase OS; 2 Hacid_dh_C domain-containing protein OS; 2-Hacid_dh_C domain-containing protein OS; 2-Hacid_dh_C domain-containing protein OS; Formate dehydrogenase, mitochondrial OS; Formate dehydrogenase, mitochondrial OS; Formate dehydrogenase, mitochondrial OS | 1327.65 | 664.83 | 1327.66 |
| | GLGGGGGSGGGGGGGL | REVERSED Uncharacterized protein OS | 1062.50 | 532.26 | 1072.49 |
| NBMi | GEGAGSEEGNNGGEGGGI | Uncharacterized protein OS | 1546.71 | 774.36 | 1546.61 |
| | VLVPIEPPHHQPL | Uncharacterized protein (Fragment) OS | 1474.83 | 492.62 | 1474.83 |
| | SFNSKFEEINR | Alpha-phaseolin OS; Cupin type-1 domain-containing protein (Fragment) OS; Phaseolin OS | 1369.66 | 457.56 | 1369.66 |
| | IEEELEEL | Charged multivesicular body protein OS; THUMP domain-containing protein 1 homolog OS; THUMP domain-containing protein OS; Reverse transcriptase domain-containing protein OS; Charged multivesicular body protein 7-like (Fragment) OS; REVERSED Cobalamin biosynthesis protein OS; REVERSED COBW domain-containing protein 1 isoform X2 OS; REVERSED COBW domain-containing protein 1 isoform X1 OS; REVERSED COBW domain-containing protein 1 OS; REVERSED CobW C-terminal domain-containing protein (Fragment) OS; REVERSED Putative cobalamin (Vitamin B12) biosynthesis CobW-like protein OS; REVERSED COBW domain-containing protein 1-like (Fragment) OS; REVERSED Uncharacterized protein OS; REVERSED COBW domain-containing protein OS; REVERSED Uncharacterized protein (Fragment) OS | 1002.42 | 502.22 | 1002.48 |
| | FLTQGDNPIFSDNQ | Cupin type-1 domain-containing protein OS; Phaseolin, alpha-type OS | 1594.72 | 798.36 | 1594.73 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| NBMi | VEEVVTCVNESDPV | Uncharacterized protein OS | 1518.56 | 760.29 | 1518.68 |
| | NVHWDPKPR | Lectin_legB domain-containing protein OS; Erythroagglutinin OS; Phytohemagglutinin OS; PHA-E protein OS; Erythroagglutinating phytohemagglutinin OS | 1147.59 | 383.54 | 1147.59 |
| | VNYPIISDPK | Peroxiredoxin OS | 1144.61 | 573.31 | 1144.61 |
| | NIDVPNNSGPADGL | Lectin_legB domain-containing protein OS; Erythroagglutinin OS; Phytohemagglutinin OS; PHA-E protein OS; Erythroagglutinating phytohemagglutinin OS | 1381.64 | 691.83 | 1381.65 |
| | AGDTAVGGGPY | Uncharacterized protein OS | 963.43 | 482.72 | 963.43 |
| | GGANANDDDGTKGANAN | RRP12-like protein OS | 1561.55 | 781.78 | 1561.62 |
| | NAGPADGL | Lectin_legB domain-containing protein OS; Leucoagglutinating phytohemagglutinin OS | 713.33 | 714.34 | 713.33 |
| | RVDEGAAEAKEDISFRSLHTGPVS | Galacturonosyltransferase-like 4-like (Fragment) OS | 2570.42 | 1286.22 | 2570.27 |
| | GPVFNPQPPKPPV | Uncharacterized protein OS;Uncharacterized protein (Fragment) OS | 1372.74 | 687.38 | 1372.75 |
| | LKDGLSVISPK | Uncharacterized protein OS; Legumin OS | 1155.68 | 578.85 | 1155.69 |
| | DLSNLTMLDLSTNN | Protein kinase domain-containing protein OS | 1550.75 | 776.38 | 1550.71 |
| | TSDNPIFSDHQ | Alpha-phaseolin OS; Cupin type-1 domain-containing protein (Fragment) OS; Phaseolin OS | 1259.54 | 630.78 | 1259.54 |
| | SFNSKFEEINRV | Alpha-phaseolin OS; Cupin type-1 domain-containing protein (Fragment) OS; Phaseolin OS | 1468.73 | 490.58 | 1468.73 |
| | KLEDYNLYENIVPICPICYVINH | Putative mitochondrial protein (Fragment) OS | 2765.36 | 922.79 | 2765.34 |
| | ATYSAFGK | Purple acid phosphatase OS | 843.41 | 422.71 | 843.41 |
| | EEEIFFEELLKE | REVERSED Maturase K OS; REVERSED Maturase-like protein (Fragment) OS; REVERSED Maturase K (Fragment) OS | 1553.68 | 518.90 | 1553.75 |
| | LTEVGPPDS | Uncharacterized protein OS | 913.43 | 457.72 | 913.44 |
| | SDNPIFSDHQ | Alpha-phaseolin OS; Cupin type-1 domain-containing protein (Fragment) OS; Phaseolin OS | 1158.49 | 580.25 | 1158.49 |
| | SASALGMACGPALAGILQTDFKI | SPX domain-containing membrane protein At4g22990 isoform X1 OS; SPX domain-containing membrane protein At4g22990 isoform X2 OS; SPX domain-containing protein OS | 2235.15 | 1118.58 | 2235.13 |
| | DSLGLPDFGYDGPL | REVERSED Chlorophyll a-b binding protein, chloroplastic OS | 1464.64 | 733.33 | 1464.68 |
| | TSDNPIFSDH | Alpha-phaseolin OS; Cupin type-1 domain-containing protein (Fragment) OS; Phaseolin OS | 1128.61 | 565.31 | 1131.48 |
| | PNNSSVFHENVT | Transcription factor PIL1 (Fragment) OS | 1343.71 | 672.86 | 1343.61 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| NBMi | ANDANEGANFGHPIRIVPGKDNE-ASR | Uncharacterized protein OS | 2749.37 | 1375.69 | 2749.32 |
| | DAGPAAALH | Pectinesterase (Fragment) OS | 821.36 | 411.69 | 821.40 |

[a] Samples are: GPCi, intestinal digested conventional cooked green pea; GPPi, intestinal digested pressure cooked green pea; GPMi, intestinal digested microwaved green pea; CPCi, intestinal digested conventional cooked chickpea; CPPi, intestinal digested pressure cooked chickpea; CPMi, intestinal digested microwaved chickpea; NBCi, intestinal digested conventional cooked navy bean; NBPi, intestinal digested pressure cooked navy bean; NBMi, intestinal digested microwaved navy bean.

[b] According to UniProt database.

[c] Observed molecular weight (Da) of the identified peptide.

[d] Calculated molecular weight (Da) of the matched peptide.

# Peptidomic profile and α-glucosidase inhibitory activity of cooked and gastrointestinal digested legumes

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**Abstract**

Legumes are a good source of bioactive compounds, including peptides with antidiabetic potential. The α-glucosidase inhibitory activity of simulated gastrointestinal digested (GID) soybean, chickpea, green pea, and navy bean was determined after using three different household cooking methods (conventional, pressure, and microwave cooking). Samples were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC), and the fractions responsible for the α-glucosidase inhibitory activity were isolated. Lastly, peptides were identified by mass spectrometry in tandem (MS/MS) and *in silico* analyses were done to hypothesise potentially bioactive sequences. The results indicated that all legume extracts exert α-glucosidase inhibitory activity after thermal treatment and GID. Peptide profiles obtained by RP-HPLC showed the highest generation of peptides after the intestinal digestion phase, with small changes between thermal treatments. Best α-glucosidase inhibitory activity was observed in fraction 2 of all gastrointestinal digested samples, with values between 40 % and 62 % inhibition. In soybean, green pea, and navy bean, conventional-cooked samples showed the highest activity, while the pressure-cooked treatment resulted in significantly higher activity in chickpea samples. Finally, 48 peptides were identified by MS/MS and 13 were found as potentially bioactive using *in silico* tools, expanding previous knowledge on antidiabetic peptides derived from legumes.

*Keywords: legume, antidiabetic, α-glucosidase inhibition, in vitro gastrointestinal digestion, peptides.*

## 1. INTRODUCTION

Legumes, belonging to the Leguminosae family, are a globally consumed non-cereal staple food (Bessada et al., 2019; Gu et al., 2023). They are a great source of protein, complex carbohydrates, fibre, minerals, vitamins, and various phytochemicals (Hu et al., 2023; Singhal et al., 2014). Their main proteins are storage proteins (albumins, globulins, and glutelins), which are abundant in lysine, leucine, aspartic acid, and arginine but are generally deficient in methionine, cysteine, and tryptophan amino acids (Bessada et al., 2019).

Legumes are gaining interest as a sustainable alternative to animal protein (FAO, 2016), but they are less digestive due to their secondary structure and the presence of antinutritional factors such as protease inhibitors, phytic acid, or tannins (Bessada et al., 2019). Nevertheless, it has been described that the application of heat treatments on legumes can inactivate thermolabile antinutritional factors and denature proteins, making them more accessible to gastrointestinal enzymes and improving their digestibility (Gu et al., 2023; Ohanenye et al., 2022).

Bioactive peptides can be obtained from legume proteins. These fragments are between 2 and 20 amino acid residues and exert their function once they are released from their parent protein after an enzymatic hydrolysis process (Acquah et al., 2022; Li et al., 2022). Bioactive peptides have the potential to positively impact health, serving as antioxidants, antihypertensives, antimicrobials, antidiabetics, etc. (Kehinde & Sharma, 2020; Valenzuela Zamudio & Segura Campos, 2022). Mojica et al. (2017) identified peptides from common beans with antidiabetic, antihypertensive, and antioxidant potential, whereas Shi et al. (2019) demonstrated the hypolipidemic activity of chickpea peptides. Nagaoka et al. (2010) identified a peptide from soybean with hypocholesterolemic activity, and Rizzello et al. (2015) demonstrated the antifungal potential of pea flour peptides.

Regarding antidiabetic activity, peptides can act as inhibitors of α-glucosidase, α-amylase, or dipeptidyl peptidase-IV (DPP-IV) enzymes, inhibitors of the glucose transporter system or insulin mimetics (Elam et al., 2021; Patil et al., 2020). Among them, reducing the digestion of carbohydrates by controlling the activity of the α-amylase and α-glucosidase enzymes is used as a common strategy in the management of type 2 diabetes. α-Amylase enzyme hydrolyses starch into oligosaccharides and disaccharides, whereas α-glucosidase enzyme hydrolyses oligosaccharides and disaccharides into glucose (Acquah et al., 2022; Iwaniak et al., 2018;). Thus, the inhibition of both enzymes would prevent complex polysaccharides from becoming hydrolysed, through different ways, and would reduce the post-prandial increase of blood glucose (Acquah et al., 2022; Kehinde & Sharma, 2020). In a previous study, different cooked and gastrointestinal digested legumes showed α-amylase inhibitory activity, being the peptide fractions <3 kDa main responsible for this bioactivity (Arnal et al., 2023). Moreover, 43 peptides were identified and predicted as probably bioactives using *in silico* tools, and quantitative peptidomics evidenced differences in the peptide profiles according to the type of legume and thermal treatment applied (Arnal et al., 2023). However, information about α-glucosidase inhibitory peptides is limited, and only a few sequences derived from cooked legumes have been reported (Li et al., 2022; Rahmi & Arcot, 2023). The aim of this study was to evaluate the α-glucosidase inhibitory activity of gastrointestinal digested soybean, chickpea, green pea, and navy bean after using three different household cooking methods (conventional, pressure, and microwave cooking), as well as to analyse their peptide profile and to identify the peptide sequences potentially responsible for the bioactivity.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Enzymes α-glucosidase from *Saccharomyces cerevisiae* (G5003-1KU), α-amylase (A3171) and pancreatin (P7545) from porcine pancreas, and

pepsin from porcine gastric mucosa (P7012), as well as bile extract bovine (B3883), p-nitrophenyl α-D-glucopyranoside (p-NPG, N1377), acarbose (PHR1253), trifluoroacetic acid (TFA), sodium phosphate monobasic, and sodium phosphate dibasic heptahydrate were acquired from Sigma-Aldrich, Co. (St. Louis, MO, USA). Acetonitrile (ACN), hydrochloric acid 37 % (HCl), sodium hydroxide (NaOH), and formic acid (FA) were purchased from Scharlau Chemie, S.A. (Sentmenat, Barcelona, Spain). All other reagents and chemicals were of analytical grade.

### 2.2. Preparation of legumes

Soybean (*Glycine max*), chickpea (*Cicer Arietinum*), green pea (*Pisum sativum*), and navy bean (*Phaseolus vulgaris*) were purchased from an online supermarket (Lantomanage S.L., Pozuelo de Alarcon, Madrid, Spain). For each type of legume, three independent batches of 1 kg were used. Dry seeds were soaked in distilled water at a legume:water ratio of 1:5 (w/v) for 16 h. After soaking, the water was drained off, and the seeds were cooked using three different types of household cooking (conventional, pressure, and microwave cooking) using distilled water at a legume:water ratio of 1:10 (w/v). Depending on the type of household cooking and type of legume, different cooking times were selected to obtain a soft texture suitable for consumption, which was considered when the seed could be easily squeezed with the fingers (Xu & Chang, 2008). In conventional cooking, soybeans were cooked for 180 min, chickpeas for 60 min, green peas for 90 min, and navy beans for 30 min using a casserole. In pressure cooking, soybeans were cooked for 10 min, chickpeas and navy beans for 5 min, and green peas for 8 min using a pressure cooker. Lastly, in microwave cooking, soybeans were cooked for 120 min, chickpeas and navy beans for 30 min, and green peas for 50 min using a domestic microwave oven (model MI 2017, Orbegozo, Murcia, Spain). Three independent batches were cooked for each type of legume and treatment. After discarding the cooking water, the legumes were ground using a hand blender to simulate the mastication and then subjected to simulated gastrointestinal digestion.

### 2.3. *In vitro* gastrointestinal digestion (GID)

Cooked legumes were subjected to *in vitro* GID using the INFOGEST standardised protocol (Brodkorb et al., 2019; Minekus et al., 2014). Firstly, the activity of digestive enzymes was determined, and the different digestive fluids (simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF)) were prepared as reported in the protocol. The oral phase was simulated by mixing 1.5 g of mashed legume at a ratio 1:1 (w/v) with SSF containing α-amylase (1.25 μkat/mL). The mixture was agitated at 40 rpm for 2 min, at 37 °C, using an Intell-MixerTM RM-2 (ELMI Ltd., Riga, Latvia) and an incubator chamber Selecta (JP Selecta, S.A., Barcelona, Spain). After 2 min, the gastric phase was carried out by diluting the oral bolus (1:1, v/v) with SGF containing pepsin (33.3 μkat/mL) and adjusting the pH to 3 with HCl (1 mol/L). This phase was incubated for 120 min as described above. Finally, the intestinal phase was simulated by diluting the gastric chyme (1:1, v/v) with SIF containing pancreatin (based on trypsin activity to achieve 1.67 μkat/mL) and bile extract (10 mmol/L), adjusting the pH to 7 with NaOH, and incubating for 120 min as described previously. After the incubation period, the digestion was stopped by heating the samples at 100 °C for 5 min and cooling them in an ice bath. Samples without digestion (named as before digestion) were obtained by mixing legumes with distilled water (1:1, w/v), whereas blank samples were obtained by blending simulated fluids, enzymes, and bile salts (without sample). All the samples were finally centrifuged at 8000 *g*, 4 °C for 10 min, and the supernatants were stored at -20 °C for the following analysis.

Separate tubes were used for each digestion phase (gastric = oral + gastric, intestinal = oral + gastric + intestinal), and each of them was performed in duplicate. Samples were named as the type of legume used: SB (soybean), CP (chickpea), GP (green pea) and NB (navy bean); followed by a letter indicating the applied cooking method: C (conventional cooking), P (pressure cooking) and M (microwave cooking); and finally, a letter indicating the digestion phase: before digestion (b), gastric (g) and intestinal (i).

### 2.4. Measurement of α-glucosidase inhibitory activity

The α-glucosidase inhibitory activity of cooked legume samples before and after digestion was determined by following the methodology described by Mora et al. (2020) with slight modifications. For that, 50 µL of the sample, in triplicate, was mixed with 25 µL of α-glucosidase enzyme (8.34 nkat /mL in sodium phosphate buffer 0.1 mol/L, pH 6.8) onto a microplate, and incubated at 25 °C for 5 min. Then, 25 µL of p-NPG (in sodium phosphate buffer 0.1 mol/L, pH 6.8) was added, and the mixture was incubated at 37 °C for 20 min. Lastly, the absorbance was measured at 405 nm using a microplate reader CLARIOstar Plus (BMG Labtech, Ortenberg, Germany). Acarbose 5 mg/mL was used as a positive control, whereas sodium phosphate buffer (0.1 mol/L, pH 6.8) was used as a negative control. The α-glucosidase inhibitory activity was calculated as a percentage of inhibition using Equation (1), where $A_c$ is the absorbance of the negative control and $A_s$ is the absorbance of the sample.

$$\%\, inhibition = [(A_c - A_s)/A_c] \times 100 \quad (1)$$

### 2.5. Isolation of the generated peptides by reversed-phase high-performance liquid chromatography (RP-HPLC)

Cooked legume samples before and after digestion were analysed by RP-HPLC to obtain the peptide profile and to isolate the peptides depending on their polarity. For that, 100 µL of sample previously filtered was injected into an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) with a column Symmetry C18 (5 µm, 4.6 × 250 mm; Waters, Milford, MA, USA). The mobile phases used were 0.1 mL/100 mL TFA as solvent A and 0.085 mL/100 mL TFA in ACN:water (60:40, v/v) as solvent B. The gradient used for peptide elution was 100 % A for 2 min and 0–50 % B for 50 min at a flow rate of 1 mL/min. The separation was monitored using a diode array detector at 214 nm. Fractions of 2 mL were collected, freeze-dried, and re-suspended in 200 µL of sodium phosphate buffer (0.1 mol/L, pH 6.8) to assay their α-glucosidase inhibitory activity as described in section 2.4. Those

fractions showing remarkable activity were analysed by tandem mass spectrometry.

### 2.6. Peptide identification of cooked and gastrointestinal digested legumes by tandem mass spectrometry.

The identification of the peptides contained in the RP-HPLC fractions of cooked and digested legume fractions with remarkable α-glucosidase inhibitory activity was done using a nanoliquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) system composed of Ekspert nanoLC 425 (Eksigent, ABSciex, Framingham, MA, USA) and a mass spectrometer nanoESI qQTOF (6600 plus TripleTOF, AB SCIEX, Framingham, MA, USA). A total of 5 µL of sample was loaded onto a trap column (3 µm C18-CL 120 Ă, 350 µm × 0.5 mm; Eksigent, Redwood City, CA, USA) and desalted with 0.1 mL/100 mL TFA at 5 µL/min for 5 min. The peptides were then loaded onto an analytical column (3 µm C18-CL 120 Ă, 75 µm × 150 mm) equilibrated in 5 mL/100 mL ACN and 0.1 mL/100 mL FA in water. Elution was carried out at a flow rate of 0.3 µL/min with a linear gradient of 5-40 % B in A for 20 min, where solvent A was 0.1 mL/100 mL FA in water and solvent B was 0.1 mL/100 mL FA in ACN. The sample was ionised in an electrospray source of Optiflow < 1 µL Nano applying 3.0 kV to the spray emitter at 200 °C. Analysis was carried out in a data-dependent acquisition mode (DDA). Survey MS1 scans were acquired from 350 to 1400 *m/z* for 250 ms, and from 100 to 1500 *m/z* for 25 ms in 'high sensitivity' mode for MS2 experiments. The following switch criteria were used: charge: 1+ to 5+; minimum intensity; 100 counts per second (cps); fragmentation after each survey scan: up to 100 ions; dynamic exclusion: 15s.

Protein Pilot v5.0. (SCIEX Framingham, MA, USA) default parameters were used to generate the peak list directly from raw data (.wiff) files. The Paragon algorithm of ProteinPilot v5.0 was used to search the SwissProt and Uniprot Prot-Algae database with the following parameters: no enzyme specificity, iodoacetamide cys-alkylation, fabaceae taxonomy restriction, and the

search effort set to throughout. Peptides were identified with a confidence ≥ 95 %.

### 2.7. *In silico* analysis of identified peptides

BIOPEP database (https://biochemia.uwm.edu.pl/biopep/start_biopep.php) was used to check if the identified peptide sequences had previously been determined as α-glucosidase inhibitors. Subsequently, the database was used to search previously identified bioactive sequences and the frequency of the occurrence of these active domains in protein sequence (A). A value is calculated as the quotient between the number of fragments with given activity in a protein sequence and the number of protein amino acid residues (Minkiewicz et al., 2019).

Then, the potential bioactivity of peptides was predicted using the Peptide Ranker software (http://distilldeep.ucd.ie/PeptideRanker/), and a score from 0 to 1 was obtained. Higher Peptide Ranker Ratio (PRR) mean a higher probability of being bioactive. This prediction is related to the specific structural characteristics of the peptide and its amino acid sequence (Mooney et al., 2012).

The peptides showing a PRR higher than 0.5 were analysed by AllerTOP v.2.0 software (https://www.ddg-pharmfac.net/AllerTOP/) to predict the potential allergenicity of peptides according to their physicochemical properties (Dimitrov et al., 2014). ToxinPred software (http://crdd.osdd.net/raghava/toxinpred) was used to predict the physicochemical properties of the peptides such as hydrophobicity, steric hindrance, sidebulk, hydropathicity, amphipathicity, hydrophilicity and charge, and the potential toxicity which is related to the amino acid composition of peptides and their position (Gupta et al., 2013). CPPpred tool (http://distilldeep.ucd.ie/CPPpred/) was used to predict the cell-penetrating probability (CPP) of the peptides with a ratio of 0 to 1, being 1 the maximum probability to pass through cellular membranes (Holton et al., 2013).

### 2.8. Statistical analysis

Statistical analysis was performed using Statgraphics Centurion XVII software (Statgraphics Technologies, Inc., The Plains, VA, USA). One-way analysis of variance (ANOVA) with Tukey's honest significance tests were used to determine significant differences in the α-glucosidase inhibitory activity of the cooked and digested legume samples and the RP-HPLC fractions. Results were expressed as the mean of replicates ± standard deviations, and differences were considered significant at $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of thermal treatment and GID on the α-glucosidase inhibitory activity of legumes

Legumes were cooked using three different thermal treatments (conventional, pressure and microwave cooking) and later subjected to *in vitro* GID. The α-glucosidase inhibitory activity was measured before GID and after gastric and intestinal phases (Table 1). According to the results, the α-glucosidase inhibitory activity of cooked legumes before digestion and after the gastric phase was, in general, significantly low (< 20 %). In all thermal-treated SB samples (Table 1), the activity significantly increased or maintained constant after the gastric phase, reaching values around 16 % of inhibition. However, in the case of GP samples (Table 1), the inhibitory activity was maintained or declined with values between 3 and 6 %. Regarding CP and NB samples (Table 1), no inhibitory activity was detected after the gastric phase, but in the case of NB samples, the activity before digestion was significantly higher, with values up to 27 % for NBP. The observed activity before GID could be attributed to the phenolic compounds found in legumes that were resistant to the thermal treatments (Ganesan & Xu, 2017), as bioactive peptides are usually small in size, encrypted within the protein sequence, and require a hydrolysis process to be released (Acquah et al., 2022). The lack of significant α-glucosidase inhibitory activity after the

gastric phase may be because the large-sized peptides generated by gastric pepsin from legume proteins could not inhibit the α-glucosidase enzyme.

**Table 1.** α-Glucosidase inhibitory activity (%) of legumes during simulated gastrointestinal digestion, previously subjected to different types of household cooking.

| Legume | Digestion phase | Types of household cooking | | |
|---|---|---|---|---|
| | | **Conventional cooking** | **Pressure cooking** | **Microwave cooking** |
| Soybean | Before | $12.22 \pm 2.13^{Bab}$ | $4.77 \pm 2.53^{Cb}$ | $22.89 \pm 3.65^{Ba}$ |
| | Gastric | $17.40 \pm 0.30^{Ba}$ | $16.61 \pm 1.01^{Bab}$ | $15.51 \pm 0.62^{Bb}$ |
| | Intestinal | $52.05 \pm 2.87^{Aab}$ | $56.56 \pm 2.83^{Aa}$ | $47.89 \pm 3.87^{Ab}$ |
| Chickpea | Before | — | $10.09 \pm 0^{B}$ | — |
| | Gastric | — | — | — |
| | Intestinal | $27.28 \pm 0.21^{b}$ | $45.02 \pm 1.96^{Aa}$ | $27.69 \pm 4.76^{b}$ |
| Green pea | Before | $10.25 \pm 2.04^{Ba}$ | $6.78 \pm 2.43^{Ba}$ | — |
| | Gastric | $3.51 \pm 0.83^{Ca}$ | $6.12 \pm 0.31^{Ba}$ | $2.92 \pm 1.86^{Ba}$ |
| | Intestinal | $22.28 \pm 0.52^{Ab}$ | $26.28 \pm 0.55^{Aa}$ | $23.57 \pm 0.59^{Ab}$ |
| Navy bean | Before | $13.79 \pm 0.2^{Bb}$ | $27.33 \pm 2.63^{Ba}$ | $9.67 \pm 1.98^{Bb}$ |
| | Gastric | — | — | — |
| | Intestinal | $42.17 \pm 0.81^{Ab}$ | $41.36 \pm 0.2^{Aa}$ | $35.87 \pm 2.8^{Ab}$ |

Capital letter indicates significant differences between digestion phases (before, gastric, and intestinal) within the same treatment ($p < 0.05$), whereas lowercase letter indicates significant differences between treated samples (conventional cooking, pressure cooking, and microwave cooking) within the same phase ($p < 0.05$).

The α-glucosidase inhibitory activity of the four legumes increased after the intestinal phase, being SB the legume showing the highest values (between 48 and 57 %) (Table 1). That increase could be related to the generation of peptides due to the proteolytic action of intestinal enzymes. The results obtained by Wang et al. (2019) showed a similar pattern for fermented and gastrointestinal digested soybean protein hydrolysates. Nevertheless, these authors observed the increase of α-glucosidase inhibitory activity during GID already after the gastric phase, probably because their study was based on a hydrolysate. González-Montoya et al. (2018) and Mojica et al. (2017) also achieved that protein isolates from soybean and common beans, respectively, exhibited α-glucosidase inhibitory activity after GID. Similar results were also obtained on hydrolysates from isolated proteins using different

commercial enzymes (Awosika & Aluko, 2019; Castañeda-Pérez et al., 2021; Jiang et al., 2018; Mojica & De Mejía, 2016; Ohara et al., 2021; Oseguera-Toledo et al., 2015; Rivero-Pino et al., 2021).

The thermal treatment used to cook the legumes had a limited impact on the α-glucosidase inhibitory activity of cooked and gastrointestinal digested samples. SBP presented the highest value (57 %), although non-significant differences were observed with SBC (Table 1). In the case of CP and GP (Table 1), pressure-cooked samples had the highest inhibitory activity, 45 % and 26 %, respectively. In NB samples (Table 1), the highest inhibitory activity was 42 % for NBC, but without significant differences with respect to NBP. Thus, the α-glucosidase inhibitory activity in the four legumes was significantly higher in those samples treated with pressure cooking than microwave, whereas it was also significantly higher than conventionally cooked samples in the case of CP and GP. The slight differences observed among the cooked samples may be attributed to distinct effects on proteins and/or food matrix structure of the different thermal treatments. The application of pressure during cooking would lead to the loss and denaturation of protein structure, facilitating the access of proteolytic enzymes to the protein and improving its digestibility (Gu et al., 2023). On the other hand, microwave cooking generates instantaneous heat in the food because of molecular motion, with changes in molecular structure but showing lower protein digestibility than ordinary processes such as conventional or pressure cooking (Habiba, 2002). So, the effect of cooking on food structure would influence the hydrolysis of proteins during GID and thereby the generation of peptides, including bioactives such as antioxidant peptides (Gallego et al., 2020). Few studies have reported α-glucosidase inhibition activity in legumes prepared using different cooking procedures and during GID. Ercan & El (2016) studied the inhibitory effect on α-glucosidase of pressure-cooked chickpeas before and after GID. These authors obtained an 18.1 % percentage inhibition that decreased to 10.9 % after GID at a concentration of 1 mg/mL, attributing this reduction in activity to the impact of digestive conditions such as temperature and pH changes. Likewise, Mojica et al. (2015) studied the inhibition

capacity of α-glucosidase in different raw and cooked gastrointestinal digested beans. The results of that study showed non-statistically significant differences between most raw and cooked samples, with inhibition percentages ranging from 40 to 70 %.

### 3.2. Fractionation of cooked and digested legume samples by RP-HPLC

Cooked legumes before and after GID were analysed by RP-HPLC to obtain their peptide profile. The obtained chromatograms are shown in Figures S1, S2, S3 and S4 for SB, CP, GP, and NB samples, respectively. As can be observed, the peptide profile was very similar between the different legumes and thermal treatments. Absorbance values increased as digestion progressed; that is, the absorbance after the intestinal phase was higher than the absorbance before digestion and after the gastric phase. Before digestion, the absorbance of all cooked legume samples was below 200 mAU, except for small peaks between minutes 35 and 45 in all the different thermal-treated CP samples and in the conventional and pressure-cooked GP samples. After the gastric phase, the absorbance was also low, although it increased in the range between 15 and 35 minutes in the SB and CP samples, suggesting that peptides after the gastric phase were mainly hydrophobic, as they elute at high ACN concentrations. After the intestinal phase, the absorbance of all cooked legume samples increased, and six defined peaks were observed (Figure S5). The first two peaks around minute 3, the third peak around minute 4, the fourth peak between minutes 8 and 10, the fifth peak between minutes 14 and 15, and the sixth peak between minutes 22 and 23. So, an increase in polar peptides was observed after the intestinal phase in all samples.

Thermal treatments appeared to have minimal impact on the peptide profile of the different legume samples since small changes could be seen in the chromatograms. However, Figure S5 shows that microwave cooking generated a higher amount of peptides in SB but a lower amount in GP, as evidenced by changes in the intensity of the peaks compared to the other two thermal treatments.

Legume samples after GID were selected for fraction separation by RP-HPLC, and the α-glucosidase inhibitory activity of each fraction was measured. Figures 1, 2, 3 and 4 show the chromatograms and the percentage of α-glucosidase inhibition of the fractions. The results showed that fraction 2 from all thermally treated and digested legumes exhibited significant α-glucosidase inhibitory activity with values between 40 % and 62 % inhibition, which was higher than the α-glucosidase inhibitory activity of the whole sample in all legumes except for SB. This fraction, which corresponds to the first two peaks eluted during minutes 3 and 4 (Figure S5), would contain mainly hydrophilic peptides. Although there were other noticeable peaks in the chromatograms, all other fractions had an activity below 20 % inhibition. The biological activity of peptides depends on primary structure, native protein composition, and sequence (Elam et al., 2021) rather than the amount in the sample. Similar results were obtained by Jiang et al. (2018), which purified the soybean protein hydrolysate using multiple techniques, showing that the hydrophilic peptide fraction from RP-HPLC had the highest α-glucosidase inhibitory activity. However, Ibrahim et al. (2018) and Mu et al. (2024) evaluated the correlation between the hydrophilicity/hydrophobicity of several peptides and their α-glucosidase inhibitory capacity, observing a non-significant relationship between both aspects

Focusing on fraction 2 of cooked and gastrointestinal digested samples, which exhibited the highest α-glucosidase inhibitory activity, Figure 5 illustrates the comparison of results among the different legumes and thermal treatments. The inhibitory activity pattern between the thermal treatments of fraction 2 within the same legume was the same as the pattern of the whole sample (Table 1), except for GP. The conventional-cooked samples had the highest activity for SB, GP, and NB, although there were no significant differences in SB between the three treatments, nor between GPC and GPP. Lastly, in CP, the pressure-cooked sample had the highest activity among the three treatments, with no significant differences with the other ones

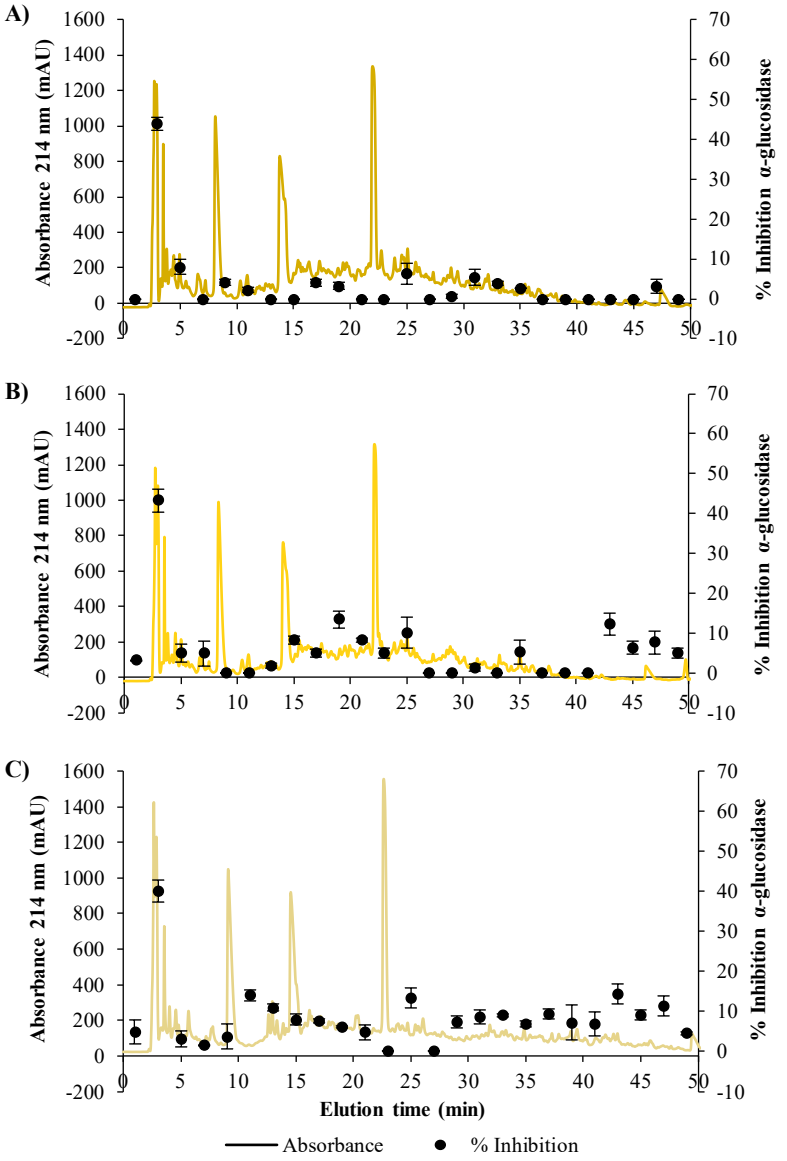


**Figure 1.** RP-HPLC profile and α-glucosidase inhibitory activity of intestinal digested soybeans with conventional (A), pressure (B) and microwave (C) thermal treatment.

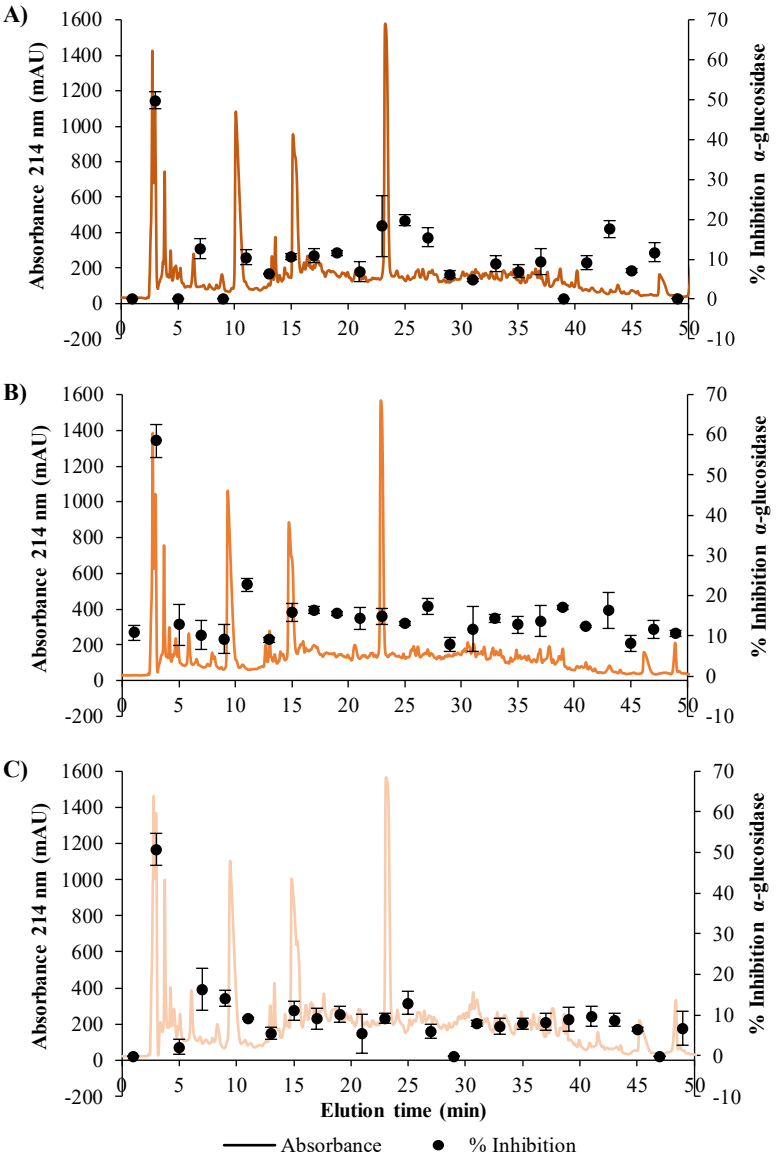


**Figure 2.** RP-HPLC profile and α-glucosidase inhibitory activity of intestinal digested chickpeas with conventional (A), pressure (B) and microwave (C) thermal treatment.

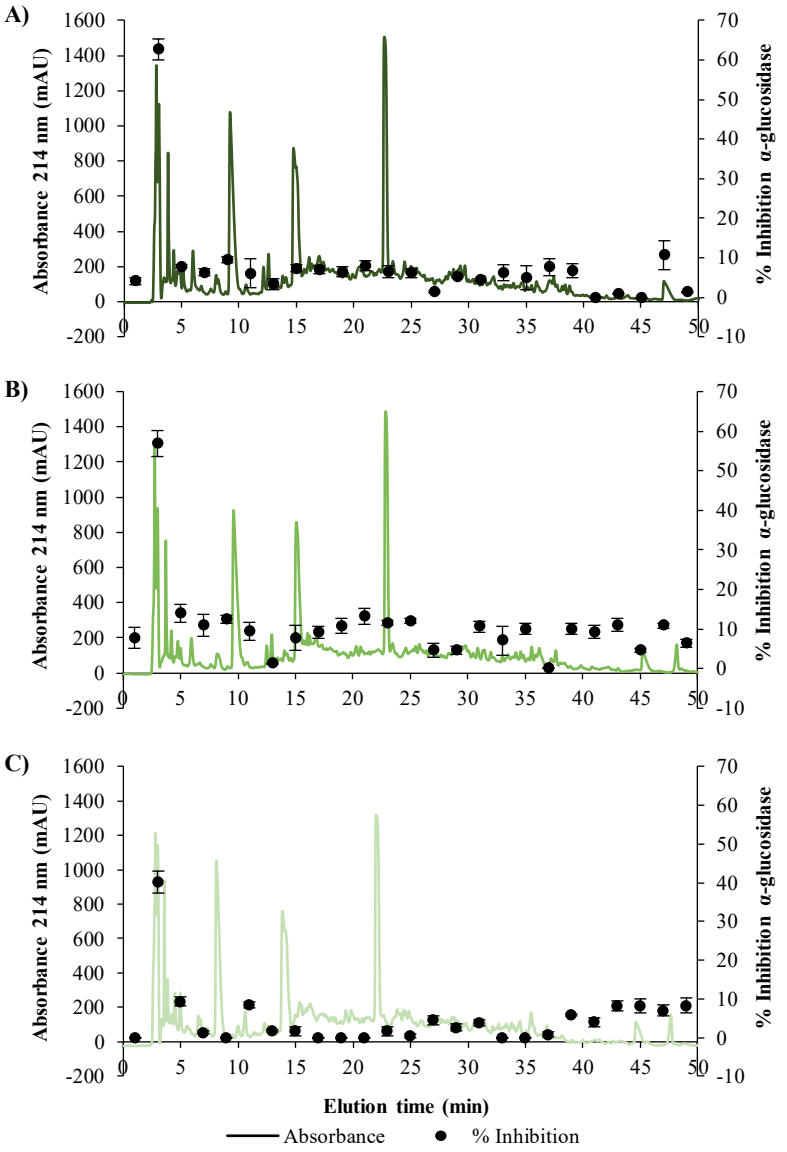


**Figure 3.** RP-HPLC profile and α-glucosidase inhibitory activity of intestinal digested green peas with conventional (A), pressure (B) and microwave (C) thermal treatment.

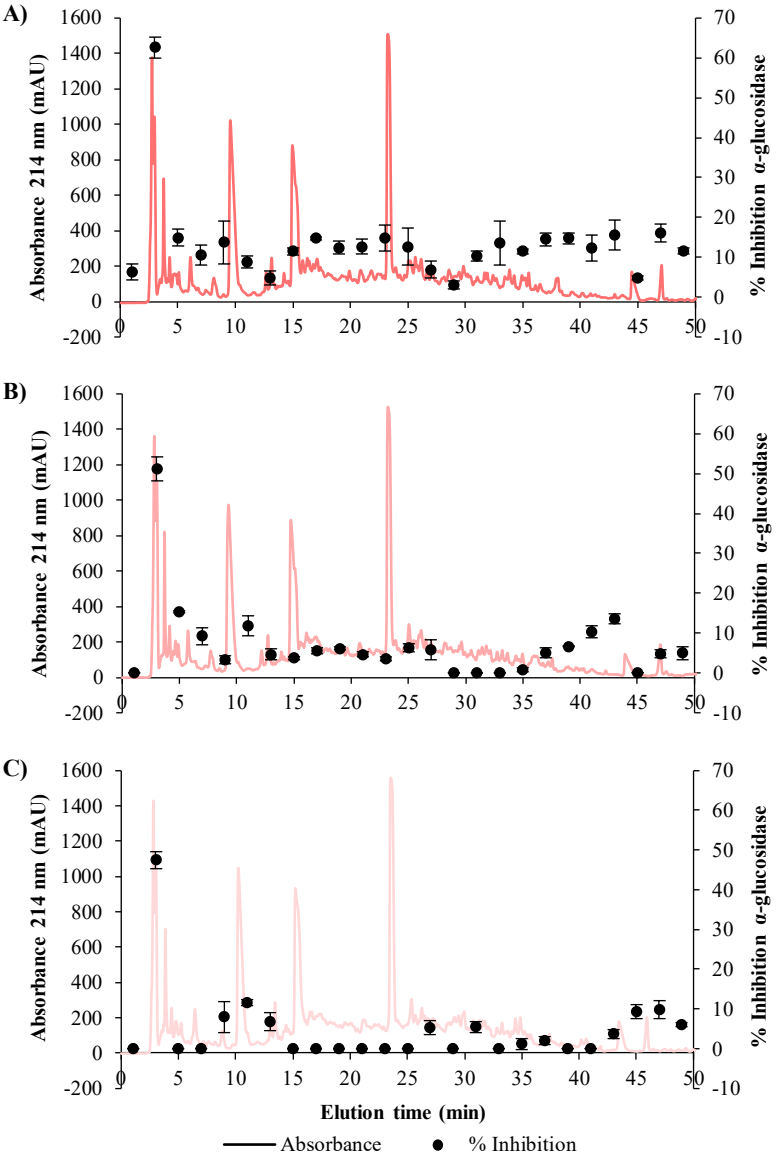


**Figure 4.** RP-HPLC profile and α-glucosidase inhibitory activity of intestinal digested navy beans with conventional (A), pressure (B) and microwave (C) thermal treatment.

By comparing within the same treatment, in conventional cooking, GP and NB showed the highest inhibitory activity (62 %), followed by CP (50 %) and SB (45 %). In pressure cooking, CP showed the most increased inhibitory activity (58 %) with no significant differences with GP (57 %) and NB (51 %). Finally, in microwave cooking, CP showed the highest inhibitory activity (50 %) with no significant differences with NB (47 %). As mentioned above, the small differences observed between the samples could be attributed to the protein structure changes resulting from heat treatments, which may impact on the degree of hydrolysis and, in turn, the generation of bioactive peptides (Gallego et al., 2020). Because conventional-cooked samples showed the highest inhibitory activity in three of the four legumes, these samples were subjected to MS/MS analysis to identify potential peptide sequences responsible for the biological activity.

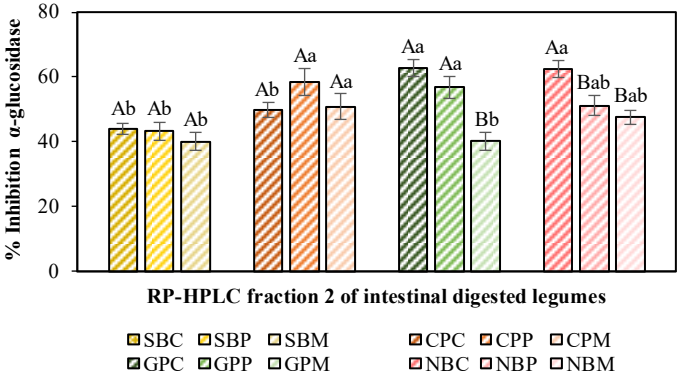


**Figure 5.** α-Glucosidase inhibitory activity of RP-HPLC fraction 2 of intestinal digested legumes (SBC, conventional cooked soybean; SBP, pressure cooked soybean; SBM, microwave cooked soybean; CPC, conventional cooked chickpea; CPP, pressure cooked chickpea; CPM, microwave cooked chickpea; GPC, conventional cooked green pea; GPP, pressure cooked green pea; GPM, microwave cooked green pea; NBC, conventional cooked navy bean; NBP, pressure cooked navy bean; NBM, microwave cooked navy bean).

Capital letter indicates significant differences between thermal treatments (conventional cooking, pressure cooking, and microwave) within the same legume ($p < 0.05$), whereas lowercase letter indicates significant differences between legumes (soybean, chickpea, green pea, and navy bean) within the same thermal treatment ($p < 0.05$).

### 3.3. Peptide identification by LC-MS/MS and *in silico* analysis

The peptides from RP-HPLC fraction 2 of intestinal digested conventional-cooked legumes (SBCi, CPCi, GPCi and NBCi) were identified using LC-MS/MS. The complete list of the peptide sequences, along with their protein accession number, observed and calculated molecular masses, and mass/charge (*m/z*), can be found in Table S1. To summarise, a total of 48 peptides were identified, including 25 in SBCi, 6 in CPCi, 10 in GPCi, and 7 in NBCi. These peptides had molecular masses between 525 and 3080 Da and ranged in length from 7 to 22 amino acids, although there does not appear to be a direct correlation between chain length and α-glucosidase inhibitory activity (Ibrahim et al., 2018; Mu et al., 2024).

The identified peptides were analysed *in silico* using different tools to predict their bioactivity, allergenicity, toxicity, physicochemical properties, and cell-penetrating probability. PeptideRanker software was used to evaluate the potential bioactivity of the identified peptides after confirming in BIOPEP that none of them had been previously reported as bioactive. Table 2 lists 13 out of a total of 48 peptides that had a PRR higher than 0.5, which were analysed with the rest of the databases. These 13 peptides had characteristics that have been reported to provide α-glucosidase inhibitory activity. For instance, the presence of S or K residues at the N-terminal end of the sequence (Di Stefano et al., 2018; Ibrahim et al., 2018) was observed in some of the identified peptides such as SQFPGPIIKVHKG, KLSMLFGGVL, and KILGICF. Other peptides featured a P or I at the 1-4 positions on the N-terminal (Mu et al., 2024), like SQFPGPIIKVHKG, FPLMHYDLLEQCLPL, QPVYALSIGGMI, PRMYPVN, CNIHGLC, KILGICF, GLIDMYSKC, PR ASGFIDVADGCCC, and LPKAAIGFIT. Some peptides had also a P closer to the C-terminal (Di Stefano et al., 2018; Ibrahim et al., 2018), such as QACLLPT, FPLMHYDLLEQCLPL, HACAGGMVQMPA, GSVGFMDK-VNSALAMCLIAGPR and PRMYPVN. Furthermore, among these listed peptides, two contained either A or R at the C-terminal, while all of them and GLIDMYSKC had P, A or S at the positions 2-4 on the C-terminal, which

seems to enhance their ability to inhibit α-glucosidase (Di Stefano et al., 2018; Ibrahim et al., 2018; Mu et al., 2024).

Regarding toxicity and allergenicity (Table 2), AllerTop and ToxinPred tools indicated that 8 of these 13 peptides would be neither allergen nor toxic. In terms of physicochemical properties, 11 out of the 13 peptides predicted as bioactive had a net charge between 0 and 1, which has been linked to strong α-glucosidase inhibitory activity of peptides (Mu et al., 2024). Moreover, it has been suggested that the ability of the peptide to exert its activity can be enhanced by low steric hindrance values and high amphipathicity since it would stabilise the enzyme conformation (Mora et al., 2020). The peptide SQFPGPIIKVHKG showed the highest value of amphipathicity (0.77) whereas the steric hindrance of all the peptides ranged between 0.54 and 0.66 (Table 2). The cell penetrating probability (CPP) of the peptides was also determined (Table 2), showing values that ranged between 0.07 and 0.37. These results indicate that the peptides would have a low probability to pass through cellular membranes. It is worth noting that although peptides with α-glucosidase inhibitory activity do not need to cross the intestinal barrier to exert their function, most of them have bioactive sequences with multiple functions that do require it.

**Table 2.** *In silico* prediction of the allergenicity, toxicity and physicochemical properties of the identified peptides with Peptide Ranker Ratio (PRR) > 0.5.

| Legume sample[a] | Peptide sequence | PRR | CPP[b] | Allergenicity prediction | Toxicity prediction | Hydrophobicity | Steric hindrance | Sidebulk | Hydropathicity | Amphipathicity | Hydrophilicity | Charge |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SBCi | QACLLPT | 0.64 | 0.37 | Non-allergen | Non-Toxic | 0.06 | 0.54 | 0.54 | 0.87 | 0.18 | -0.76 | 0 |
| | SQFPGPIIKVHKG | 0.63 | 0.09 | Non-allergen | Non-Toxic | -0.06 | 0.57 | 0.57 | -0.25 | 0.77 | -0.12 | 2.5 |
| | FPLMHYDLLEQCLPL | 0.62 | 0.13 | Allergen | Non-Toxic | 0.07 | 0.55 | 0.55 | 0.53 | 0.26 | -0.69 | -1.5 |
| | HACAGGMVQMPA | 0.51 | 0.15 | Non-allergen | Non-Toxic | 0.08 | 0.57 | 0.57 | 0.57 | 0.23 | -0.57 | 0.5 |
| CPCi | GSVGFMDKVNSALAMCLIAGPR | 0.83 | 0.17 | Allergen | Non-Toxic | 0.02 | 0.63 | 0.63 | 0.69 | 0.28 | -0.28 | 1 |
| | QPVYALSIGGMI | 0.71 | 0.08 | Non-allergen | Non-Toxic | 0.2 | 0.63 | 0.63 | 1.06 | 0.1 | -0.88 | 0 |
| | PRMYPVN | 0.58 | 0.20 | Non-allergen | Non-Toxic | -0.25 | 0.62 | 0.62 | -0.91 | 0.35 | -0.27 | 1 |
| GPCi | CNIHGLC | 0.77 | 0.07 | Allergen | Non-Toxic | 0.07 | 0.56 | 0.56 | 0.89 | 0.21 | -0.84 | 0.5 |
| | KILGICF | 0.77 | 0.09 | Non-allergen | Non-Toxic | 0.24 | 0.66 | 0.66 | 1.97 | 0.52 | -0.84 | 1 |
| | GLIDMYSKC | 0.56 | 0.07 | Allergen | Non-Toxic | -0.04 | 0.66 | 0.66 | 0.31 | 0.41 | -0.21 | 0 |
| NBCi | PRASGFIDVADGCCC | 0.81 | 0.08 | Allergen | Toxic | -0.05 | 0.63 | 0.63 | 0.53 | 0.16 | -0.03 | -1 |
| | KLSMLFGGVL | 0.70 | 0.23 | Non-allergen | Non-Toxic | 0.2 | 0.63 | 0.63 | 1.48 | 0.37 | -0.74 | 1 |
| | LPKAAIGFIT | 0.57 | 0.13 | Non-allergen | Non-Toxic | 0.19 | 0.59 | 0.59 | 1.26 | 0.37 | -0.63 | 1 |

[a] Legume samples are: SBCi, intestinal digested conventional cooked soybean; CPCi, intestinal digested conventional cooked chickpea; GPCi, intestinal digested conventional cooked green pea; NBCi, intestinal digested conventional cooked navy bean.

[b] CPP: cell-penetrating probability of peptides.

In addition, the peptides with a PRR > 0.5 were analysed using the BIOPEP database to identify any previously reported active domains. Table 3 displays the bioactive sequences contained in the identified peptides as well as the biological activities and frequency of occurrence in protein sequence (A) of the active domains. Results showed that the identified peptides contained several dipeptides reported as bioactives encrypted in their amino acid sequences. Based on the number of bioactive sequences and the A value, ACE-I inhibitor and DPP-IV inhibitor were the main biological activities predicted for these sequences. DPP-IV inhibitory activity is related to antidiabetic activity since these inhibitors prevent the fast breakdown of the GLP-1 hormone, which elevates the level of active GLP-1 after a meal. This leads to a decrease in glucagon production in the liver and an increase in insulin levels by stimulating the beta cells of the pancreas (Patil et al., 2020). Furthermore, the sequence YP encrypted in the peptide PRMYPVN of CPCi and the sequence AD in the peptide PRASGFIDVADGCCC of NBCi were reported as α-glucosidase inhibitors according to the BIOPEP database.

**Table 3.** Potential biological activity according to BIOPEP database of the identified peptides with Peptide Ranker Ratio >0.5.

| Legume sample[a] | Peptide sequence | Bioactive sequence | Biological activity[b] | A[c] |
|---|---|---|---|---|
| SBCi | QACLLPT | LLP, PT, LP | ACE | 0.4286 |
| | | LL | STI | 0.1429 |
| | | LP, LL, PT, QA | DPP-IV | 0.5714 |
| | SQFPGPIIKVH-KG | PGP, PG, GP | AM | 0.2308 |
| | | FP, GP, KG, PG, HK | ACE | 0.3846 |
| | | GP, PGP, PG | AT | 0.2308 |
| | | II | STI | 0.0769 |
| | | GP, PG, PGP | RE | 0.2308 |
| | | PGP | INH | 0.0769 |
| | | PGP | CHE | 0.0769 |
| | | GP, FP, II, KG, KV, PG, PI, QF, VH | DPP-IV | 0.6923 |
| | | HK | DPP-III | 0.0769 |
| | | QF | REN | 0.0769 |
| | FPLMHYDLLE-QCLPL | HY, FP, PL, LPL, LP, DL | ACE | 0.4667 |
| | | LL | STI | 0.0667 |
| | | MH | NE | 0.0667 |
| | | QCL, EQC, LPL | AO | 0.2000 |
| | | HY | AI | 0.0667 |
| | | LP, LL, FP, LPL, PL, HY, LM, MH, YD | DPP-IV | 0.6667 |
| | | LPL | REN | 0.0667 |
| | HACAGGMVQ-MPA | GM, AG, GG | ACE | 0.25 |
| | | ACA | AO | 0.0833 |
| | | MP, PA, HA, AG, GG, MV, VQ | DPP-IV | 0.5833 |
| CPCi | GSVGFMDKV-NSALAMCLI-AGPR | GP | AM | 0.0455 |
| | | PR, GP, IA, LA, GF, VG, AG, GS, AGP | ACE | 0.4091 |
| | | GPR, GP | AT | 0.0909 |
| | | LI | STI | 0.0455 |
| | | GP | RE | 0.0455 |
| | | SALAM | AO | 0.0455 |
| | | LA | APR | 0.0455 |
| | | GP, LA, IA, AL, AG, GF, KV, LI, SV, VG, VN | DPP-IV | 0.5 |
| | | GF, PR, LA FM | DPP-III | 0.1818 |
| | QPVYALSIGG-MI | VY, YA, IG, GM, GG, QP | ACE | 0.5 |
| | | VY | AO | 0.0833 |
| | | QP, AL, GG, MI, PV, SI, VY, YA | DPP-IV | 0.6667 |

**Table 3.** Cont.

| | | | | |
|---|---|---|---|---|
| CPCi | QPVYALSIGG-MI | VY | DPP-III | 0.0833 |
| | | YA | REN | 0.0833 |
| | PRMYPVN | MY, PR, YP | ACE | 0.4286 |
| | | MY | AO | 0.1429 |
| | | YP, MY, PV, RM, VN | DPP-IV | 0.7143 |
| | | YP | GLUI | 0.1429 |
| | | PR | DPP-III | 0.1429 |
| GPCi | CNIHGLC | GL, HG | ACE | 0.2857 |
| | | GL, IH | DPP-IV | 0.2857 |
| | | IH | DPP-III | 0.1429 |
| | KILGICF | GI, LG, CF, LGI, IL | ACE | 0.7143 |
| | | IL | STI | 0.1429 |
| | | GI, IL, KI | DPP-IV | 0.4286 |
| | GLIDMYSKC | MY, GL, DM | ACE | 0.3333 |
| | | LI | STI | 0.1111 |
| | | MY | AO | 0.1111 |
| | | GL, LI, MY, SK, YS | DPP-IV | 0.5556 |
| NBCi | PRASGFIDVA-DGCCC | PR, RA, GF, SG, DG | ACE | 0.3333 |
| | | RA | APR | 0.0667 |
| | | VA, RA, AD, AS, GF | DPP-IV | 0.3333 |
| | | AD | GLUI | 0.0667 |
| | | GF, PR | DPP-III | 0.1333 |
| | KLSMLFGGVL | LF, FG, GV, GG, KL, FGG | ACE | 0.6 |
| | | VL | STI | 0.1 |
| | | GG, GV, ML, VL | DPP-IV | 0.4 |
| | | GGV | HMGI | 0.1 |
| | | SM | DPP-III | 0.1 |
| | LPKAAIGFIT | AA, GF, IG, AI, KA, LP | ACE | 0.6 |
| | | AA | HYP | 0.1 |
| | | KA, LP, AA, GF, PK | DPP-IV | 0.5 |
| | | GF, KA | DPP-III | 0.2 |

[a] Legume samples are: SBCi, intestinal digested conventional cooked soybean; CPCi, intestinal digested conventional cooked chickpea; GPCi, intestinal digested conventional cooked green pea; NBCi, intestinal digested conventional cooked navy bean.
[b] Biological activities are: ACE, ACE inhibitor; AI, anti-inflammatory; AM, antiamnestic; AO: antioxidative; APR, activating ubiquitin-mediated proteolysis; AT, antithrombotic; CHE, chemotactic; DPP-III, dipeptidyl peptidase III inhibitor; DPP-IV, dipeptidyl peptidase IV inhibitor; GLUI, alpha-glucosidase inhibitor; HMGI, HMG-CoA reductase inhibitor; HYP, hypotensive; INH, inhibitor; NE: neuropeptide; RE: regulating; REN, renin inhibitor; STI, stimulating.
[c] A: frequency of the occurrence of the active domain in protein sequence.

## 4. CONCLUSIONS

This study proved that conventional-cooked, pressure-cooked and microwaved-cooked samples of SB, CP, GP, and NB displayed α-glucosidase inhibitory activity after GID, with values up to 60 % inhibition. In all gastrointestinal digested legumes, the activity was concentrated in a polar fraction from RP-HPLC and, in general, the fractionation of the samples increased the inhibitory activity with values ranging from 40 % to 62 %. The type of thermal treatment did not greatly influence the α-glucosidase inhibitory activity, although the highest activity was observed in conventional-cooked SB, GP, and NB, and in pressure-cooked CP. A total of 48 peptides were identified by LC-MS/MS in the conventional-cooked and digested legume samples, 13 of which were found to be potentially bioactive using *in silico* tools. The present study proved that the α-glucosidase inhibitory activity of legumes increased after the GID, which would suggest their potential antidiabetic activity. However, it would be necessary to synthesise the main peptide sequences to confirm their *in vitro* α-glucosidase inhibitory activity as well as to validate the antidiabetic properties of these peptides through *in vivo* assays to ensure their effectiveness and safety.

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**Author contribution**

Milagros Arnal: Conceptualization, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. Marta Gallego: Conceptualization, Supervision, Writing – Review & Editing. Pau Talens: Conceptualization, Supervision, Writing – Review & Editing, Project

administration, Funding acquisition. Leticia Mora: Conceptualization, Formal analysis, Investigation, Supervision, Writing – Review & Editing, Project administration, Funding acquisition.

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## Supplementary data

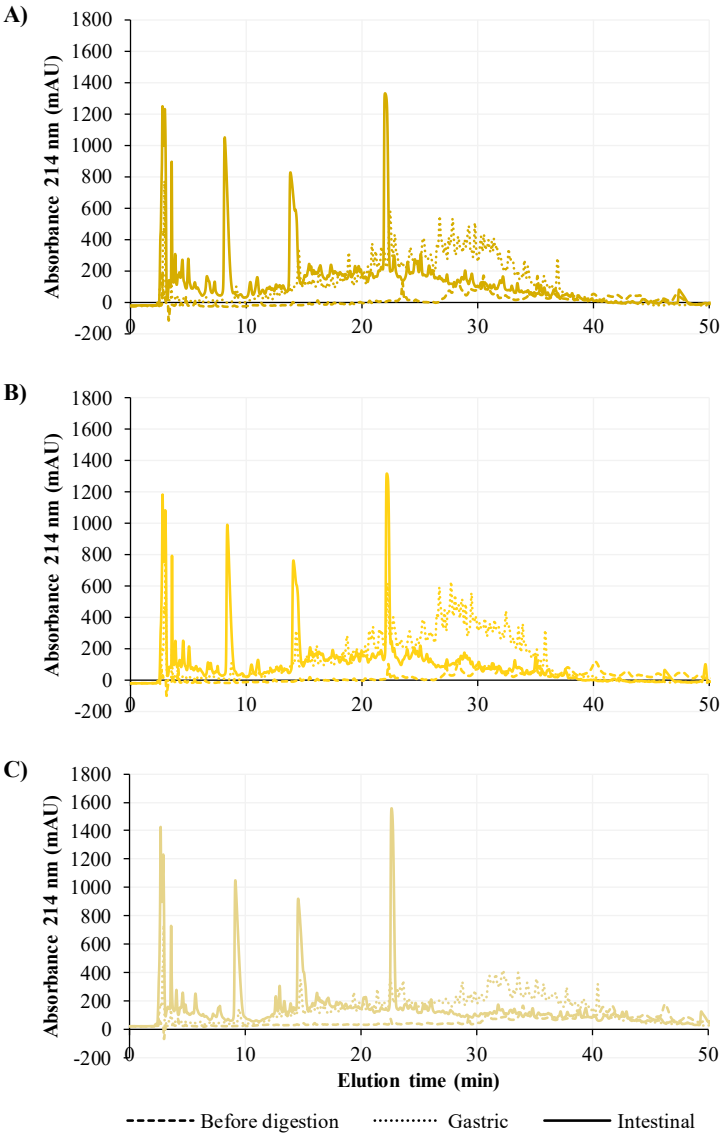


**Figure S1.** RP-HPLC profile of conventional (A), pressure (B) and microwaved (C) cooked soybeans at different phases of gastrointestinal digestion.

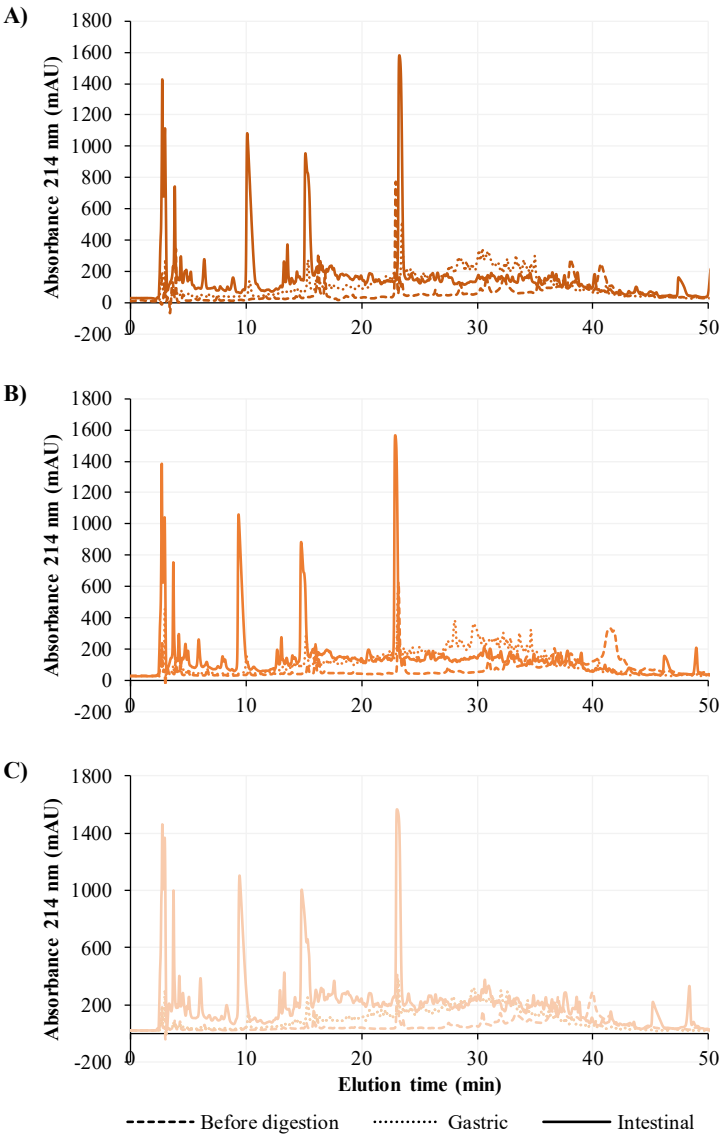


**Figure S2.** RP-HPLC profile of conventional (A), pressure (B) and microwaved (C) cooked chickpeas at different phases of gastrointestinal digestion.

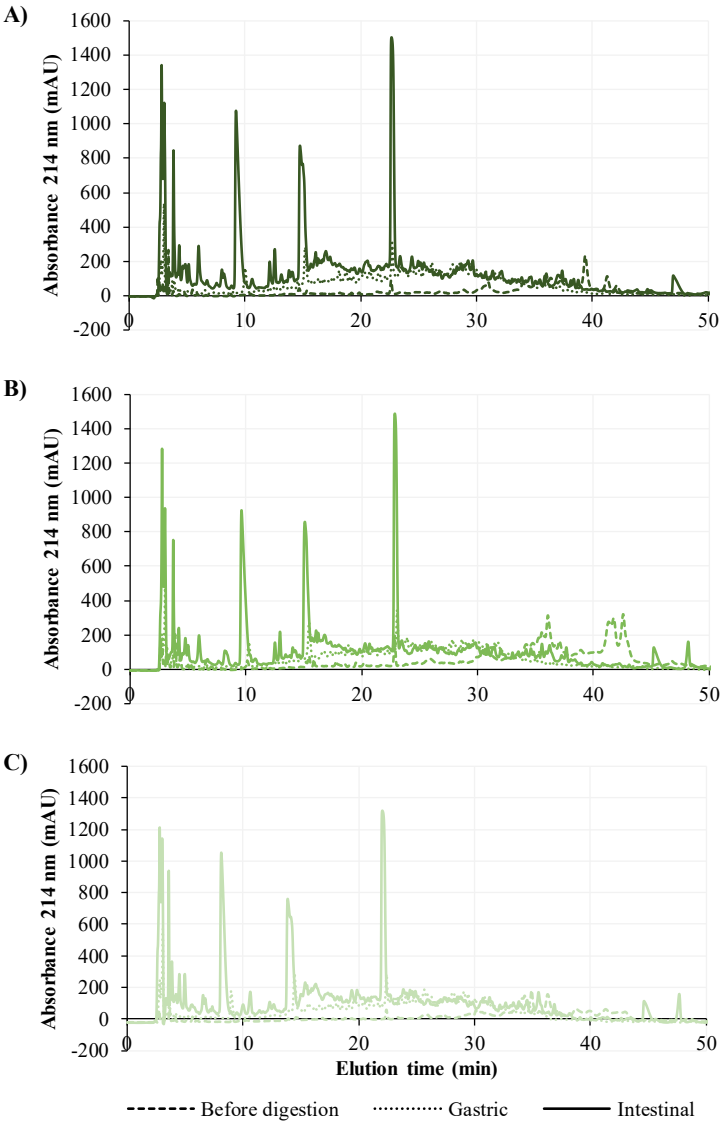


**Figure S3.** RP-HPLC profile of conventional (A), pressure (B) and microwaved (C) cooked green peas at different phases of gastrointestinal digestion.

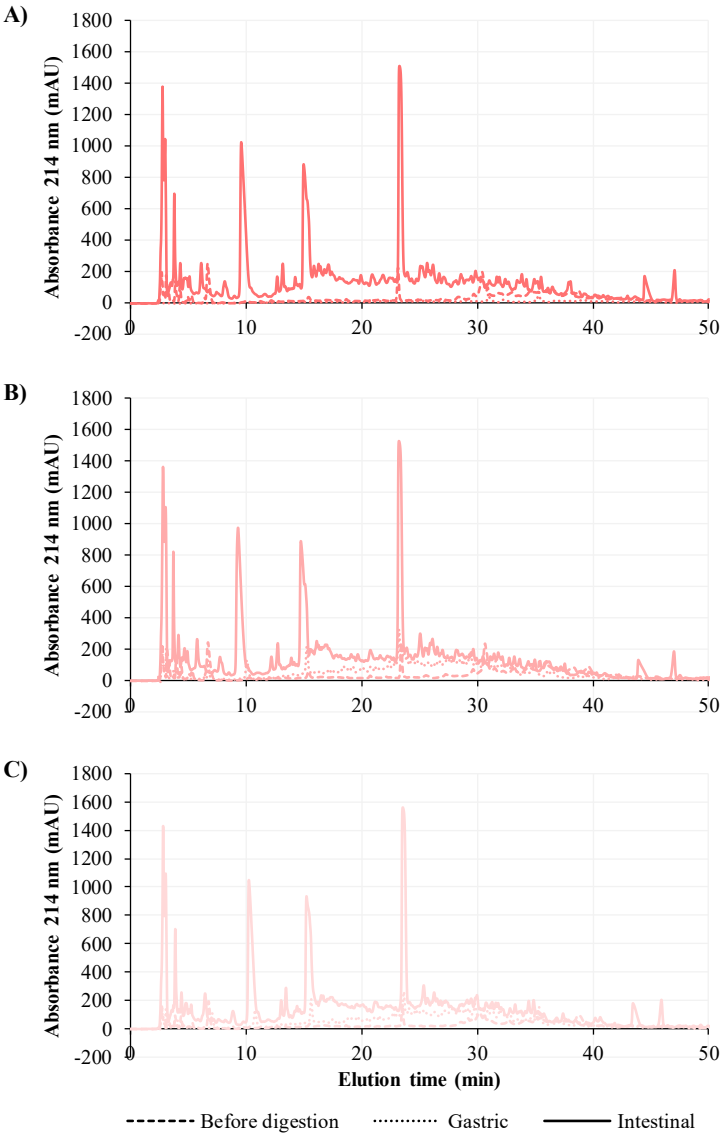


**Figure S4.** RP-HPLC profile of conventional (A), pressure (B) and microwaved (C) cooked navy beans at different phases of gastrointestinal digestion.

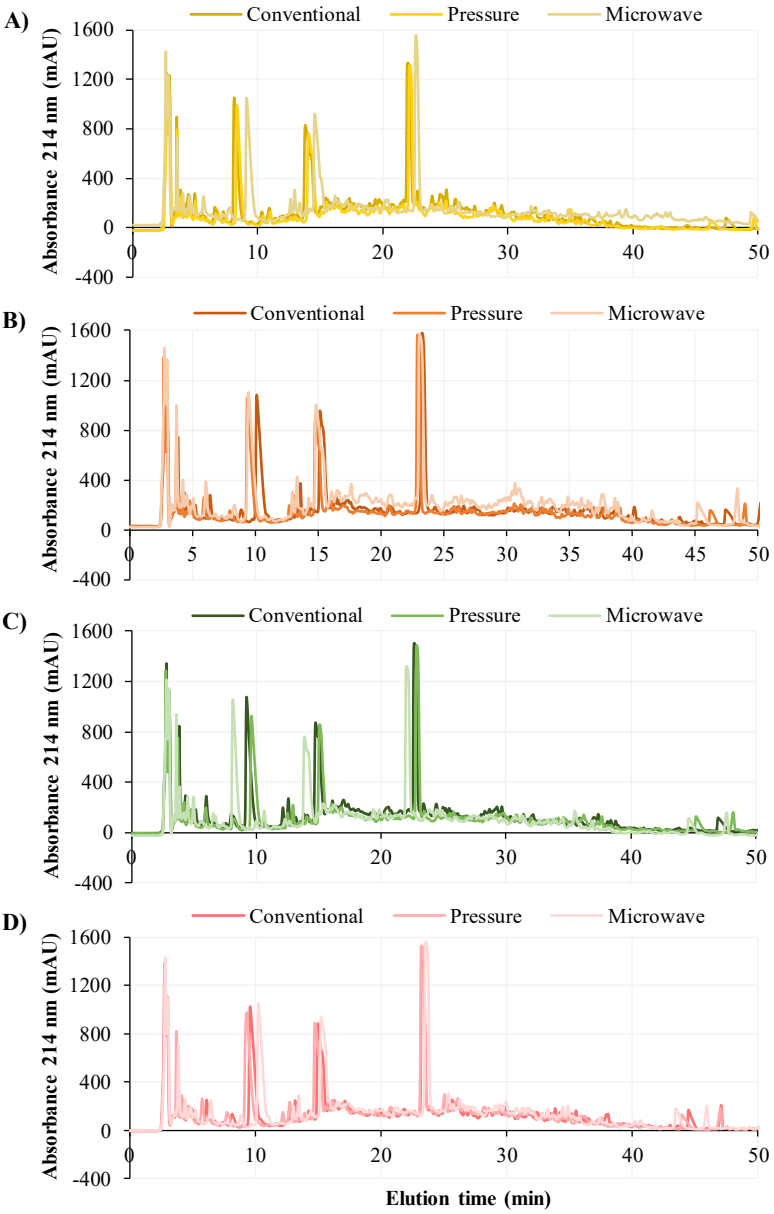


**Figure S5**. RP-HPLC profile of intestinal digested legumes from soybean (A), chickpea (B), green pea (C) and navy bean (D) with different thermal treatments.

**Table S1.** Peptide sequences identified by MS/MS in RP-HPLC fraction 2 of gastro-intestinal digested conventional cooked legumes.

| Legume sample[a] | Peptide Sequence | Protein accession number[b] | Observed MW (Da)[c] | Observed *m/z* | Calculated MW (Da)[d] |
|---|---|---|---|---|---|
| SBCi | QACLLPT | ANM15_ARATH | 744.40 | 373.20 | 744.38 |
| | FPLMHYDLLEQCLPL | BCAL2_ARATH | 1878.87 | 627.30 | 1878.89 |
| | HACAGGMVQMPA | ARFV_ORYSJ | 1187.50 | 396.84 | 1187.49 |
| | ETAAVMQEFT | PSA2_ORYSI | 1141.54 | 571.78 | 1141.50 |
| | MDDDDDMAEMYLTEKK | MRS25_ARATH | 2028.61 | 677.21 | 2028.75 |
| | PPAGAEEE | CFA46_CHLRE | 798.29 | 400.15 | 798.34 |
| | GRVAIRQ | CIPKL_ORYSJ | 798.42 | 400.22 | 798.48 |
| | SMVLSPKRHKHC | FB55_ARATH | 1437.87 | 480.30 | 1437.73 |
| | MVEEADR | CML20_ARATH | 864.42 | 289.15 | 864.36 |
| | SQFPGPIIKVHKG | LAC15_ARATH | 1422.91 | 475.31 | 1422.80 |
| | VPATECN | Y1054_ORYSJ | 732.30 | 367.16 | 732.31 |
| | RGFGFVTMSTAA | CP29A_ARATH | 1275.66 | 426.23 | 1275.59 |
| | HGGGAGA | NDRP1_ARATH | 525.21 | 263.61 | 525.23 |
| | PKSHGQTQS | EIN4_ARATH | 968.53 | 485.27 | 968.47 |
| | GLPRTPE | CDP1_ARATH | 768.44 | 385.23 | 768.41 |
| | HLGKGHVVLGVPSSIHPVI-TDGVKP | SPL6_ORYSJ | 2558.56 | 853.86 | 2558.43 |
| | EFLSPSRVEQLH | C82A2_SOYBN | 1440.87 | 721.44 | 1440.74 |
| | HMEVFSPGTKNM | TAF1_ORYSJ | 1424.70 | 713.36 | 1424.61 |
| | GSASRAH | WRK68_ARATH | 684.37 | 343.19 | 684.33 |
| | PANVSSYQR | PRL1_ARATH | 1020.45 | 511.23 | 1020.50 |
| | SGGYVIH | AMT22_ORYSJ | 747.42 | 250.15 | 747.36 |
| | KNCYAALPEHGK | COMT1_CLABR | 1329.67 | 444.23 | 1329.65 |
| | TCLCGEK | HEM21_ARATH | 752.34 | 377.18 | 752.32 |
| | DIGQGNTYPDI | Y1054_ORYSJ | 1191.45 | 398.16 | 1191.54 |
| | GFAMMSGTSMAT | SBT3A_ARATH | 1206.50 | 604.26 | 1206.47 |
| CPCi | LASNGSRVELLDSGN | Y1614_ARATH | 1530.63 | 511.22 | 1530.76 |
| | GSVGFMDKVNSALAMCLI-AGPR | IYO_ARATH | 2268.00 | 757.01 | 2268.11 |
| | PRMYPVN | CHT5B_MEDTR | 891.51 | 446.76 | 891.43 |
| | QPVYALSIGGMI | AB8B_ARATH | 1247.78 | 416.93 | 1247.66 |
| | PLIVGTGGAMTYIMSKKA-QRV | AB7B_ARATH | 2267.99 | 757.00 | 2268.20 |
| | SLKTSRVGIMGPEKKNSM | OBP2A_ARATH | 1978.23 | 660.42 | 1978.03 |
| GPCi | GGLSMND | ATP1_ARATH | 692.34 | 347.18 | 692.28 |
| | KILGICF | GGP1_ARATH | 792.39 | 265.14 | 792.46 |
| | LNGQAEN | MB3R4_ARATH | 744.30 | 249.11 | 744.34 |
| | CNIHGLC | KPRO_MAIZE | 774.25 | 259.09 | 774.32 |

**Table S1.** Cont.

| | | | | | |
|---|---|---|---|---|---|
| GPCi | RIPHHLSSEELKKDLALKFFP | SDN4_ARATH | 2536.59 | 846.54 | 2536.38 |
| | GLIDMYSKC | PP354_ARATH | 1044.38 | 349.13 | 1044.46 |
| | MMMVKKEEDGGGN | RGA_ARATH | 1488.46 | 497.16 | 1488.59 |
| | TLLANSDLKPLPV | ATPI_OEDCA | 1379.91 | 690.96 | 1379.80 |
| | KLSKSGKHAE | HIP34_ARATH | 1099.51 | 550.76 | 1099.60 |
| | YRRRSSPIRTTTGGSKSVNFS | FUT1_ARATH | 2356.45 | 786.49 | 2356.24 |
| NBCi | DEMGGIAIPM | NU4C_VITVI | 1032.52 | 345.18 | 1032.46 |
| | NESVSGEKQNFWDTSGFKI-VVSMSMLI | NEAP2_ARATH | 3080.51 | 1027.84 | 3080.45 |
| | KLSMLFGGVL | STC_RICCO | 1079.51 | 540.76 | 1079.60 |
| | PRASGFIDVADGCCC | GDL5_ARATH | 1512.47 | 505.16 | 1512.62 |
| | PGKGPIVLH | MVD2_ARATH | 932.50 | 311.84 | 932.54 |
| | LPKAAIGFIT | UBC24_ARATH | 1029.68 | 344.23 | 1029.62 |
| | FHRKSPSSVSHLVSNF | AP4E_ARATH | 1843.95 | 615.66 | 1843.93 |

[a] Legume samples are: SBCi, intestinal digested conventional cooked soybean; CPCi, intestinal digested conventional cooked chickpea; GPCi, intestinal digested conventional cooked green pea; NBCi, intestinal digested conventional cooked navy bean.
[b] According to UniProt database.
[c] Observed molecular weight (Da) of the identified peptide.
[d] Calculated molecular weight (Da) of the matched peptide.

# Capítulo 2

## Harinas enriquecidas en hierro

**2.1. Artículo 3**

Arnal, M., Gallego, M., Mora, L., & Talens, P. (2023). Vacuum impregnation as a sustainable technology to obtain iron-fortified broad bean (*Vicia faba*) flours. *Food & Function*, *14*(11), 5429-5441.

**2.2. Artículo 4**

Arnal, M., Gallego, M., Mora, L., & Talens, P. (2025). Effect of iron-fortification process on the stability and iron bioaccessibility of broad bean flours. *LWT – Food Science and Technology*, *223*, 117766.

# Vacuum impregnation as a sustainable technology to obtain iron-fortified broad bean (*Vicia faba*) flours

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**Abstract**

Iron-fortified broad bean flours were obtained by vacuum impregnation during soaking. The impact of vacuum impregnation and iron fortification on the hydration kinetics of broad beans, as well as the processing (soaking, autoclaving, and dehulling) on the iron-absorption inhibitors (phytic acid and tannins), iron content, iron bioaccessibility, and physicochemical and techno-functional properties of flours was investigated. Results showed that the use of vacuum impregnation during soaking reduced the broad beans' soaking time by 77 %, and using iron solution instead of water did not affect the hydration kinetics. After soaking, iron-fortified broad bean flours increased twice (without hull) or more (with hull) the iron and bioaccessible iron content regarding non-fortified flours. Cooking broad beans by autoclaving modified the tannin content, the iron content and its bioaccessible fraction, and the physicochemical and techno-functional properties of the flours. Autoclaving increased the water holding capacity and absorption rate, swelling capacity, bulk density, and particle size, while decreased the solubility index, whiteness index, emulsifying capacity, emulsion stability, and gelling capacity. Finally, dehulling did not practically affect the physicochemical and techno-functional properties of flours, but showed a decrease in iron content, although increased iron bioaccessibility was observed, occurred mainly due to the reduction in tannin concentrations. The results obtained in this study demonstrated that vacuum impregnation is a useful technology for obtaining iron-fortified broad bean flours with different physicochemical and techno-functional properties depending on the production process used.

*Keywords: legume, flour, processing, fortification, iron, bioaccessibility.*

## 1. INTRODUCTION

Broad bean is a nutrient-rich legume since it is high in protein, complex carbohydrates, and dietary fibre. It also contains antinutritional factors such as lectins, phytic acid, tannins, vicine, and convicine, which reduce its biological value (Dhull et al., 2021), although these compounds can be reduced with simple treatments or bioprocessing techniques like dehulling, soaking, cooking, extrusion, germination, or fermentation (Rahate et al., 2021).

Dry broad beans can be consumed in different ways, either whole after soaking and cooking treatments or as flour after a milling process (L'Hocine et al., 2020). The flour offers a versatile and highly nutritious source of protein and can be easily added to many food products such as pasta, crackers, mayonnaise, sausages, and meat analogues (Millar et al., 2019; Sharan et al., 2021). To produce flour, the seeds can be directly milled or pretreated, for example by soaking or cooking, to improve their nutritional, physicochemical, and organoleptic properties (L'Hocine et al., 2020). Soaking is one of the most common pretreatments because it shortens the cooking time and reduces the content of antinutritional factors through activation of endogenous enzymes or leaching into the soaking medium (Dhull et al., 2021; L'Hocine et al., 2020). Likewise, cooking is also widely used because inactivates thermolabile antinutritional factors, increases protein and starch digestibility, reduces the beany flavour, and changes the physicochemical and technofunctional properties of flours (L'Hocine et al., 2020).

Soaking is a time-consuming part of the processing, so it would be interesting to shorten it by using technologies such as ultrasounds, high hydrostatic pressures or vacuum impregnation (Chigwedere et al., 2019; Miano & Augusto, 2018a). This latest technology is based on depressurisation and, later, the reestablishment of the atmospheric pressure of a solid product-solution system. During depressurisation, the air inside the product is expelled through the pores. When the atmospheric pressure is reestablished, the solution flows into the pores as a result of the pressure gradient generated,

accelerating the entry of the solution into the product (Zanella-Díaz et al., 2014).

Even though the soaking is shortened, it takes up a significant amount of time that can be used to incorporate water-soluble compounds and fortify legumes (Miano & Augusto, 2018b). In this sense, iron is an interesting compound as iron deficiency is one of the main causes of micronutrient malnutrition worldwide (Man et al., 2021; Dary & Hurrell, 2006). Nevertheless, adding iron to legumes may not have the expected effect because they contain iron-absorption inhibitors such as phytic acid and tannins that could chelate it and form insoluble complexes. In these cases, it is advisable to add already chelated iron, such as NaFeEDTA, which has been shown to have two to three times greater absorption level than ferrous sulphate (Mattar et al., 2022; Dary & Hurrell, 2006).

Iron fortification of staple food such as cereals, legumes, flours, and bread made from these flours would be an effective way to treat or prevent iron deficiency (Man et al., 2021). Furthermore, foods of high nutritional value, such as legumes and their flours, could help to prevent protein-energy malnutrition and to ensure an optimal uptake of essential micronutrients (Bessada et al., 2019; Temba et al., 2016).

Thus, the main purpose of this study was to obtain iron-fortified broad bean flours using vacuum impregnation during soaking. The specific objectives were (a) to evaluate the effect of vacuum impregnation and iron fortification on the hydration kinetics of broad beans, and (b) to study the impact of processing on the iron-absorption inhibitors, iron content, iron bioaccessibility, and the physicochemical and techno-functional properties of the flours.

## 2. MATERIALS AND METHODS

### 2.1. Samples and reagents

Broad beans (*Vicia faba*), purchased from a supermarket (Madrid, Spain), were manually cleaned to remove damaged seeds and foreign materials, and stored in plastic bags until use.

Enzymes α-amylase (A3171) and pancreatin (P7545) from porcine pancreas and pepsin from porcine gastric mucosa (P7012), bovine bile extract (B3883), ethylenediaminetetraacetic acid iron (III) sodium salt hydrate (NaFeEDTA), hydroxylamine monohydrochloride, 1,10-phenanthroline, 5-sulfosalicylic acid dihydrate, tannic acid, and phytic acid sodium salt hydrate were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) and sodium acetate trihydrate were purchased from Labbox Labware S.L. (Premià de Dalt, Barcelona, Spain). Folin-Ciocalteu's Reagent was purchased from Panreac Química S.L.U. (Castellar del Vallès, Barcelona, Spain) and ferric chloride was purchased from MP Biomedicals (Illkirch, France). All other reagents and chemicals were of analytical grade.

### 2.2. Broad bean hydration kinetics during soaking

The determination of the hydration rate of the broad beans during soaking was carried out at atmospheric pressure (atmospheric soaking, AS) and using vacuum impregnation during the first 5 min of soaking and re-establishing the atmospheric pressure after that time (vacuum soaking, VS). In both cases, broad beans were soaked in a ratio of 1:3 (w/v) with deionised water at room temperature (22 ± 2 °C) and were periodically removed, superficially dried with paper and weighed. For AS, the beans were immersed in deionised water for 16 h and the water gain was measured for the initial 6 h (at 1 h interval) and up to 10 h (at 2 h interval). Likewise, for VS, the beans were soaked for 3 h and the water gain was measured for the first hour (at 10 min interval), for the second hour (at 30 min interval), and finally at 3h. The absorbed water was calculated using Equation 1.

$$H_t(\%) = \frac{M_t - M_0}{M_0} \times 100 \quad (1)$$

where $H_t$ is the water absorbed at time $t$, $M_t$ is the weight of the sample at time $t$, and $M_0$ is the initial weight of the sample.

To study if the iron solution affected the hydration kinetics, the VS was repeated but replacing the soaking water with an iron solution, which contained 0.5 mg NaFeEDTA/mL. The selected concentration was 0.5 mg NaFeEDTA/mL since the EFSA recommends not exceeding the amount of 1.9 mg EDTA/kg b.w./day, which is established as safe, and the maximum amount of NaFeEDTA allowed in foodstuff is not legislated in the European Union (EFSA, 2010). All soaking tests were duplicated, and independent samples (50 g of dry broad beans) were prepared each time.

The Peleg's equation was used to model the hydration kinetics of broad beans because it is one of the most common equations used to model the hydration or rehydration of different food products (Miano & Augusto, 2018a; Peleg, 1988). However, the water gained by the beans instead of the moisture content was modelled as shown in the Equation 2 (Zanella-Díaz et al., 2014):

$$H_t = H_0 + t/(k_1 + k_2 t) \quad (2)$$

where $H_t$ is the water absorbed at time t (g $H_2O$/g sample); $H_0$ is the water absorbed at t = 0 min (considered 0 g $H_2O$/g sample); $t$ is the soaking time; $k_1$ is the Peleg constant 1 (min· g sample/g $H_2O$), which is inversely related to the initial water absorption rate; $k_2$ is the Peleg constant 2 (g sample/g $H_2O$), which is inversely associated with the maximum water absorption capacity.

### 2.3. Broad bean flours processing

Figure 1 shows the processing of the broad bean flours. Broad beans were vacuum soaked (1:3, w/v) with water or with the iron solution (0.5 mg NaFeEDTA/mL) until reached 100 % hydration. After draining the soaking medium, half of the water-soaked beans and half of the iron-soaked beans

were cooked by steaming using an autoclave for 5 min at 121 °C. To obtain the flours, the soaked and soaked-autoclaved broad beans were dried in a convection dryer at 50 °C until constant weight, and then milled using a ball mill (Mixer Mill MM400, Retsch GmbH, Haan, Germany). Flours were processed with and without hull. A total of 8 types of flour, 4 non-fortified flours (soaked broad bean flour, BS; soaked broad bean flour with hull, BS-H; soaked-autoclaved broad bean flour, BSA; soaked-autoclaved broad bean flour with hull, BSA-H) and 4 fortified flours (iron-soaked broad bean flour, BSFe; iron-soaked broad bean flour with hull, BSFe-H; iron-soaked-autoclaved broad bean flour, BSFeA; iron-soaked-autoclaved broad beans flour with hull, BSFeA-H) were obtained. Two batches per flour were processed.

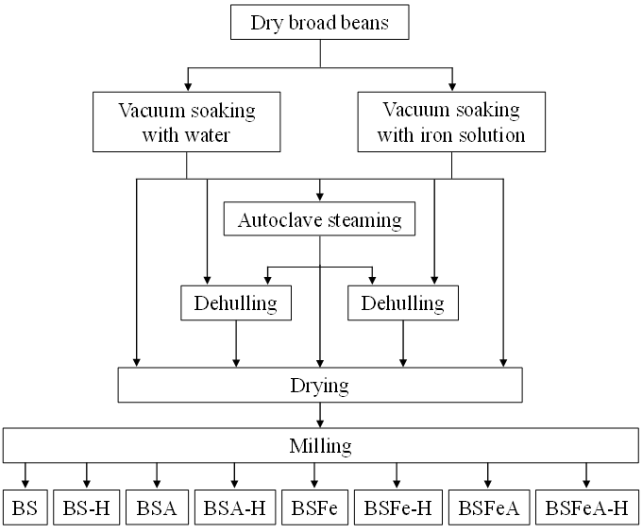


**Figure 1.** Broad bean flours processing. BS, soaked broad bean flour; BS-H, soaked broad bean flour with hull; BSA, soaked-autoclaved broad bean flour; BSA-H, soaked-autoclaved broad bean flour with hull; BSFe, iron-soaked broad bean flour; BSFe-H, iron-soaked broad bean flour with hull; BSFeA, iron-soaked-autoclaved broad bean flour; BSFeA-H, iron-soaked-autoclaved broad bean flour with hull.

### 2.4. Phytic acid and tannin determination of broad bean flours

The phytic acid content was determined following the method described by Embaby (2010) with some modifications. For that, 5 mL of HCl (2.4 %) were added to 0.1 g of broad bean flours in tubes of 15 mL, and incubated with agitation for 1 h at room T for phytic acid extraction. Then, the samples were centrifugated at 6000 *g*, 4 °C for 10 min and 0.75 mL of the supernatants were mixed with 0.25 mL of wade reagent (0.03% solution of FeC13·6H2O containing 0.3% sulfosalicylic acid in water). The mixture was centrifuged at 7000 *g* for 10 min and the absorbance of the collected supernatant was measured at 500 nm using a UV–Visible spectrophotometer (Helios Zeta, Thermo Scientific, UK). Results were expressed as mg phytic acid/g flour.

The determination of tannins was performed according to the method standardised by the AOAC (1995). So, 0.2 g of flour were mixed with 70 mL of acetone 70 % and incubated with agitation for 24 h at room T for tannins extraction. Samples were then centrifuged at 8000 *g* for 10 min, and 0.1 mL of these supernatants were mixed with 0.2 mL of $Na_2CO_3$ 20 %, 1.6 mL of distilled water and 0.1 mL of Folin-Ciocalteu reagent. After 30 min of incubation, the absorbance was measured at 760 nm. Results were expressed as mg tannic acid/g flour.

For the extractions of both compounds, an Intell-MixerTM RM-2 (ELMI Ltd., Riga, Latvia) at 30 rpm was used to agitate the samples. The extractions were performed in duplicate and the determinations were done in triplicate.

### 2.5. Iron determination of broad bean flours

The iron content was determined spectrophotometrically according to the method of Kosse et al. (2001) slightly modified by Zunin et al. (2017). Kosse et al. (2001) proved that the total $Fe^{2+}$ determination provides an accurate measure of iron content in most fortified and unfortified foods.

The iron-extracting solution was prepared by dissolving 10 g of hydroxylamine monohydrochloride in 40 mL of water and adding 20 mL of

concentrated HCl and 20 g of TCA to the solution, which was finally brought to 200 mL with water. Note that $Fe^{3+}$ is reduced to $Fe^{2+}$ with an excess of hydroxylamine monohydrochloride. To prepare the chromogen solution, 36 mg of 1,10-phenanthroline were added to 10 mL of water and heated to dissolve it. After cooling, the solution was brought to 100 mL with 3 M sodium acetate and cold stored in the dark until use. To obtain the standard curve, iron standard solutions of 0, 5, 10, 20, 25 and 50 µg Fe/mL were prepared from an iron stock solution of 0.5 mg Fe/mL using NaFeEDTA.

In order to extract iron from samples, 0.5 g of flour and 5 mL of iron-extracting solution were added to glass tubes and mixed. The tubes were put in a boiling water bath for 15 min. After incubation, the tubes were cooled and centrifuged at 8228 *g*, 8 °C for 10 min, and the supernatant was filtered.

Finally, for iron determination, 0.5 mL of filtrated sample or standard solution was mixed with 1.5 mL of chromogen solution and incubated at room temperature in the dark for 15 min. The absorbance was measured at 510 nm against a blank containing 0.5 mL iron-extracting solution and 1.5 mL 3 M sodium acetate. A sample blank was also prepared for each sample by adding 0.5 mL filtrated sample to 1.5 mL 3 M sodium acetate. The absorbance obtained was subtracted from the absorbance obtained for the corresponding sample. Iron extraction and determination was done in duplicate, and the results were expressed as mg Fe/ 100 g flour.

### 2.6. Iron bioaccessibility of broad bean flours

In order to determine the bioaccessible iron content of broad bean flours, a simulation of gastrointestinal digestion (GID) was performed following the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014), and the soluble iron content in the supernatant digest was determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7900 ICP-MS (Agilent Technologies, Inc. Headquarters, Santa Clara, California, US).

The GID was performed in duplicate at 37 °C under constant gentle mixing using an Intell-MixerTM RM-2 and an incubator chamber Selecta (JP

Selecta, S.A., Barcelona, Spain). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared (Brodkorb et al., 2019). For the oral phase, 0.5 g of flour was reconstituted with 0.5 g of water, and the paste was diluted 1:1 (w/v) with SSF containing α-amylase enzyme (75 U/mL). After 2 min, the phase was stopped by decreasing the pH to 3 with 2 M HCl, and the gastric phase was started by diluting the oral bolus 1:1 (v/v) with SGF containing pepsin (2000 U/mL). After 120 min, the gastric phase was stopped by increasing the pH to 7 with 1 M NaOH. Lastly, for the intestinal phase, the gastric chyme was diluted 1:1 (v/v) with SIF containing pancreatin (based on trypsin activity to achieve 100 U/mL) and bovine bile extract (10 mM). The digestion was stopped after 120 min by heating (100 °C, 5 min), and the samples were cooled in an ice bath and centrifugated at 8228 *g*, 4 °C for 10 min. The supernatants were lyophilised, and the total iron was determined by ICP-MS after digestion in a microwave oven because the spectrophotometric method presented interferences probably resulting from the compounds added during GID. To obtain blank samples of the digestion, 0.5 g of flour was replaced by 0.5 g of water.

## 2.7. Physicochemical properties of broad bean flours

### *2.7.1. Colour*

A Minolta CM-700d spectrophotometer (Konica Minolta Sensing, Inc., Osaka, Japan) was used to measure, in triplicate, the colour of the different broad bean flours. CIELAB colour coordinates L*, a*, and b* were obtained using the standard light source D65 and standard observer 10°. The whiteness index (WI) was calculated using Equation 3 (Marta et al., 2022).

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \quad (3)$$

### *2.7.2. Particle size distribution*

The particle size distribution of broad bean flours was measured using a Mastersizer 2000 laser scattering (Malvern Instruments, Malvern, UK) equipped with a dry powder unit Scirocco (Jiang et al., 2016). The

measurement conditions were an absorption index of 0.1 and a refractive index of 1.52. Six replicates of each sample were analysed. Results were expressed as the maximum particle diameter below 50 % of sample is contained (d(0.5)) and equivalent spherical diameter (D[4,3]).

*2.7.3. Bulk density*

The bulk density (BD) of broad bean flours was determined according to Du et al. (2014). The flour sample was transferred into a 10 mL graduated cylinder that was previously tared. The cylinder was gently tapped on a flat surface several times until the sample level was not diminished after filling to the 10 mL mark. BD was measured in triplicate and calculated as the weight of the sample (g)/volume (mL).

*2.7.4. Water absorption index, water solubility index, and swelling power*

The water absorption index (WAI), water solubility index (WSI), and swelling power (SP) were determined following the methods of Du et al. (2014) and Cornejo and Rosell (2015) with some modifications. Briefly, 0.83 g of bean flour was mixed with 10 mL of deionised water and cooked in a water bath at 60 °C for 15 min. The cooked flour was cooled at room temperature and centrifuged at 3000 $g$, 4 °C for 10 min. The supernatant was transferred into a pre-weighed recipient, lyophilised, and weighed to determine the solid content, and the weight of the sediment was taken. WAI, WSI, and SP were measured in triplicate and calculated using Equations 4, 5 and 6.

$$WAI\ (g/g) = \frac{Weight\ of\ sediment}{Sample\ weight} \quad (4)$$

$$WSI\ (g/100\ g) = \frac{Weight\ of\ dissolved\ solids\ in\ supernatant}{Sample\ weight} \quad (5)$$

$$SP\ (g/g) = \frac{Weight\ of\ sediment}{Sample\ weight - Weight\ of\ dissolved\ solids\ in\ supernatant} \quad (6)$$

## 2.8. Techno-functional properties of broad bean flours

### *2.8.1. Water-holding capacity and oil-binding capacity*

Water-holding capacity (WHC) and oil-binding capacity (OBC) were determined according to Setia et al. (2019) in triplicate. To measure the WHC, 1 g of flour was suspended in 5 mL of deionised water in a centrifuge tube and kept vertically for 20 min, with agitation for 10 s at time intervals of 5 min. After the 20 min, the suspension was centrifuged at 1000 *g*, 22 °C for 15 min and the supernatant was carefully decanted. The weight of the sediment was recorded, and WHC was expressed as the weight of retained water over the dry weight of flour (g water/ g sample).

The procedure to determine OBC was very similar to that of WHC, but 1 g of flour was suspended in 10 mL of sunflower oil and kept for 30 min. After agitation and centrifugation, the sediment was weighted and OBC was expressed as the weight of bound oil over the dry weight of flour (g oil/ g sample).

### *2.8.2. Emulsifying activity and emulsion stability*

The emulsifying activity (EA) and emulsion stability (ES) of broad bean flours were determined in duplicated using the method described by Stone et al. (2021) with slight modifications. Briefly, 1.75 g of flour was dispersed in 25 mL of deionised water. The pH was then adjusted to 7 using 0.1 M HCl/NaOH and left overnight stirring. The following day, the pH was readjusted to 7, and 25 mL of sunflower oil was added to the flour suspension. The mixture was homogenised for 1 min at level 4 speed using an Ultraturrax, and then divided into four centrifuge tubes. Two tubes were centrifugated at 1300 *g* for 5 min to measure the EA, and another two tubes were heated to 80 °C in a water bath for 30 min, cooled to room temperature, and centrifuged at 1300 *g* for 5 min to determine the ES. EA and ES were expressed in percentages and calculated as shown in Equations 7 and 8.

$$EA\ (\%) = \frac{Height\ of\ emulsified\ layer\ (cm)}{Height\ of\ entire\ layer\ in\ tube\ (cm)} \times 100 \quad (7)$$

$$ES\ (\%) = \frac{Height\ of\ emulsified\ layer\ after\ heating\ (cm)}{Height\ of\ entire\ layer\ in\ tube\ (cm)} \times 100 \quad (8)$$

*2.8.3. Gelation capacity*

The gelation capacity of flours was determined in duplicate according to Sosa et al. (2020). Suspensions between 2-20 % (w/v) were prepared, and 5 mL of each suspension was put in a test tube and heated for 1 h in a boiling water bath. After the incubation, the tubes were tempered with running water and cooled at 4 °C for 2 h. The least gelation concentration (LGC) was taken when the sample did not fall or slip when the tube was inverted.

### 2.9. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's honest significance tests was carry out to evaluate the differences in means of phytic acid, tannins, and iron content, and the physicochemical and techno-functional properties studied, while Pearson product moment correlations were carried out to measure the strength and direction of the linear relationship between the physicochemical and techno-functional properties of the broad bean flours. Both statistical analyses were performed using Statgraphics Centurion XVII software (Statgraphics Technologies, Inc., The Plains, VA, USA). Results were expressed as the mean of replicates ± standard deviations, and differences were considered significant at $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of vacuum impregnation on broad bean hydration kinetics

Water absorption curves of dry broad beans during AS and VS are shown in Figure 2A. Water absorption increased with soaking time until reaching the equilibrium moisture, but the behaviour of the hydration kinetics was different depending on the type of soaking (AS or VS). The hydration kinetics of atmospheric-soaked broad beans had a sigmoidal shape probably because the hull is impermeable to water and water would only enter through the hilum. Nevertheless, once the hull is hydrated from the inside, it becomes

permeable to water and the moisture content steadily increases until the equilibrium moisture is reached (Miano & Augusto, 2018a). Conversely, vacuum-soaked broad beans had a downward concave shape and a faster water imbibition in the initial phase than atmospheric-soaked broad beans. So, during the first hour of soaking, vacuum-soaked broad beans absorbed 85.7 % water, while atmospheric-soaked beans reached only 15.5 %. The rapid initial water absorption of vacuum-soaked broad beans may be due to the internal structure of the seeds and the hydrodynamic mechanism generated by the vacuum impregnation. At the initial stages of the hydration, the water absorption is mainly through the hilum to fill the spaces between the hull and the cotyledon, and later the gap between the two cotyledons (Miano & Augusto, 2018a; Sasikala et al., 2011). Broad beans are one of the legumes with higher porosity, approximately 10.9 ± 2.5 % (Arnal et al., 2023), probably because it is one of the largest legumes and the spaces between the hull and the cotyledon and the two cotyledons are bigger. The air occluded in these spaces and pores is pressurised with the advance of the wetting front, which could exert resistance to hydration. Therefore, when vacuum impregnation is applied during soaking, the air occluded is expelled, and when the pressure is restored, the water flows inwards accelerating the legume hydration (Chigwedere et al., 2019) and the permeation of the hull. Therefore, as shown in Figure 2A, broad beans with AS required 840 min to reach the equilibrium moisture whereas broad beans with VS required only 90 min, significantly shortening the soaking time.

In this regard, few studies have researched the effect of vacuum impregnation or vacuum pressure soaking on the legumes' hydration kinetics. Xiao et al. (2015) studied the impact of vacuum soaking in soybeans after 8 and 16 h of soaking, obtaining a significant increase in the water absorption of vacuum-soaked soybeans with regard to atmospheric pressure-soaked soybeans. Similarly, Zanella-Díaz et al. (2014) studied the shortening of the soaking time of tepary and pinto beans by applying pressure gradients at room temperature, obtaining the best results at high-pressure gradients. Li et al. (2021) and Tian et al. (2014) used vacuum soaking to hydrate rice, and both studies

observed an increase in the water absorption rate of vacuum-soaked rice during the first hour of soaking compared to rice soaked at atmospheric pressure.

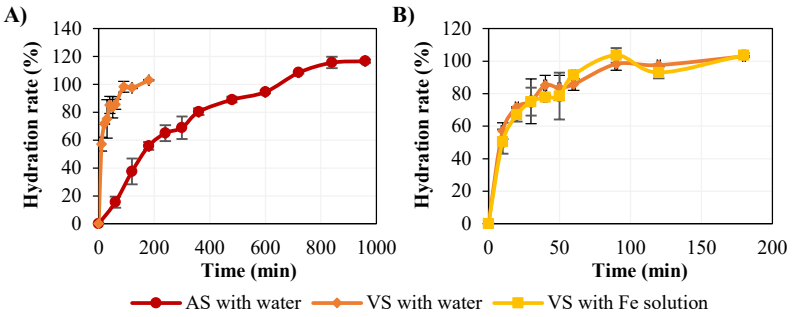


**Figure 2.** Hydration curves of dry broad beans during soaking. (A) Water absorption of broad beans during atmospheric pressure soaking (AS) and vacuum pressure soaking (VS). (B) Water or Fe solution absorption of broad beans during VS.

The experimental hydration curves were modelled with Peleg's equation for predicting the water absorption rate at any soaking time. Equations 11 and 12 were obtained, which describe, respectively, the hydration level ($H_t$) of broad beans with AS and broad beans with VS as a function of the soaking time.

$$\frac{t}{H_t - H_0} = 0.5605t + 267.09 \quad (11)$$

$$\frac{t}{H_t - H_0} = 0.9181t + 11.16 \quad (12)$$

As seen in the equations, both constants were affected by the vacuum impregnation, but $k_1$ to a greater extent. This constant is inversely related to the initial water absorption rate, which fits with the experimental results obtained. The broad beans soaked at atmospheric pressure, with a $k_1$ value of 267.09, had a low initial water absorption rate. In contrast, the broad beans soaked at vacuum pressure, with a $k_1$ value of 11.16, had a high initial water absorption rate (Figure 2A). The $k_2$ values obtained also agree with the experimental data. Broad beans soaked at atmospheric pressure showed a $k_2$ value slightly lower than broad beans soaked at vacuum pressure. This means

that atmospheric-soaked broad beans had a higher maximum water absorption capacity than those vacuum-soaked broad beans, as shown in Figure 2A. In other food matrices, the maximum water absorption capacity did not change or increase after vacuum impregnation (García-Segovia et al., 2011; Li et al., 2021).

Using the equations obtained after modelling (Equations 11 and 12), atmospheric-soaked broad beans required 608 min to reach 100 % water hydration rate, whereas vacuum-soaked broad beans required only 136 min.

### 3.2. Effect of iron fortification on broad bean hydration kinetics

To obtain iron-fortified broad beans, the water used during soaking was replaced by an iron solution, and it was tested whether the change of the soaking medium affected the hydration kinetics. The iron salt used to prepare the iron solution was NaFeEDTA because it is water-soluble and has two to three times better absorption than ferrous sulphate in food such as legumes (Mattar et al., 2022). Furthermore, it is stable during processing and storage and causes few organoleptic problems (Martínez-Navarrete et al., 2002).

Figure 2B compares the hydration curves of broad beans using water and Fe solution during vacuum soaking. According to the results, the iron solution did not alter the hydration kinetics since there were no significant differences between the % absorption of water and iron solution at any soaking time. Similar results were obtained by Miano and Augusto (2018b) when comparing the hydration kinetics of carioca kidney beans soaked with water and soaked with ferrous sulphate solution. Ferrous sulphate solution did not alter the hydration kinetic at neither of the two concentrations tested (0.271% and 0.542%, w/v), probably due to these concentrations did not modify variables that might affect the hydration kinetics, such as the pH and ionic strength of the medium.

### 3.3. Effect of processing on the phytic acid and tannin content of broad bean flours

Phytic acid and tannins were determined due to they are able to chelate and form insoluble complexes with iron, affecting its bioaccessibility. The results obtained are shown in Figure 3. The phytic acid content was around 18.2 and 20.2 mg of phytic acid per g of flour, so it was not greatly affected by processing conditions neither with hull or without hull flours (indicated by capital letters in Figure 3A). Nevertheless, statistically significant differences were observed between flours with hull and without hull within the same treated flour (indicated by lowercase letters in Figure 3A). Dehulling significantly increased the phytic acid content which could be due to this compound is mainly located in the cotyledon (Alonso et al., 2000; Luo & Xie, 2013).

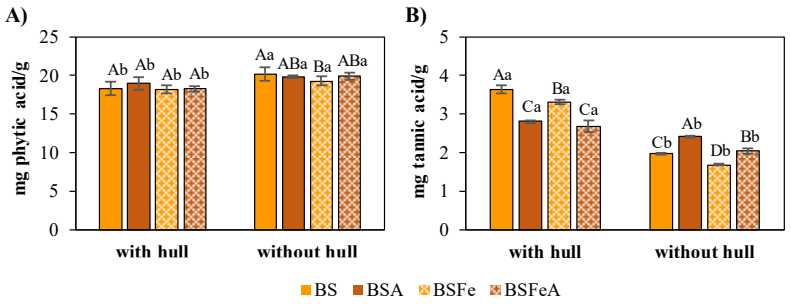


**Figure 3.** (A) Phytic acid content and (B) tannin content of broad bean flours. BS, soaked broad bean flour; BSA, soaked–autoclaved broad bean flour; BSFe, iron-soaked broad bean flour; BSFeA, iron-soaked–autoclaved broad bean flour. Capital letters indicate significant differences between treated flours within flours with hull or without hull, whereas lowercase letters indicate significant differences between flours with hull or without hull within the same treated flour.

Regarding the tannin content, the results are presented in the Figure 3B. Dehulling also had the greatest impact on the tannin content. The flours with hull presented between 2.68 and 3.63 mg of tannic acid/g of flour, whereas the flours without hull showed between 1.68 and 2.42 mg/g. The decrease observed with dehulling is due to tannins are mainly located in the hull

(Alonso et al., 2000; Luo & Xie, 2013). Moreover, it was observed that after autoclaving, the tannin content of flours with hull decreased whereas the content of flours without hull increased. These results could be due to hull's tannins, during autoclaving, diffused into the condensed steam around the beans and to the cotyledon causing an increase in tannin content when the hull was removed.

### 3.4. Effect of processing on the iron content and iron bioaccessibility of broad bean flours

The iron content of the different types of broad bean flours (BS, BSA, BSFe, BSFeA with hull and without hull) was determined to study how the production process of the flour affected the iron content, and results are shown in Figure 4A. In all cases except in the iron-soaked-autoclaved flours, the iron content of the flours decreased when the hull was removed, which would mean that a significant part of the iron, both naturally present and added, was found in the hull. Similar results were obtained by Luo et al. (2010), which showed that the total iron content of hulls (5.26 mg/100 g d.m.) was significantly higher than that of dehulled broad beans (2.38 mg/100 g d.m.). In the case of iron-soaked-autoclaved flours, it was observed that the iron content of BSFeA flour was significantly higher than that of BSFeA-H but lower than the content of BSFe-H. This increase after dehulling could be because, as the autoclaving was carried out on broad beans with hull, part of the added iron in the hull diffuses towards the cotyledon.

Regarding how the soaking and cooking could affect the iron content of broad bean flours (Figure 4A), the content of iron increased approximately twice in samples without hull (BSFe) and almost three times in those with hull (BSFe-H) after soaking with iron solution. Nevertheless, the results after soaking and autoclaving were different in presence and absence of hull. The iron content of BSFeA-H flour decreased after autoclaving, probably because an amount of the iron contained in the hull was lost through the steam condensed on the beans. However, the iron content of BSFeA flour increased

because, as mentioned above, some of the iron contained in the hull could diffuse into the cotyledon. Even so, both fortified flours after autoclaving (BSFeA-H and BSFeA) had higher iron content than their controls (BSA-H and BSA), in particular, BSFeA-H twice as much as BSA-H and BSFeA almost three times more than BSA. As regards non-fortified flours, the cooking by steam-autoclaving did not affect the iron content of broad bean flours neither with the hull (BS-H, BSA-H) nor without the hull (BS, BSA).

Nevertheless, not all the iron in broad bean flours is usually available to be absorbed because it could form insoluble complexes with tannins and phytates (Man et al., 2021; Shubham et al., 2020). Therefore, the soluble iron after GID was determined as it is a good indicator of bioaccessibility, which is defined as the fraction of a compound that is released from its food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (Cilla et al., 2018; Mattar et al., 2022). The results of bioaccessible iron in broad bean flours are shown in Figure 4B. As expected, the iron concentration in the different flours after GID was much lower than that before GID (Figure 4). However, fortified flours maintained higher iron content than non-fortified flours, specifically, BSFe-H presented three times more bioaccessible iron than BS-H, BSFeA-H almost three times more than BSA-H, BSFe two times more than BS, and BSFeA four times more than BSA. Moreover, dehulling significantly impacted on the bioaccessible iron content. Each flour without hull had a higher bioaccessible iron content than the flour with hull, except for soaked-autoclaved broad bean flours (BSA, BSA-H) in which no significant differences were observed. The reduction of bioaccessible iron in the flours with hull could be due to the large proportion of tannins present in the hull of broad beans (Figure 3B), which could act as chelating agents of iron and form stable complexes reducing iron bioaccessibility (Luo et al., 2010; Multari et al., 2015). Krishnan et al. (2012) studied the influence of dehulling on the iron bioaccessibility of finger millet, obtaining that the bioaccessible iron increased in samples without hull. However, Gwala et al. (2020) observed that dehulling did not reduce or improve the iron bioaccessibility of Bambara groundnuts. In addition, the effect of autoclaving

depended on the presence of the hull. The soaked-autoclaved flours with hull had higher bioaccessible iron content than their corresponding soaked flours. Nevertheless, the soaked-autoclaved flours without hull presented lower bioaccessible iron content than the soaked flours. These results were in concordance with those of tannin content (Figure 3B) because flours with higher tannin content had lower bioaccessible iron content. Hemalatha et al. (Hemalatha et al., 2007) obtained the same results when they studied the iron bioaccessibility of raw and pressure-cooked chickpeas with and without hulls. Nevertheless, they showed an increased iron bioaccessibility after pressure cooking the green gram in both with and without hull.

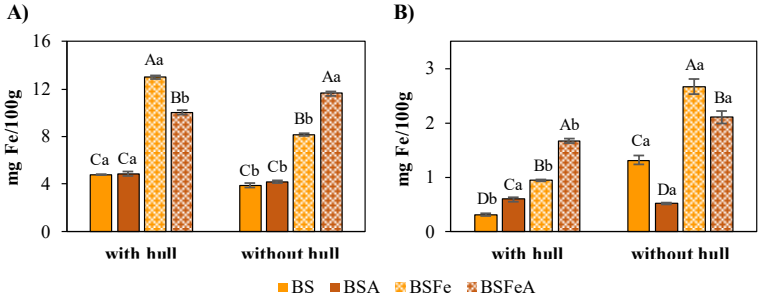


**Figure 4.** Iron content of broad bean flours (A) before gastrointestinal digestion and (B) after gastrointestinal digestion (bioaccessible iron). BS, soaked broad bean flour; BSA, soaked–autoclaved broad bean flour; BSFe, iron-soaked broad bean flour; BSFeA, iron-soaked–autoclaved broad bean flour.
Capital letters indicate significant differences between treated flours within flours with hull or without hull, whereas lowercase letters indicate significant differences between flours with hull or without hull within the same treated flour.

### 3.5. Effect of processing on the physicochemical and techno-functional properties of broad bean flours

The physicochemical properties of the different treated broad bean flours, including colour through the whiteness index (WI), particle size distribution expressed as d(0.5) and D[4,3], bulk density (BD), water absorption index (WAI), water solubility index (WSI) and swelling power (SP), were determined and are presented in Table 1. Generally, all physicochemical properties

have been affected by the thermal treatment but not by iron fortification or dehulling.

**Table 1.** Physico-chemical properties of broad bean flours.

| Flour | WI | d(0.5) (µm) | D[4,3] (µm) | BD (g/mL) | WAI (g/g) | WSI (g/100 g) | SP (g/g) |
|---|---|---|---|---|---|---|---|
| **BS** | $86.0 \pm 0.1^{b}$ | $54.9 \pm 1.7^{e}$ | $106.1 \pm 1.8^{d}$ | $0.763 \pm 0.007^{b}$ | $2.21 \pm 0.04^{d}$ | $27.4 \pm 0.3^{a}$ | $3.05 \pm 0.05^{c}$ |
| **BSFe** | $85.9 \pm 0.2^{b}$ | $64.0 \pm 2.3^{d}$ | $129.9 \pm 2.9^{c}$ | $0.791 \pm 0.024^{b}$ | $2.20 \pm 0.02^{d}$ | $27.7 \pm 0.1^{a}$ | $3.05 \pm 0.03^{c}$ |
| **BSA** | $73.5 \pm 0.2^{f}$ | $164.9 \pm 8.1^{a}$ | $211.9 \pm 6.0^{a}$ | $0.955 \pm 0.008^{a}$ | $3.85 \pm 0.03^{a}$ | $13.7 \pm 0.1^{e}$ | $4.46 \pm 0.03^{a}$ |
| **BSFeA** | $75.1 \pm 0.3^{d}$ | $134.3 \pm 2.3^{c}$ | $178.8 \pm 2.7^{b}$ | $0.944 \pm 0.003^{a}$ | $3.74 \pm 0.01^{b}$ | $14.1 \pm 0.2^{de}$ | $4.36 \pm 0.01^{a}$ |
| **BS-H** | $84.6 \pm 0.2^{c}$ | $44.7 \pm 3.3^{f}$ | $132.1 \pm 6.4^{c}$ | $0.786 \pm 0.005^{b}$ | $2.59 \pm 0.01^{c}$ | $25.0 \pm 0.3^{b}$ | $3.45 \pm 0.01^{b}$ |
| **BSFe-H** | $86.9 \pm 0.2^{a}$ | $45.4 \pm 5.2^{f}$ | $171.5 \pm 24.9^{b}$ | $0.776 \pm 0.014^{b}$ | $2.64 \pm 0.03^{c}$ | $24.0 \pm 0.5^{c}$ | $3.48 \pm 0.02^{b}$ |
| **BSA-H** | $70.2 \pm 0.3^{g}$ | $155.7 \pm 6.9^{b}$ | $212.7 \pm 4.0^{a}$ | $0.930 \pm 0.003^{a}$ | $3.80 \pm 0.04^{ab}$ | $14.1 \pm 0.2^{de}$ | $4.42 \pm 0.04^{a}$ |
| **BSFeA-H** | $74.4 \pm 0.3^{e}$ | $127.2 \pm 4.8^{c}$ | $180.3 \pm 4.4^{b}$ | $0.952 \pm 0.012^{a}$ | $3.77 \pm 0.04^{b}$ | $14.7 \pm 0.2^{d}$ | $4.42 \pm 0.06^{a}$ |

Physicochemical properties are: WI, whiteness index; d(0.5), maximum particle diameter below 50 % of sample is contained; D[4,3], equivalent spherical diameter; BD, bulk density; WAI, water absorption index; WSI, water solubility index; SP, swelling power.
Flours are: BS, soaked broad bean flour; BS-H, soaked broad bean flour with hull; BSA, soaked-autoclaved broad bean flour; BSA-H, soaked-autoclaved broad bean flour with hull; BSFe, iron-soaked broad bean flour; BSFe-H, iron-soaked broad bean flour with hull; BSFeA, iron-soaked-autoclaved broad bean flour; BSFeA-H, iron-soaked-autoclaved broad bean flour with hull.
Different letters in each column indicate significant differences between the types of flour.

The WI of broad bean flours varied between 70.2 and 86.9. Although statistically significant differences were observed between practically all samples, two groups of samples could be differentiated. So, the WI of soaked-autoclaved flours were lower than those of soaked flours whether or not flours had added iron and/or hull. The darkening of soaked-autoclaved flours could be due to the compounds derived from Maillard reactions generated during the thermal treatment (Marta et al., 2022).

The particle size distributions of broad bean flours are represented in Figure 5 and the results obtained of d(0.5) and D[4,3] are shown in Table 1. Soaked-autoclaved flours had a bimodal distribution with a small first peak in the region of 91 to 120 µm and a defined second peak in the region of 316 to 363 µm. Soaked flours presented a trimodal distribution with a defined first peak around 3-4 µm, a defined second peak around 23-26 µm, and a broader and less defined third peak in the region of 240 to 417 µm. Taking into account the values of d(0.5) and D[4,3], the flours with larger particle sizes were BSA and BSA-H, followed by BSFeA and BSFeA-H, BSFe and BSFe-H, and BS and BS-H, the latter being the smallest in particle size. Jiang et al. (2016) reported the particle size distributions of conventional thermal treated and microwave treated broad bean flours, showing a bimodal distribution and d(0.5) values in the range of 430-530 µm. These differences between results would be due to the particle size distribution of flours depends not only on the type of legume and the treatment carried out, but also on the type of milling and the conditions used (Thakur et al., 2019).

The BD of flours were in the range of 0.763-0.955 g/mL, as shown in Table 1. Thermal treatment affected this property since the values of soaked-autoclaved flours were higher than those of the soaked flours. In fact, the first ones were in the range of 0.930 to 0.955 g/mL, whereas the second ones were between 0.763 and 0.791 g/mL. These results suggest that soaked-autoclaved flours have a denser structure than soaked flours (Du et al., 2014; Kumar et al., 2022). Kumar et al. (2022) who measured the BD of soaked broad bean flour and pressure-cooked broad bean flour, obtained results with the same

tendency but with lower values (0.58 and 0.71 g/mL, respectively). Naiker et al. (2020) also reported the same tendency but with hyacinth bean flour.

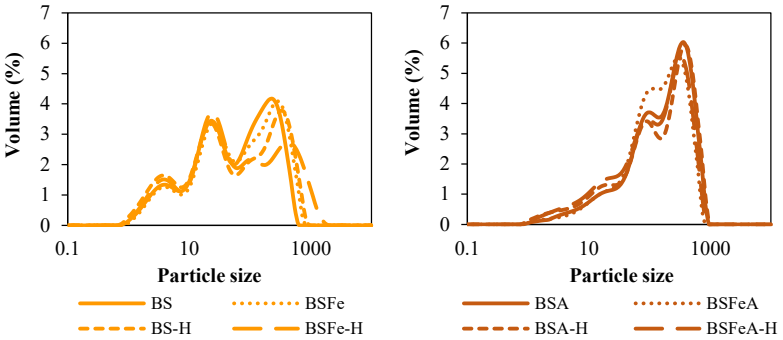


**Figure 5.** Particle size distribution of broad bean flours. BS, soaked broad bean flour; BS-H, soaked broad bean flour with hull; BSA, soaked–autoclaved broad bean flour; BSA-H, soaked–autoclaved broad bean flour with hull; BSFe, iron-soaked broad bean flour; BSFe-H, iron-soaked broad bean flour with hull; BSFeA, iron-soaked–autoclaved broad bean flour; BSFeA-H, iron-soaked–autoclaved broad bean flour with hull.

The values of WAI and SP were, respectively, in the range of 2.2-2.64 g/g and 3.05-3.48 g/g in soaked flours and in the range of 3.74-3.85 g/g and 4.36-4.46 g/g in soaked-autoclaved flours. The WAI is related to the capacity of flour to be associated with water molecules, which in turn is related to the hydrophilicity and gelation capacity of biomolecules (Bento et al., 2021; Du et al., 2014). The increase in WAI and SP values after the soaking-autoclaving treatment could be due to starch gelatinisation during the heat treatment (Ahmed et al., 2016; Simons & Hall, 2018). Gelatinised starch loses the crystalline structure and increases the number of amorphous structures, leading to an increase of sites and groups taking part in water binding (Lv et al., 2011). The WSI presented an opposite tendency to WAI and SP. Soaked flours had a WSI of 24-27.7 g/100 g, whereas soaked-autoclaved flours presented a WSI of 13.7-14.7 g/100 g. The WSI is an indicator of the degree of leaching of soluble compounds from flour (Ahmed et al., 2016). The lower WSI of soaked-autoclaved flours may be attributed to the denaturation of proteins and the fact that before obtaining the flour, during the cooking of seeds,

the soluble compounds of the broad beans could be leached out in the steam that condenses around them (Aguilera et al., 2009; Simons & Hall, 2018). Results with the same tendency were reported by Naiker et al. (2020) and Simons and Hall (2018). Naiker et al. (2020) determined the WSI and SP of soaked and soaked-autoclaved hyacinth bean flours at different temperatures, and Simons and Hall (2018) determined the WAI and WSI of raw and cooked pinto bean flours.

As regards the results of WAI, WSI and SP, it can be seen in Table 1 that WAI and SP were higher in soaked-autoclaved flours than in soaked flours. Table 2 presents the determined techno-functional properties (water-holding capacity (WHC), oil-binding capacity (OBC), emulsifying activity (EA), emulsion stability (ES) and gelation capacity expressed as least gelation concentration (LGC)), and Table 3 the Pearson's correlation coefficients of physicochemical and techno-functional properties of the broad bean flours. As with the physicochemical properties, the techno-functional properties are mainly affected by the heat treatment.

**Table 2.** Techno-functional properties of broad bean flours.

| Flour | WHC (g/g) | OBC (g/g) | EA (%) | ES (%) | LGC (%) |
|---|---|---|---|---|---|
| **BS** | $1.07 \pm 0.05^{d}$ | $0.80 \pm 0.04^{ab}$ | $46.5 \pm 0.8^{a}$ | $68.3 \pm 0.7^{a}$ | $8^{b}$ |
| **BSFe** | $1.28 \pm 0.09^{c}$ | $0.84 \pm 0.05^{a}$ | $45.5 \pm 0.4^{a}$ | $65.4 \pm 0.1^{a}$ | $8^{b}$ |
| **BSA** | $1.79 \pm 0.10^{ab}$ | $0.86 \pm 0.14^{a}$ | $8.6 \pm 0.3^{bc}$ | $5.5 \pm 0.4^{c}$ | $20^{a}$ |
| **BSFeA** | $1.65 \pm 0.04^{b}$ | $0.78 \pm 0.05^{ab}$ | $4.5 \pm 0.4^{c}$ | $3.5 \pm 0.0^{c}$ | $20^{a}$ |
| **BS-H** | $1.34 \pm 0.09^{c}$ | $0.81 \pm 0.04^{ab}$ | $43.9 \pm 3.4^{a}$ | $55.7 \pm 1.3^{b}$ | $8^{b}$ |
| **BSFe-H** | $1.44 \pm 0.02^{c}$ | $0.83 \pm 0.03^{a}$ | $46.5 \pm 0.8^{a}$ | $53.9 \pm 2.1^{b}$ | $8^{b}$ |
| **BSA-H** | $1.84 \pm 0.05^{a}$ | $0.66 \pm 0.02^{b}$ | $10.3 \pm 0.9^{b}$ | $6.0 \pm 0.1^{c}$ | $20^{a}$ |
| **BSFeA-H** | $1.80 \pm 0.02^{ab}$ | $0.69 \pm 0.04^{ab}$ | $10.1 \pm 0.8^{b}$ | $6.0 \pm 0.1^{c}$ | $20^{a}$ |

Techno-functional properties are: WHC, water-holding capacity; OAC, oil-binding capacity; EA, emulsifying activity; ES, emulsion stability; LGC, least gelation concentration.
Flours are: BS, soaked broad bean flour; BS-H, soaked broad bean flour with hull; BSA, soaked-autoclaved broad bean flour; BSA-H, soaked-autoclaved broad bean flour with hull; BSFe, iron-soaked broad bean flour; BSFe-H, iron-soaked broad bean flour with hull; BSFeA, iron-soaked-autoclaved broad bean flour; BSFeA-H, iron-soaked-autoclaved broad bean flour with hull.
Different letters in each column indicate significant differences between the types of flour.

The WHC is the water that 1 g of flour imbibes and retains under low-speed centrifugation. Results obtained (Table 2) showed that the thermal treatment increased the WHC. In fortified flours, the WHC increased around 1.26 times whereas in non-fortified flours increased 1.67 times in samples without hull and 1.37 times in those with hull. These results agree with several previous studies where flours from pigeon pea, dolichos bean, jack bean, cannellini bean, common bean, and hyacinth bean were analysed (Acevedo et al., 2017; Aguilera et al., 2011; Lin & Fernández-Fraguas, 2020; Naiker et al., 2020). WHC was positively correlated with WAI (r 0.9563, $p < 0.001$) and SP (r 0.9611, $p < 0.001$), and negatively correlated with WSI (r -0.9477, $p < 0.001$) (Table 3). Furthermore, samples with hulls presented slightly higher values than samples without hulls. BSA-H had the highest WHC with 1.84 g/g, and BS had the lowest WHC with 1.07 g/g. Similar results were obtained by Kumar et al. (2022). The increase of WHC after autoclaving could be related to protein denaturation and starch gelatinisation (Kumar et al., 2022). During denaturation, proteins are unfolded and some polar side chains and hidden peptide bonds are exposed, which could increase the ability to hold water. Moreover, as mentioned above, gelatinised starch loses its crystalline structure, favouring water-holding (Lin & Fernández-Fraguas, 2020).

The high values of WHC, WAI, and SP of the soaked-autoclaved flours would make them suitable for doughs and meat products because it probably imbibes water without proteins dissolution, thereby attaining a thickening and viscous body (Aguilera et al., 2011). Moreover, the low WSI would make them suitable for pasta processing as they will have less soluble solids losses in the cooking water (Bento et al., 2021).

The OBC is related to mouthfeel and flavour retention (Lin & Fernández-Fraguas, 2020). The OBC of broad bean flours was around 0.8 g/g (Table 2), which was lower than the values reported in other legume flours (Acevedo et al., 2017; Aguilera et al., 2011; Lin & Fernández-Fraguas, 2020; Naiker et al., 2020). This would make them suitable for preparing fried products as they

would not cause a greasy mouthfeel (Aguilera et al., 2011). No significant differences were observed between the samples except for the BSA-H, which had lower OBC, 0.66 g/g. The obtained results are close to those of Kumar et al. (2022), who measured the OBC of soaked and thermal treated broad bean flours and obtained results around 0.6 g/g. The OBC has been attributed to the physical entrapment of oil within the protein through non-covalent bonds such as hydrophobic, electrostatic, and hydrogen bonds (Acevedo et al., 2017). The differences between samples could be related to the protein conformation change due to flour processing.

The EA and ES results of broad bean flours are also presented in Table 2. The EA is a techno-functional property related to the ability of proteins and other surface-active molecules to adsorb the oil-water interface, contributing to the formation of emulsions, whereas ES is a property related to the stability of this adsorbed layer over a period of time (Lin & Fernández-Fraguas, 2020). Both properties were strongly affected by the autoclaving process carried out during the processing of flours. The soaked flours had an EA of approximately 45 %, with and without hull and iron, which is similar to that obtained by Setia et al. (2019) (47.2 %). After autoclaving, the EA was reduced by 78 to 89 %, depending on the type of flour, which has been reported in previous studies (Aguilera et al., 2011; Bento et al., 2021; Medhe et al., 2019). As regards ES, soaked flours without hull presented values of 65-68 %, and soaked flours with hull of 53-55 %, which is similar to the result (54.2 %) of Setia et al. (2019). Soaked-autoclaved flours had a much lower ES, around 5 %. Other studies with colourful beans (*Phaseolus vulgaris* L.) and hyacinth beans have also shown a decrease in ES after thermal treatment, but not as abruptly (Bento et al., 2021; Naiker et al., 2020). The high EA and ES of soaked flours could be attributed to starch and fibre, which increase the viscosity of the continuous phase, as well as to the strength of the protein adsorbed in the interface (Lin & Fernández-Fraguas, 2020). Likewise, the low EA and ES of soaked-autoclaved flours may be due to the protein denaturation resulting from the autoclaving process (Bento et al., 2021). These results

agree with the correlations obtained as EA and ES were negatively correlated with WAI, SP, and WHC but positively correlated with WSI (Table 3).

**Table 3.** Pearson's correlation coefficients of physicochemical and techno-functional properties of broad bean flours.

| | WAI | WSI | SP | BD | WI | d(0.5) | D[4,3] | WHC | OBC | EA | ES | LGC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **WAI** | 1 | | | | | | | | | | | |
| **WSI** | **-0.9987** | 1 | | | | | | | | | | |
| **SP** | **0.9989** | **-0.9956** | 1 | | | | | | | | | |
| **BD** | **0.9739** | **-0.9761** | **0.9655** | 1 | | | | | | | | |
| **WI** | **-0.9555** | **0.9570** | **-0.9470** | **-0.9574** | 1 | | | | | | | |
| **d(0.5)** | **0.9318** | **-0.9399** | **0.9164** | **0.9599** | **-0.9689** | 1 | | | | | | |
| **D[3,4]** | **0.8966** | **-0.8944** | **0.8994** | **0.8304** | **-0.8431** | **0.8483** | 1 | | | | | |
| **WHC** | **0.9563** | **-0.9477** | **0.9611** | **0.9217** | **-0.9134** | **0.8799** | **0.9493** | 1 | | | | |
| **OBC** | -0.5406 | 0.5330 | -0.5430 | -0.5104 | 0.6438 | -0.4655 | -0.3959 | -0.5545 | 1 | | | |
| **EA** | **-0.9731** | **0.9794** | **-0.9630** | **-0.9911** | **0.9576** | **-0.9547** | **-0.8100** | **-0.8924** | 0.5221 | 1 | | |
| **ES** | **-0.9965** | **0.9978** | **-0.9927** | **-0.9847** | **0.9587** | **-0.9391** | **-0.8767** | **-0.9463** | 0.5473 | **0.9867** | 1 | |
| **LGC** | **0.9777** | **-0.9826** | **0.9678** | **0.9926** | **-0.9740** | **0.9675** | **0.8333** | **0.9127** | -0.5655 | **-0.9944** | **-0.9876** | 1 |

WAI: water absorption index; WSI: water solubility index; SP: swelling power; BD: bulk density; WI: whiteness index; d(0.5), maximum particle diameter below 50 % of sample is contained; D[4,3], equivalent spherical diameter; WHC, water-holding capacity; OBC, oil-binding capacity; EA, emulsifying activity; ES, emulsion stability; LGC, least gelation concentration.
Values in bold type correspond to $p < 0.05$.

Finally, the gelation capacity of broad bean flours was measured through the LGC. The lower the value of the LGC of the flour, the better its gelation capacity. The LGC of soaked and soaked-autoclaved flours were 8 % and 20 %, respectively (Table 2). This property was positively correlated with WAI, SP, and WHC, while it was negatively correlated with WSI, EA, and ES (Table 3). However, it means the opposite because the higher the LGC, the lower the CG. Like other techno-functional properties, gelation capacity was affected by the thermal treatment of the flours, but fortification and dehulling did not affect it. Kumar et al. (2022) and Aguilera et al. (2009) observed the same effect after thermal treatment. However, Kumar et al. (2022), who studied the LGC of soaked and pressure-cooked broad bean flour, obtained lower LGC, with values of 6 and 10 %, respectively. The decrease in the gelation capacity after autoclaving might be due to the denaturation and aggregation of proteins (Aguilera et al., 2009).

## 4. CONCLUSIONS

Results of hydration kinetics showed that the use of vacuum impregnation during soaking reduced by 77 % the time to reach 100 % hydration of the broad beans in comparison to soaking without vacuum impregnation. Furthermore, using iron solution as a soaking medium did not affect the hydration kinetics of the broad beans. Iron content and its bioaccessibility are influenced by flour processing. Soaking with iron increased iron content and its bioaccessibility, whereas autoclaving process affects tannin, iron content, and its bioaccessible fraction. Finally, dehulling showed a decrease in iron content, although increased iron bioaccessibility was observed, occurred mainly due to the reduction in tannin concentrations. Regarding the physicochemical and techno-functional properties of flours, autoclaving was responsible of significant changes whereas iron fortification and dehulling did not affect practically any of them. The thermal process increased the water holding capacity and absorption rate, swelling capacity, bulk density, and particle size, while decreased the solubility index, whiteness index,

emulsifying activity, emulsion stability, and gelling capacity of the flours. This study shows that vacuum impregnation can be used as a suitable technology to obtain iron-fortified broad bean flours with a higher amount of bioaccessible iron. Physicochemical and technological properties of the flours will depend on the applied processing conditions, which could be selected depending on the desired characteristics when the flours are added to food products.

**Author contributions**

Milagros Arnal: Conceptualization, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. Marta Gallego: Conceptualization, Supervision, Writing – Review & Editing. Leticia Mora: Conceptualization, Supervision, Writing – Review & Editing, Project administration. Pau Talens: Conceptualization, Formal analysis, Supervision, Writing – Review & Editing, Project administration, Funding acquisition.

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# Effect of iron-fortification process on the stability and iron bioaccessibility of broad bean flours

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**Abstract**

Iron-fortified flours are traditionally prepared by blending a micronutrient premix with milled flour until a homogeneous mixture is obtained. In this study, an alternative method using vacuum impregnation during the soaking stage was used to obtain iron-fortified broad bean flours. The stability of iron-fortified broad bean flours obtained using vacuum impregnation or conventional addition, as well as the effect of cooking and gastrointestinal digestion on the produced fresh pasta, was evaluated. The results showed that the use of vacuum impregnation to obtain iron-fortified broad bean flours resulted in higher lipid and protein oxidation compared to the conventional method of iron addition. However, it did not significantly affect the stability of the flours as lipid and protein oxidation remained stable during 7 weeks of storage. In addition, vacuum impregnation to fortify the broad bean flour prevented the loss of added iron during cooking and improved its bioaccessibility when making pasta from 100 % broad bean flour.

*Keywords: Vicia faba, soaking, vacuum impregnation, iron bioaccessibility.*

## 1. INTRODUCTION

Iron deficiency is a leading cause of micronutrient malnutrition globally (Dary and Hurrell, 2006; Man et al., 2021). Fortifying staple foods like cereals, legumes, flour, and bread could effectively treat or prevent it (Man et al., 2021). Iron-fortified flours are usually made by blending a micronutrient premix with milled flour until a homogeneous mixture is obtained (Hurrell, 2022; Peña-Rosas et al., 2014).

In the case of legumes, the time-consuming soaking step required for their processing could be used for fortification. Moreover, this process could be shortened by applying vacuum impregnation technology since the vacuum facilitates the removal of air from inside the legumes, and the re-establishment of atmospheric pressure accelerates the entry of the soaking medium (Arnal et al., 2023). However, it is important to note that the iron compound added can cause undesirable organoleptic problems due to, for example, the oxidation of fats, and can interact with iron-absorption inhibitors present in legumes such as phytic acid and tannins (Dary and Hurrell, 2006). For this reason, new approaches for micronutrient incorporation, such as encapsulation, nanoparticles and chelates, have been proposed to avoid interactions between the food matrix and the fortifier (Mattar et al., 2022).

In a previous study, the application of vacuum impregnation during soaking allowed the incorporation of a chelated form of iron, NaFeEDTA, to obtain iron-fortified broad bean flour (Arnal et al., 2023). This study investigated the impact of vacuum impregnation and iron fortification on the hydration kinetics of broad beans and assessed how processing methods such as soaking, autoclaving or dehulling influenced iron-absorption inhibitors (phytic acid and tannins), content and bioaccessibility of iron, and the physicochemical and techno-functional properties of the resulting flours. The findings highlight that vacuum impregnation is an effective technology for producing iron-fortified broad bean flour, with the advantage over the conventional blending method in which iron is incorporated into the broad bean matrix (Arnal et al., 2023). However, the impact of iron integration into flour

through vacuum impregnation and its behaviour during food processing is still unknown. In this regard, the stability of flour during storage could be affected due to the prooxidative behaviour of iron (Barden & Decker, 2016; Xiong & Guo, 2021), and the use of vacuum impregnation could prevent iron losses during cooking. Therefore, the aim of this study was to assess the oxidative stability of the flour over time and to evaluate the behaviour of added iron during cooking and subsequent gastrointestinal digestion when the flour is used to make fresh pasta.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

The ethylenediaminetetraacetic acid iron (III) sodium salt hydrate (NaFeEDTA) from Sigma-Aldrich, Co. (St. Louis, MO, USA) was used to fortify broad beans. 1,1,3,3-tetramethoxypropan (TMP) and 2-thiobarbituric acid (TBA) from Sigma-Aldrich, Co. (St. Louis, MO, USA), and trichloroacetic acid (TCA) from Scharlau Chemie S.A. (Sentmenat, Barcelona, Spain) were used to evaluate lipid oxidation. Sodium pyrophosphate tetrabasic (Na4O7P2), TRIS maleate salt, ethylene glycol-bis(2-amino-ethilether)-N,N,N',N'-tetra-acetic acid (EGTA), ethyl acetate, guanidine hydrochloride, sodium phosphate dibasic, and sodium phosphate monobasic from Sigma-Aldrich, Co. (St. Louis, MO, USA) and TCA, potassium chloride (KCl), magnesium chloride (MgCl2), and, ethanol absolute from Scharlau Chemie S.A. (Sentmenat, Barcelona, Spain) were used to determine protein oxidation. Finally, α-amylase (A3171) and pancreatin (P7545) from porcine pancreas, pepsin from porcine gastric mucosa (P7012) and bovine bile extract (B3883) from Sigma-Aldrich, Co. (St. Louis, MO, USA) and KCl, MgCl2, potassium dihydrogen phosphate (KH2PO4), sodium hydrogen carbonate (NaHCO3), sodium chloride (NaCl), calcium chloride dihydrate (CaCl2·2H2O), and ammonium carbonate ((NH4)2CO3) from Scharlau Chemie S.A. (Sentmenat, Barcelona, Spain) were used to simulate the gastrointestinal digestion.

## 2.2. Process for obtaining iron-fortified broad bean flours using vacuum impregnation and conventional addition

Broad beans (*Vicia faba*) were purchased from a supermarket (Madrid, Spain) and classified to remove damaged seeds. Then, broad beans were superficially washed with water and soaked in a ratio 1:3 (w/v) in water or iron solution (0.5 mg NaFeEDTA/ mL) for 180 min, using vacuum impregnation during the first 5 min and re-storing the atmospheric pressure after that time. After that, the soaking medium was drained, and the broad beans were cooked by steaming using an autoclave for 5 min at 121 °C, followed by dehulling, drying at 50 °C until constant weight, and milling using a ball mill (Mixer Mill MM400, Retsch GmbH, Haan, Germany). The flour obtained from beans soaked in water was the control flour (B), and the flour obtained from beans soaked in iron solution was the iron-fortified flour obtained by vacuum impregnation (BFeVI). To obtain the iron-fortified broad bean flour by addition (BFeA), the B was mixed with solid NaFeEDTA until the same iron concentration of BFeVI was achieved. Two batches of each type of flour were prepared.time.

## 2.3. Determination of the stability of fortified flours

Control and iron-fortified flours were stored at 25 °C and 37 °C during 7 weeks to study their stability during storage. For that, the levels of lipid and protein oxidation were measured after 1, 3 and 7 weeks.

### *2.3.1. Lipid oxidation*

Lipid oxidation was evaluated in triplicate using the thiobarbituric acid reactive substances (TBARS) method (Salih et al., 1987) with some modifications according to Gallego et al., 2020. Briefly, 20 mL of TCA (50 mg/mL) was added to 5 g of flour, homogenised and centrifuged at 12,000 *g*, 4 °C for 10 min. The supernatant was collected and filtered through a 10-12 µm cellulose membrane filter. The assay was performed by mixing 4 mL of sample and 4 mL of 0.02 M TBA solution and heating at 95 °C for 1 h. After cooling,

the absorbance was measured at 532 nm. The results were expressed as µg malonaldehyde (MDA) per g of sample from a standard curve of TMP (0.5-12 µM), since it is a precursor of MDA, and 1 mol TMP is equivalent to 1 mol MDA (Equation 1).

$$\frac{\mu g\ MDA}{g\ flour} = \frac{\mu M\ MDA \times molecular\ weight\ MDA \times V\ extraction}{g\ sample\ extraction} \quad (1)$$

*2.3.2. Protein oxidation*

Protein oxidation was evaluated by the DNPH method according to (Armenteros et al., 2009) with slight modifications. The total carbonyl content was determined, in duplicate, by derivatisation with DNPH. Flours were homogenised in a 1:4 (w/v) ratio in pyrophosphate buffer (pH 7.4; 2 mmol/L $Na_4P_2O_7$, 10 mmol/L TRIS maleate salt, 2 mmol/L EGTA, 100 mmol/L KCl, 2 mmol/L $MgCl_2$) for 30 s. The homogenates were divided into two aliquots of 100 µL, and proteins were precipitated by adding 1 mL of TCA (0.1 g/mL) and centrifuging at 10,000 *g* for 5 min. After removing the supernatants, one pellet was treated with 1 mL HCl 2 mol/L for protein quantification and the other with 1 mL of 2 mg/mL DNPH in HCl 2 mol/L for carbonyl determination. Both samples were incubated for 1 h at room temperature, shaking every 15 min, and then precipitated with 1 mL of TCA (0.1 g/mL) and centrifuged at 10,000 *g* for 5 min. The pellets were taken and washed twice with 1 mL of 1:1 ethanol/ethyl acetate (v/v), shaken, and centrifuged for 5 min at 10,000 *g*. Finally, the pellets were dissolved in 1.5 mL of 20 mmol/L sodium phosphate buffer (pH 6.5) containing 6 mol/L guanidine hydrochloride, and centrifuged for 5 min at 10,000 *g* to remove insoluble fragments. The protein concentration was determined by measuring the absorbance at 280 nm, using a standard curve of BSA (0.5-2 mg/mL). The carbonyl content was measured at 370 nm and calculated using the Lambert-Beer law, considering the absorption coefficient for the protein hydrazones. The results were expressed as nmol of carbonyl per mg of protein (Equation 2).

$$\frac{nmol\ carbonyl}{mg\ protein} = \frac{nmol\ carbonyl/mL}{mg\ protein/mL} = \frac{(Abs\ 370\ nm/\Sigma\ x\ l)\times 1000}{mg\ BSA/mL} \quad (2)$$

Where ξ is the adsorption coefficient for the protein hydrazones (21 L $mmol^{-1}$ $cm^{-1}$) and l is the length travelled by the light in the medium (1 cm).

### 2.4. Preparation of fresh 100 % broad bean pasta

Fresh pasta was prepared by mixing 70 g of flour, 65 g of egg, and 1 g of salt for 2 min in a food processor (Mycook One, Taurus Group, Oliana, Spain). The dough was kneaded for 1 min, coated with plastic wrap, and then allowed to stand for 30 min at 4 °C. A pasta-making machine (CASA International NV, Itegem, Belgium) was used to obtain dough sheets (2 mm thick) and, subsequently, fettuccines (6.6 mm wide and 15 cm long).

Fresh pasta was prepared with the three different flours (B, BFeVI and BFeA), obtaining three different types of pasta: pasta made with flour B (PB), pasta made with flour BFeVI (PBFeVI) and pasta made with flour BFeA (PBFeA). Two batches of each type of pasta were prepared.

### 2.5. Determination of iron losses during cooking

Total iron was determined in fresh uncooked and cooked pasta to evaluate losses during cooking. For this purpose, 25 g of freshly prepared pasta was cooked in 500 mL boiling water for 4 min, which was the time needed for the white core of the pasta to disappear when gently squeezed between two glass plates (Arribas et al., 2020). Then, the uncooked and cooked pasta were lyophilised and digested to determine the total iron content in triplicate, using inductively coupled plasma mass spectrometry (ICP-MS) with an Agilent 7900 ICP-MS instrument (Agilent Technologies, Inc. Headquarters, Santa Clara, CA, USA). Results were expressed as mg of iron per 100 g of pasta on dry basis (d.b.).

### 2.6. Determination of iron bioaccessibility of different types of cooked pasta

To assess the percentage of bioaccessible iron in each type of cooked broad bean pasta (PB, PBFeVI, PBFeA), *in vitro* gastrointestinal digestion

(GID) using the INFOGEST protocol was performed (Brodkorb et al., 2019; Minekus et al., 2014). An Intelli-Mixer™ RM-2 (Biogen Cientifica S.L., Madrid, Spain) and an incubator chamber Selecta (JP Selecta, S.A., Barcelona, Spain) were used to maintain samples in constant gentle mixing at 37 °C during the GID. Firstly, simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared (Minekus et al., 2014). The GID was started by mixing 1 g of cooked pasta with SSF (pH 7) containing α-amylase enzyme (1.25 μkat/mL) at a ratio of 1:1 (w/v), and incubated for 2 min to simulate the oral phase. In the gastric phase, the pH was decreased to 3 and the oral bolus was diluted 1:1 (v/v) with SGF containing pepsin (33.3 μkat/mL). After 120 min of incubation, the intestinal phase was started by increasing the pH to 7 and diluting the gastric chyme 1:1 (v/v) with SIF containing pancreatin (based on trypsin activity to achieve 1,67 μkat/mL) and bovine bile extract (10 mmol/L). Finally, after incubating the samples for another 120 min, the digestion was stopped by thermal shock (100 °C for 5 min) followed by cooling in an ice bath. After that, the samples were centrifuged at 8000 g, 4 °C for 10 min, and the supernatants were recovered and lyophilised to finally determine the total iron content as previously described. Control samples of the GID process were obtained by replacing pasta with water. The percentage of bioaccessible iron was calculated as total iron in the supernatant divided by the total iron in the sample.

### 2.7. Statistical analysis

Statistical analyses were performed using Statgraphics Centurion XIX software (Statgraphics Technologies, Inc., The Plains, VA, USA). A multifactorial ANOVA was used to analyse the results of lipid and protein oxidation of flours, with sample type and storage time and temperature as factors. In those cases where the factor was significant ($p < 0.05$), a one-way ANOVA with Tukey's Honest Significance Difference test was used to evaluate differences between the means ($p < 0.05$). One-way ANOVA with Tukey's Honest Significance Difference test was also used to evaluate the differences in the means of iron content and iron bioaccessibility of the flours.

Results were expressed as the mean of replicates ± standard deviation, and differences were considered significant at $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of iron addition on flour stability

To evaluate the stability of the flours stored at 25 °C and 37 °C, the values of lipid and protein oxidation of B, BFeVI and BFeA were determined immediately after obtaining the flours as well as after 1, 3 and 7 weeks of storage. The multifactorial ANOVA indicated a significant ($p < 0.05$) effect of the type of sample, storage time, and temperature. So, a simple ANOVA was performed grouping each factor, and the results are shown in Figure 1. At 0 weeks, B exhibited a lipid oxidation level of 0.62 μg MDA/g sample (Figure 1A) and a protein oxidation level of 8.6 nmol carbonyls/mg protein (Figure 1B). These results could be due to oxidation produced by enzymes naturally present in broad beans, such as lipoxygenase, or to the flour production process, which involved drying at 50 °C and milling (Sharan et al., 2021; Xiong & Guo, 2021). BFeVI exhibited significantly ($p < 0.05$) higher levels of lipid and protein oxidation during storage compared to B and BFeA, which could be attributed to the flour production process. In BFeVI, the dissolved iron was incorporated into the bean tissue during soaking, and beans were exposed to high temperatures to be dried after iron incorporation. This might have contributed to increased oxidation since both metals and high temperatures can initiate lipid oxidation and then accelerate it through reactions that produce free radicals and/or reactive oxygen species (ROS) (Barden & Decker, 2016). Moreover, these ROS catalysed by iron could be causing the observed increase in protein oxidation by interacting with the reactive side chain groups and converting them to carbonyls and other derivatives (Xiong & Guo, 2021). Lastly, BFeA showed significantly ($p < 0.05$) higher lipid oxidation than B but a similar level of protein oxidation. In this case, iron was added after drying and milling the broad beans, and it was not integrated into the tissue. Therefore, the contact between the iron and the flour

would be lower than in the case of BFeVI. Similarly, Rebellato et al. (2018) obtained iron-fortified refined wheat flour by the addition of NaFeEDTA, observing slightly higher but not significant ($p > 0.05$) lipid oxidation compared to the control flour.

Regarding the progression of oxidation over time, B and BFeA showed a slight but significant ($p < 0.05$) increase in lipid oxidation at the two storage temperatures. However, in general, lipid oxidation in BFeVI and protein oxidation in the three flours at both storage temperatures did not increase over the studied time. In this regard, few studies have evaluated lipid and protein oxidation of flours and iron-fortified flours from pulses. Duque-Estrada et al. (2020) observed a moderate increase in the carbonyl content of a full-fat soybean flour after two months of storage at room temperature, with values ranging from 5.9 to approximately 8.5 nmol carbonyls/mg protein, although this increase was not statistically significant ($p > 0.05$). Likewise, Rebellato et al. (2018) observed an increase in lipid oxidation in iron-fortified refined wheat flour stored for 120 days at room temperature.

As mentioned above, high temperatures can promote oxidation. By comparing the two storage temperatures (25 °C and 37 °C) for the same sample and storage time, significant ($p < 0.05$) differences in lipid oxidation were only observed at the end of the storage period for the three types of flours, as well as in BFeA after 1 and 7 weeks of storage for protein oxidation. The absence of major differences between both temperatures may be advantageous for storing the flours over a wide range of ambient temperatures. However, further studies could consider other variables, such as relative humidity, a wider range of temperatures, or packaging material, to confirm that the level of oxidation remains stable throughout the product's shelf life. In addition, a sensory analysis could be carried out to assess whether the process could affect the organoleptic characteristics of the flours.

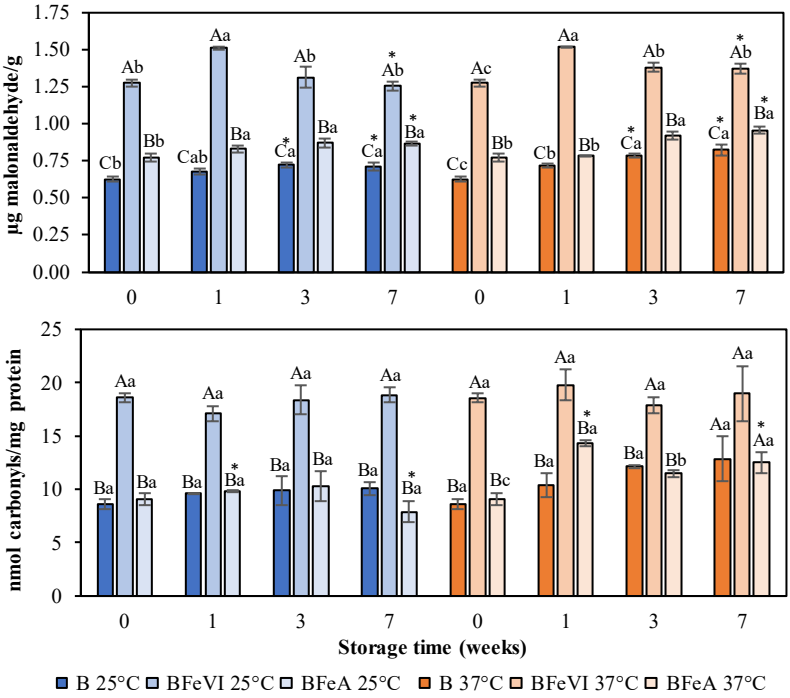


**Figure 1.** Lipid (A) and protein (B) oxidation of control and iron-fortified broad bean flours stored at 25 °C and 37 °C for 7 weeks. B, broad bean flour; BFeVI, iron-fortified broad bean flour by vacuum impregnation; BFeA, iron-fortified flour by addition. Results are shown as mean (n) ± standard deviation (lipid oxidation n = 3, protein oxidation n = 2). Different capital letters indicate significant differences between different sample types within the same storage time and temperature ($p < 0.05$), lowercase letters indicate significant differences between different storage weeks within the same sample type and storage temperature ($p < 0.05$), and "*" indicates significant differences between different storage temperatures within the same sample type and storage week ($p < 0.05$).

### 3.2. Effect of the fortification process of broad bean flours on iron added during cooking and *in vitro* gastrointestinal digestion

To evaluate the iron fortification process of broad bean flour, a fresh 100 % broad bean pasta was prepared, and iron losses during cooking were determined. As shown in Figure 2, samples PBFeVI and PBFeA presented the same iron content (10.4 mg Fe/100 g d.b), being significantly ($p < 0.05$) higher than that of PB (5.3 mg Fe/100 g d.b.). Cooking the pasta did not

modify the iron contents of the raw PB and PBFeVI, but caused a reduction of approximately 17 % in the value of PBFeA. Therefore, it is crucial to assess the amount of fortifier lost during the processing of the product, as the quantity added is not necessarily equal to the amount ultimately ingested.

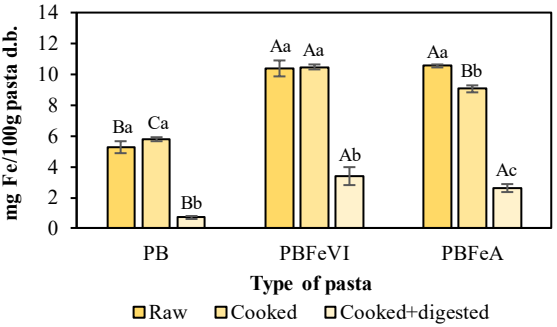


**Figure 2.** Iron content of fresh 100 % broad bean pasta before and after cooking, and after cooking and *in vitro* gastrointestinal digestion. PB, pasta of broad bean flour; PBFeVI, pasta of iron-fortified broad bean flour by vacuum impregnation; PBFeA, pasta of iron-fortified flour by addition.
Results are shown as mean (n = 3) ± standard deviation. Different capital letters indicate significant differences between different types of pasta ($p < 0.05$), and lowercase letters indicate significant differences between raw, cooked, and cooked and digested pasta ($p < 0.05$).

No studies have been found that evaluate iron loss during cooking of iron-fortified legume pasta, but a similar trend was observed in cereal-based pasta. Tazrart et al. (2016) determined the iron losses after cooking maccheroncini pasta made with 50 % broad bean flour, proving that there was no effect of cooking and that the iron content remained constant. Nevertheless, Kumari et al. (2023) fortified a pasta based on apple pomace and finger millet with iron and ascorbic acid, reporting a decrease in iron concentration after cooking. The reason why PBFeVI did not lose iron during cooking and behaved similarly to PB could be because iron would be integrated into the cellular structure of beans during the fortification process, which would help its retention during processing and cooking. Furthermore, the iron content of cooked pasta after GID was significantly ($p < 0.05$) lower than the content before digestion in all types of pasta (Figure 2). Broad beans contain anti-

nutritional factors such as phytic acid and tannins that can form complexes with iron during digestion and hinder its subsequent absorption (Man et al., 2021; Shubham et al., 2020). However, the fortification process increased the percentage of iron bioaccessibility. As shown in Figure 3, PB had a 12 % bioaccessible iron, whereas PBFeVI and PBFeA reached values of 32 % and 29 %, respectively.

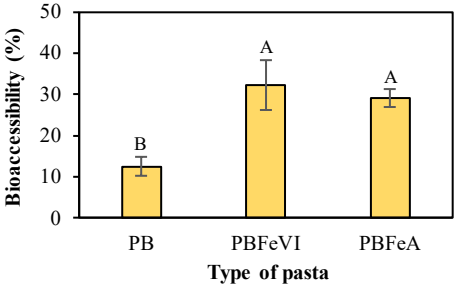


**Figure 3.** Iron bioaccessibility of cooked fresh 100 % broad bean pasta. PB, pasta of broad bean flour; PBFeVI, pasta of iron-fortified broad bean flour by vacuum impregnation; PBFeA, pasta of iron-fortified flour by addition.
Results are shown as mean (n = 3) ± standard deviation. Different capital letters indicate significant differences between different types of pasta ($p < 0.05$).

Although no studies have been found evaluating the iron bioaccessibility in legume- or cereal-based fortified pasta, similar results were observed in several works carried out on iron-fortified cereal-based products. Tripathi and Platel (2013) fortified sorghum and pearl millet flours with iron and iron + EDTA, observing in both cases an increase in the iron bioaccessibility in the flour and derived products. Bryszewska et al. (2019) showed an increase in iron bioaccessibility and transport efficiency in wheat bread fortified with microencapsulated iron. Similarly, Rebellato et al. (2017a) and Rebellato et al. (2017b) investigated the effect of different iron compounds used to fortify wheat flour for making whole wheat roll bread and French bread, respectively, observing that NaFeEDTA increased the iron bioaccessibility compared to control and samples fortified with ferrous sulphate, ferrous fumarate, reduced iron, or microencapsulated ferrous compounds. Therefore, the fact that in this study fortified flours had a higher percentage of

bioaccessible iron could be related to the use of NaFeEDTA as iron fortification compound.

## 4. CONCLUSIONS

Iron-fortification did not affect the stability of broad bean flours during 7 weeks of storage, although higher lipid and protein oxidation was observed when vacuum impregnation was applied compared to conventional fortification. However, the use of vacuum impregnation to fortify the broad bean flour prevented the loss of added iron during the cooking of a pasta made from 100 % broad bean flour and increased its bioaccessibility, probing to be an interesting alternative for obtaining fortified flours. Additional studies could be carried out to confirm that the oxidation level remains stable throughout the shelf life of the product and to ensure that no undesirable organoleptic alterations occur at the sensory level.

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**Author contributions: CRediT**

Milagros Arnal: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. Marta Gallego: Writing – review & editing, Supervision, Conceptualization. Leticia Mora: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. Pau Talens: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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# Capítulo 3

## Harinas hidrolizadas con enzimas

**Artículo 5**

Arnal, M., Gallego, M., Mora, L., & Talens, P. (2025). Antinutritional factors and protein digestibility of broad bean flours hydrolysed during soaking using vacuum enzyme impregnation. *Food Research International*, *199*, 115353.

# Antinutritional factors and protein digestibility of broad bean flours hydrolysed during soaking using vacuum enzyme impregnation

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**Abstract**

The hydrolysis of legume proteins improves their nutritional and functional properties. Usually done by mixing flour with an enzyme solution, the process can be simplified using vacuum enzyme impregnation during soaking. This study used vacuum impregnation with papain or bromelain to obtain hydrolysed broad bean flours. It examined the impact of vacuum impregnation and enzyme incorporation on hydration kinetics and the impact of hydrolysis and dehulling on antinutritional factors and protein digestibility. Vacuum impregnation accelerated hydration and enzyme incorporation did not alter the hydration rate. Maximum hydrolysis degrees were 9.1 % with papain and 8.8 % with bromelain after 4 h of soaking. Hydrolysis increased phytic acid, total phenolics, and tannins content while decreasing trypsin inhibitors. Dehulling increased phytic acid and trypsin inhibitors, reduced tannins, and enhanced protein digestibility. Vacuum enzyme impregnation during soaking was effective for hydration and protein hydrolysis, modifying the nutritional properties of broad bean flours.

*Keywords: legumes, hydrolysed flours, vacuum impregnation soaking, papain, bromelain.*

## 1. INTRODUCTION

Broad beans (*Vicia faba*), which belong to the *Leguminosae* (or *Fabaceae*) family, are rich in nutrients such as proteins, carbohydrates, dietary fibre, and minerals (Bessada et al., 2019). They contain an average protein content of 26 % (Bessada et al., 2019; Dhull et al., 2021), making them one of the most affordable sources of protein in developing countries (Rahate et al., 2021). However, the digestibility of these proteins is generally lower than that of animal proteins mainly due to the protein structure and the presence of some antinutritional factors such as trypsin inhibitors, phytic acid, and condensed tannins, among others (Bessada et al., 2019). These compounds can be effectively reduced through bioprocessing techniques such as sprouting or fermentation, as well as through simple treatments like soaking, cooking, autoclaving, or dehulling (Rahate et al., 2021). Another interesting treatment could be the enzymatic hydrolysis of legume proteins, which has been described as an alternative to improve their nutritional and functional properties (Gouseti et al., 2023; Mookerjee & Tanaka, 2023). A variety of proteases derived from plants, animals, microorganisms, and fungi are commercially available for hydrolysis. Among those of plant origin, papain and bromelain are two of the most widely used enzymes, obtained from papaya and fruit or steam of pineapple, respectively (Gouseti et al., 2023). For example, Konieczny et al. (2020) hydrolysed commercial pea protein-enriched flour with papain and reduced the contents of condensed tannins and total phenolics, as well as trypsin and chymotrypsin inhibitory activity. Goertzen et al. (2021) hydrolysed isolated chickpea protein with papain and reduced trypsin and chymotrypsin inhibitory activities. Therefore, up to now, the hydrolysates have been commonly obtained by blending flour or protein isolate with an enzyme solution, followed by an incubation period and a drying step (Eckert et al., 2019; Goertzen et al., 2021; Konieczny et al., 2020; Mokni Ghribi et al., 2015; Samaei et al., 2020). However, in the case of legumes, the hydrolysis process could be simplified using vacuum enzyme impregnation during soaking. Soaking is a batch and time-consuming part of the processing, but it can be reduced significantly using vacuum impregnation. This process

involves depressurisation and subsequent re-establishment of atmospheric pressure in a solid product-solution system. During depressurisation, the air is expelled from the product's pores, and upon return to atmospheric pressure, the solution enters the pores due to the pressure gradient (Zanella-Díaz et al., 2014). In a previous study, it was found that using vacuum impregnation when soaking broad beans could reduce the soaking time to about 12 h (Arnal et al., 2023). This fact allows considering the use of an enzyme solution as a soaking medium to hydrolyse bean proteins during soaking.

This study aimed to obtain hydrolysed broad bean flours through the incorporation of papain or bromelain using vacuum impregnation during soaking. The impact of vacuum impregnation and enzyme incorporation on the hydration kinetics, and the effect of the degree of hydrolysis and dehulling on the content of antinutritional factors and protein digestibility of the flours was evaluated.

## 2. MATERIALS AND METHODS

### 2.1. Samples and reagents

Broad beans to obtain flour were purchased from an online supermarket (Pozuelo de Alarcón, Madrid, Spain). The enzymes bromelain 2000 and papain 30000 used to hydrolyse the flour were acquired from Cygyc Biocon S.L. (Les Franqueses del Vallés, Barcelona, Spain).

The reagents used were trinitrobenzenesulfonic acid (TNBS), sodium phosphate, L-Leucine, sulfosalicylic acid, sodium carbonate, tannic acid, phytic acid, Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA), trypsin from bovine pancreas (T8003), α-amylase (A3171) and pancreatin (P7545) from porcine pancreas, pepsin from porcine gastric mucosa (P7012) and, bovine bile extract (B3883) from Sigma Aldrich Co. (St Louis, MO, USA); sodium biphosphate, tris-(hydroxymethyl)-aminomethane, calcium chloride dihydrate, dimethyl sulfoxide (DMSO) and, gallic acid from Scharlau Chemie S.A. (Sentmenat, Barcelona, Spain); ferric

chloride from MP Biomedicals LLC, (Solon, OH, USA); and Folin-Ciocateu reagent from Panreac Química S.L.U. (Castellar del Vallès, Barcelona, Spain). All other reagents and chemicals were of analytical grade.

### 2.2. Broad bean hydration kinetics during soaking at 55 °C

The hydration rate of the broad beans during soaking at 55 °C was determined at atmospheric pressure (atmospheric soaking, AS) and after using 5 min of vacuum impregnation during soaking (vacuum impregnation soaking, VS). In both cases, broad beans were soaked in distilled water at a ratio of 1:3 (w/v). For VS, the beans were soaked in vacuum for the first 5 min, and then the atmospheric pressure was restored for the remaining time. In this case, broad beans were soaked for 4 h and the water gain was measured immediately after 5 min of vacuum impregnation, at intervals of 10 min for the first hour and every 30 min for the remaining 3 h. Likewise, for AS, the beans were immersed in distilled water for 10 h, and the water absorbed was measured at intervals of 1 h for the first 6 h and at intervals of 2 h for the remaining 4 h. After soaking, broad beans were weighed. The hydration rate was calculated using Equation 1.

$$H_t(\%) = [(M_t - M_0)/M_0] \times 100 \quad (1)$$

where $H_t$ is the water absorbed at time t, $M_t$ is the weight of the sample at time t, and $M_0$ is the initial weight of the sample.

To study the effect of enzyme incorporation on the hydration kinetics, the water of the soaking medium was replaced by an enzymatic solution. So, the VS was also performed using 0.01 g/mL papain solution or 0.01 g/mL bromelain solution, which were the concentrations recommended by the supplier. All soaking tests were carried out in triplicate, and independent samples (30 g of dry broad beans) were prepared each time.

### 2.3. Processing of hydrolysed broad bean flours

To obtain the hydrolysed broad bean flours, the broad beans were soaked (1:3, w/v) using VS with 0.01 g/mL papain or bromelain solutions and incubated for 1, 2, 4 or 6 h at 55 °C. The temperature was selected considering the optimal working temperature range of both enzymes. The pH of the solutions was between 5 and 6, so it was comprised in the optimal range for the action of the enzymes. After the incubation period, the soaking medium was drained, and the beans were cooked by steaming using an autoclave for 10 min at 121 °C to inactivate the enzymes. After cooking, half of each batch was dehulled and all broad beans were dried in a convection dryer at 55 °C until constant weight. Finally, broad beans were milled using a Mixer Mill MM400 (Retsch GmbH, Haan, Germany). In parallel, control flours were prepared by soaking the broad beans in water as previously described in section 2.2. A total of 24 types of flour were obtained, which are beans soaked in water (control), beans soaked in 0.01 g/mL papain solution, and beans soaked in 0.01 g/mL bromelain solution, at different soaking times (1, 2, 4 or 6 h), and with and without hull. Two batches per flour were processed.

### 2.4. Determination of the degree of protein hydrolysis of flour

The degree of protein hydrolysis (DH) is the proportion of peptide bonds cleaved during hydrolysis and was determined using the method of Adler-Nissen (1979) by the reaction of TNBS with free amino groups (Rutherfurd, 2010). Briefly, 40 µL of sample or standard, was mixed with 320 µL of sodium phosphate buffer (0.2 mol/L, pH 8.2) and 320 µL of TNBS (3.41 mmol/L), and incubated at 50 °C for 1 h. Then, to stop the reaction, 640 µL of HCl 0.1 mol/L was added and left for 30 min to cool down. The absorbance was measured at 340 nm and results were expressed as mg L-leucine/g sample. The DH was calculated as shown in the Equation 2.

$$DH\ (\%) = [(h - h_0)/(h_{tot} - h_0)] \times 100 \quad (2)$$

where $h$ is the concentration of amino groups in the hydrolysed sample, $h_0$ is the concentration of amino groups in the sample without hydrolysis, and $h_{tot}$

is the concentration of amino groups in the sample hydrolysed with 6 mol/L HCl at 100 °C for 24 h.

## 2.5. Determination of antinutritional factors of broad bean flours

### *2.5.1. Phytic acid content*

The determination of phytic acid content was performed according to Embaby (2010) with some modifications. For the phytic acid extraction, 0.1 g of broad bean flour was mixed with 5 mL of HCl (2.4 g/100 g) and agitated at room temperature for 1 h. Then, the samples were centrifuged at 6000 *g* and 4 °C for 10 min, and the supernatant was recovered. A total of 250 µL of Wade reagent (solution of 0.3 mg/mL of $FeCl_3 \cdot 6H_2O$ containing 3 mg/mL sulfosalicylic acid in water) was added to 750 µL of supernatant, and the mixture was centrifuged at 7000 *g* for 10 min. The absorbance of the supernatants was measured at 500 nm using a UV-Visible spectrophotometer (Helios Zeta, Thermo Scientific, UK), and the results were expressed as mg phytic acid/ g flour.

### *2.5.2. Trypsin inhibitors content*

The trypsin inhibitors were determined based on the method of Hamerstrand et al. (1981) with some modifications. So, 1 g of broad bean flour was mixed with 50 mL of 5 mmol/L NaOH and incubated with agitation at room temperature for the extraction of trypsin inhibitors. After 3 h of agitation, samples were centrifuged at 8000 *g* for 10 min and the supernatants were collected. Later, BAPNA substrate was prepared dissolving 40 mg BAPNA in 1 mL DMSO and diluted to 100 mL with Tris-buffer (0.05 M, pH 8.2 with 0.02 mol/L $CaCl_2$), and prewarmed to 37 °C. Trypsin standard solution was prepared at 0.02 mg/mL with HCl. For determination of trypsin inhibitors, 0.4 mL distilled water as reagent blank, 0.4 mL trypsin standard solution + 0.4 mL distilled water as standard, 0.2 mL sample + 0.2 mL distilled water as sample blank, and 0.2 mL sample + 0.2 mL distilled water + 0.4 mL trypsin standard solution as sample, were prepared. The four samples were incubated at 37 °C for 10 min and then, 1 mL of BAPNA substrate was added to each

sample and incubated again for 10 min. To stop the reaction, 0.2 mL of acetic acid (30 mL/100 mL) was added to each sample and 0.4 mL trypsin standard solution was added to the reagent blank and sample blank. Finally, the samples were centrifuged at 8000 *g* for 10 min, and the absorbance of supernatants was measured at 410 nm. The results were calculated as shown in Equation 3 and expressed as mg trypsin inhibitors/g sample.

$$mg\ tripsin\ inhibitors/g\ sample = \frac{[(A_{ST} - A_{RB}) - (A_S - A_{SB})] \times D \times V_E}{0.019 \times g \times 1000 \times V_S} \quad (3)$$

where $A_{ST}$ is the absorbance of the standard, $A_{RB}$ is the absorbance of the reagent blank, $A_s$ is the absorbance of the sample, $A_{SB}$ is the absorbance of the sample blank, D is the dilution factor, $V_E$ is the extraction volume, g are the grams of sample, and $V_S$ is the sample volume used to the determination.

*2.5.3. Total phenolic content*

Total phenolic content was determined following the method of Teixeira-Guedes et al. (2019). For the extraction, 2 mL of methanol (80 mL/100 mL) was added to 0.2 g of broad bean flour, and incubated for 24 h at room temperature in the dark with stirring. The samples were then centrifuged at 8000 *g* for 10 min, and the supernatants were collected. For the assay, in triplicate, 20 µL of extracts or standard was mixed with 100 µL of Folin-Ciocalteu reagent and 80 µL of 75 mg/mL of sodium carbonate. The mixture was incubated at 42 °C for 30 min and after that, the absorbance was measured at 765 nm using a VANTAstar microplate reader (BMG Labtech, Ortenberg, Germany). The results were expressed as mg gallic acid/ g flour.

*2.5.4. Tannin content*

The AOAC standardised method was used to determine the tannin content (Embaby, 2010). For tannin extraction, 0.2 g of broad bean flours was mixed with 70 mL acetone (70 mL/100 mL) and incubated with agitation for 24 h at room temperature. Then, samples were centrifuged at 8000 *g* for 10 min, and the supernatants were collected. To determine the content of tannins, 0.1 mL of supernatant was mixed with 1.6 mL of distilled water, 0.2 mL of sodium

carbonate (0.2 g/mL), and 0.1 mL of Folin–Ciocalteu reagent, and the mixture was incubated for 30 min. Then, the absorbance was measured at 760 nm, and the results were expressed as mg tannic acid/ g flour.

### 2.6. Determination of *in vitro* protein digestibility

The in *vitro* protein digestibility was determined by measuring the DH after *in vitro* gastrointestinal digestion (GID) as described by Purwandari et al. (2023). For that, the free amino groups after GID were determined by the TNBS method as described in the section 2.4. The DH was calculated as shown in the Equation 2 but, in this case, $h$ was the concentration of amino groups in the sample after GID, and $h_0$ was the concentration of amino groups in the sample before GID.

The GID was performed, in duplicate, following the INFOGEST protocol (Brodkorb et al., 2019; Minekus et al., 2014). First of all, simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were prepared according to the protocol. To simulate the oral phase, 0.5 g of flour was mixed with 0.5 g of water, and the resulting paste was diluted 1:1 (w/v) with SSF that contained α-amylase enzyme at 1.25 μkat/mL. After 2 min under constant gentle mixing (Intell-MixerTM RM-2, ELMI Ltd., Riga, Latvia) at 37 °C (Incubator chamber, JP Selecta, S.A., Barcelona, Spain), the oral phase was stopped by decreasing the pH to 3 with 2 mol/L HCl. The gastric phase began by mixing the oral bolus with SGF, which contained pepsin (33.3 μkat/mL), in 1:1 (v/v) ratio. After 120 min of incubation with the same conditions as the oral phase, the pH was increased to 7 with 1 mol/L NaOH to terminate the gastric phase. To complete the intestinal phase, the gastric chyme was diluted 1:1 (v/v) with SIF containing pancreatin (based on trypsin activity to achieve 1.67 μkat/mL) and bovine bile extract (10 mmol/L), and was incubated for 120 min as described in the previous phases. The digestion was stopped by heating the samples at 100 °C for 5 min and immediately cooling them in ice. Finally, the samples were centrifuged at 8000 *g* and 4 °C for 10 min, and the content of free amino groups was

determined in the supernatants. Sample blanks with all enzymes and bile (without sample) were also performed for subtracting the obtained values from the results of samples.

### 2.7. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's honest significance tests was performed using Statgraphics Centurion XIX software (Statgraphics Technologies, Inc., The Plains, VA, USA) to evaluate the differences of means between control and hydrolysed broad bean flours, soaking-hydrolysis times, and dehulled and non-dehulled flours. Results were expressed as the mean of replicates ± standard deviations, and differences were considered significant at $p < 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of vacuum impregnation and enzyme solution on broad beans hydration kinetics

The process of soaking beans using enzyme solutions to obtain hydrolysed flours was carried out at 55 ºC, which is in the optimal temperature range for both papain and bromelain. So, the hydration kinetics of broad beans were studied at 55 °C under atmospheric pressure (AS) and applying vacuum impregnation (VS) (Figure 1A). The results showed that water absorption increased until moisture equilibrium was reached, but the hydration kinetics varied according to the type of soaking (AS or VS). The hydration kinetics of broad beans with AS had a slightly sigmoidal shape, whereas the broad beans with VS had a downward concave shape. The same trend of the results was obtained in our previous study where the broad beans were soaked at room temperature (Arnal et al., 2023). However, increasing the temperature of the soaking medium accelerated the hydration kinetics of broad beans in both types of soaking. Comparing both studies, after 60 min of soaking, atmospheric-soaked broad beans had a hydration rate of 15 % at room temperature whereas 32 % was reached at 55 °C. Similarly, broad beans with VS

had a hydration rate of 86 % at room temperature and 102 % at 55 °C. This observed increase in the hydration rate may be attributed to several factors, such as acceleration of reaction rates, expansion of tissues and pores, reduction of water viscosity improving capillary flow, or partial dissolution of certain components leading to an increase in pore size (Miano & Augusto, 2018). Nevertheless, it seems that temperature could increase or decrease the water hydration capacity of the broad beans. With AS, broad beans soaked at room temperature reached a hydration rate of 115 %, whereas broad beans soaked at 55 °C reached a value of 105 %. Similar results were obtained by Li et al. (2020) and Miano et al. (2018), who studied the hydration kinetics of common beans and black beans, respectively. The decrease in the hydration capacity of legumes could be due to several reasons such as excessive loss of water-soluble components, damage to cell membranes and walls, or decrease in the driving force of mass transfer caused by the quick saturation of the outer layer of grains with water (Miano & Augusto, 2018). Different results were obtained with VS since the temperature increased the hydration capacity of broad beans. A hydration rate of 100 % was achieved at room temperature, whereas it increased to 110-120 % at 55 °C. The observed increase in hydration capacity could be due to increased capacity to hold water by expanding the pores and internal spaces, or by opening the pores due to increased solubility of components (Miano & Augusto, 2018).

To evaluate the effect of enzyme solutions on hydration kinetics, the VS was repeated using 0.01 g/mL papain or bromelain solutions. Figure 1B displays the results obtained by comparing VS in water with the enzyme solutions, showing that the incorporation of enzymes into the soaking medium did not significantly affect the hydration kinetics of the broad beans. The hydration curves were similar in the three cases, so the soaking step could be used not only to hydrate the legumes but also to hydrolyse broad bean proteins in shorter times.

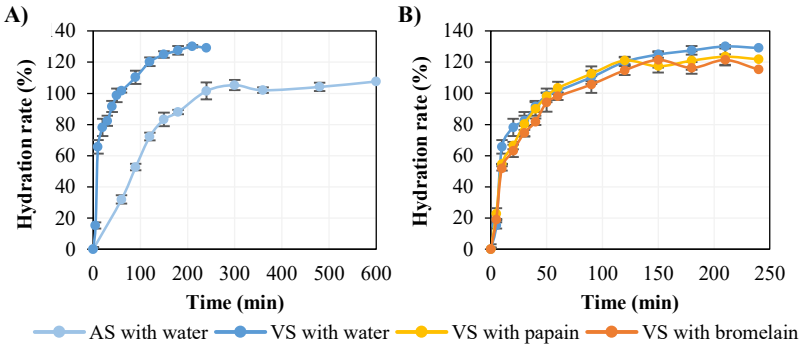


**Figure 1.** Hydration curves of dry broad beans during soaking at 55 ◦C. (A) Water absorption of broad beans during atmospheric pressure soaking (AS) and vacuum impregnation soaking (VS). (B) Water or enzymatic solution absorption of broad beans during VS.

### 3.2. Effect of soaking-hydrolysis on DH of broad bean flour

The objective of soaking broad beans with enzymes was to achieve a low to moderate degree of protein hydrolysis, which has been reported to cause positive changes in the functional and nutritional properties of plant proteins (Gouseti et al., 2023; Mookerjee & Tanaka, 2023).

Broad beans were soaked in 0.01 g/mL papain or bromelain solution for 1, 2, 4, and 6 h, and then cooked, dried, and ground into flour. Another batch was soaked in water to evaluate the DH of the flours. As can be seen in Figure 2, the DH increased as the soaking time increased. The DH obtained with papain was almost double than that obtained with bromelain during the first and second hours of soaking. However, the DH was similar after 4 h of soaking, reaching a maximum of 9.1 % with papain and 8.8 % with bromelain. After 6 h of soaking, the DH remained constant or decreased slightly. Papain was the most active enzyme during the first 2 h of soaking since differences in the accessibility and specificity of both enzymes towards substrate would affect the DH obtained. However, both papain and bromelain are endopeptidases and have structural and chemical similarities (Mazorra-Manzano et al., 2018; Gurumallesh et al., 2019), which ultimately results in hydrolysed flours with comparable DH values.

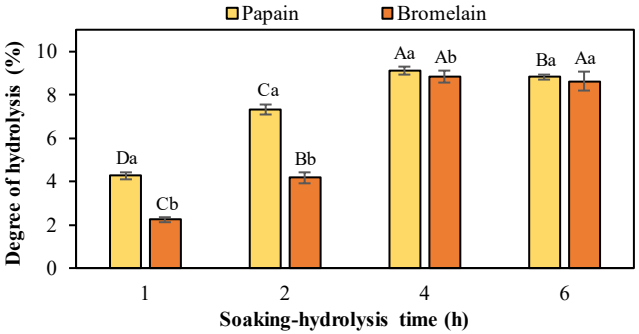


**Figure 2.** Degree of protein hydrolysis of broad bean flours hydrolysed with papain and bromelain during soaking.
Capital letters indicate significant differences between the time of soaking-hydrolysis within flour with the same enzyme soaking, whereas lowercase letters indicate significant differences between types of enzyme soaking within the same time of soaking-hydrolysis.

To date, enzymatic hydrolysates of legume proteins have been obtained by mixing the flour or protein isolate with enzyme solutions. So, Eckert et al. (2019) hydrolysed protein isolated from broad bean with pepsin, trypsin, flavourzyme, and neutrase at an E/S ratio of 1/20 and optimum pH and temperatures, obtaining DH values of 16.9 %, 9.9 %, 12.2 % and 6.4 %, respectively, after 60 min of hydrolysis. Ribéreau et al. (2018) hydrolysed yellow pea flour using the enzymes papain and bromelain, at pH of 6.5 and 8 and E/S ratios of 1/50 and 1/25, respectively, reaching DH values of 17.5 % with papain and 10.2 % with bromelain after 5 min at 40 °C. Likewise, Konieczny et al. (2020) hydrolysed pea protein-enriched flour with papain (pH 6.2, E/S 1/2500, 45 °C) for 40 min, obtaining a DH of 11.3 %. The DH values reported in these studies were higher than those obtained in the present work, probably because the enzymes would have easier access to the substrate in flours or protein isolates. It should also be noted that, in this case, the hydrolysis took place during the soaking of the legume, when the seeds were whole, which would initially hinder the activity of the enzyme. Furthermore, the differences in the DH values could be attributed to variations in the legume type, enzyme activity and accessibility, E/S ratio, and hydrolysis conditions (time, temperature, and pH) (Vogelsang-O'Dwyer et al., 2022).

### 3.3. Effect of the soaking-hydrolysis and dehulling on the antinutritional factors of broad bean flours

Some antinutritional factors present in legumes can interact with proteins and reduce their digestibility. However, it has been shown that enzymatic hydrolysis could improve their nutritional value (Gouseti et al., 2023; Mookerjee & Tanaka, 2023). In the present study, the contents of phytic acid, trypsin inhibitors, total phenolic, and tannin were determined in the control broad bean flours and in those hydrolysed with papain or bromelain (Table 1).

The phytic acid content (Table 1) of the flours ranged from 18.4 to 26.2 mg /g flour. The use of enzymes during soaking decreased or increased the content of phytic acid depending on the soaking time. After 1 h of soaking, the amount of phytic acid in the hydrolysed flours decreased compared to control flours, but it increased after 2 and 4 h of soaking. Phytic acid can bind to proteins and form complexes (Bessada et al., 2019; Dhull et al., 2021). However, the enzymatic hydrolysis could affect these complexes and favour the release of phytic acid (Ribéreau et al., 2018), and once it is in free form, it could be more easily leached into the soaking medium. Therefore, after 1 h of soaking, when the DH was still low, the phytic acid released have would been leach into the soaking medium. However, when the DH increased after 2 h of soaking, there would be a higher proportion of phytic acid released than leached into the medium, resulting in an increase in phytic acid content. No statistically significant differences were observed between 2 and 4 h of soaking, probably because more compound was leached into the soaking medium than released by protein hydrolysis. Ribéreau et al. (2018) also observed an increase in phytic acid content after the hydrolysis of pea flours with papain and bromelain. In addition, results showed that dehulling broad beans before obtaining flours significantly increased the phytic acid content. The flours with hull presented values between 18.4 and 25.5 mg phytic acid/g flour, while the flours without hull had values between 19.2 and 26.2 mg/g. Phytic acid is evenly distributed in the cotyledon, so its content would

increase when the hull is removed (Luo & Xie, 2013). Similar results were reported by Luo et al. (2010), who observed a higher phytic acid content in dehulled broad beans than in those with hulls.

Regarding trypsin inhibitors (Table 1), the content of the control flours ranged from 3.5 to 5.7 mg/g. However, the trypsin inhibitor content of the flours decreased from 4.2 to 2.6 mg/g after hydrolysis with papain and from 4.8 to 2.8 mg/g after hydrolysis with bromelain. In all cases, hydrolysed flours (with and without hull) had a lower trypsin inhibitor content than control flours. The observed reduction could be due to the hydrolytic activity of the enzymes on trypsin inhibitors or to the effect of heat, since they would be more exposed when proteins are hydrolysed and consequently more susceptible to denaturation (Goertzen et al., 2021; Konieczny et al., 2020; Ribéreau et al., 2018). Furthermore, protein hydrolysis would facilitate the leaching of trypsin inhibitors into the soaking medium (Konieczny et al., 2020). The reduction in trypsin inhibitor content became more significant as the soaking time increased, which would correspond to an increase in DH. This fact was observed in all cases except for flours hydrolysed with papain for 4 h (with and without hull), where the content remained constant. During soaking with papain, the DH increased significantly after 1 and 2 h , but not as much after 4 h of soaking compared to the hydrolysis with bromelain. Therefore, a greater amount of trypsin inhibitors may have been hydrolysed or leached into the soaking medium during the first hours of soaking. Several authors have reported similar results. Ribéreau et al. (2018) observed a reduction in trypsin inhibitors in yellow pea flours hydrolysed with bromelain, papain, and trypsin in comparison to non-hydrolysed flours. Konieczny et al. (2020) used pepsin, papain, trypsin, and savinase enzymes to obtain different hydrolysates of pea protein enriched flour, showing that only the type of enzyme, and not the DH, had a significant impact on the reduction of trypsin inhibitors. Goertzen et al. (2021) also observed a reduction in trypsin inhibitor content in chickpea protein isolate hydrolysed with pepsin, papain, and trypsin, but in this case, it was significantly influenced by both enzyme type and DH. In addition, it can be observed that all the flours without hull had a higher trypsin

inhibitor content than flours with hull. Like phytic acid, trypsin inhibitors are mostly located in the cotyledon of broad beans (Avilés-Gaxiola et al., 2018), so removing the hull would lead to an increase in these compounds.

Total phenolics were also determined in the control and hydrolysed flours (Table 1). The content of these compounds ranged from 1.3 to 2.9 mg gallic acid/g flour, with an increase in the hydrolysed flours compared to the control. These results suggest that hydrolysis of proteins would break the covalent bonds formed between phenolics and proteins (Ribéreau et al., 2018), leaving the phenolic compounds free but retained in the structure of broad beans. Furthermore, flours treated with papain showed the highest total phenolic content, which could be related to the higher DH obtained in these flours. It was also observed that the content of phenolic compounds in the control flours decreased as the soaking time increased, which could be due to the leaching of phenolic compounds into the soaking medium (Luo & Xie, 2013). Siah et al. (2014) reported a reduction in total phenolic content after soaking different varieties of broad beans in water for 12 h. Nevertheless, no significant changes were observed in the total phenolic content of hydrolysed flours with increasing soaking time, except for a slight increase after 4 h of soaking in the papain hydrolysed flours without hull and in the bromelain hydrolysed flours with hull. Similar results were obtained by Ribéreau et al. (2018) in yellow pea flour hydrolysates. However, the hydrolysed flours obtained by Konieczny et al. (2020) and Goertzen et al. (2021) had a lower content of total phenolic content than the untreated flours, which could be because they were obtained from protein-rich flour or protein isolate. On the other hand, in general, no significant differences were observed between samples with and without hull. These results were unexpected because the hull contains a large amount of phenolic compounds (Rahate et al., 2021), so they would be reduced by removing the hull. However, the amount of phenolics remained constant, possibly because some of these compounds could have diffused into the cotyledon during cooking, which was carried out before the dehulling process.

Finally, the tannin content was also determined in the different flour hydrolysates (Table 1). Tannins are a type of phenolic compounds found mainly in the hull of legumes, which can form complexes with polysaccharides, alkaloids, and proteins (Singh et al., 2017). The content of these compounds in the control and hydrolysed flours ranged from 3 to 6.9 mg tannic acid/g sample. The flours treated with papain or bromelain presented a higher tannin content than the control flours. Specifically, flours treated with papain had the highest tannin content, which could be due to these samples reached a higher DH than those treated with bromelain, as discussed above. Moreover, the tannin content was influenced by the soaking time. In the control flours, the tannin content decreased after 2 h of soaking and remained constant after 4 h, suggesting that these compounds leached into the soaking medium during the first 2 h of soaking (Luo et al., 2010). These results agree with Luo and Xie (2013), which reported that the tannin content of two varieties of broad beans remained constant after 12, 24 and 36 h of soaking. In the hydrolysed flours, the tannin content remained constant after 1 and 2 h of soaking but increased significantly after 4 h of soaking. These results could be explained by the hydrolysis of proteins that would form complexes with tannins, so that tannins would be released and leached into the soaking medium during the first 2 h. However, more tannins were released than leached after 2 h, resulting in an increased content at 4h of soaking. In other studies on legume hydrolysates obtained from flours or protein isolates, the concentration of tannins was not detectable after hydrolysis, but different methods of determination were used (Goertzen et al., 2021; Konieczny et al., 2020). Finally, dehulling reduced the content of tannins in both control and hydrolysed flours, which could be because tannins are mainly located in the hull, as mentioned above (Singh et al., 2017).

**Table 1.** Degree of protein hydrolysis, content of antinutritional factors, and protein digestibility of control and hydrolysed broad bean flours with and without hulls.

| Type of flour | Soaking hydrolysis time (h) | Hull | Hydrolysis degree (%) | Phytic acid (mg/g) | Trypsin inhibitors (mg/g) | Phenolic compounds (mg/g) | Tannins (mg/g) | Protein digestibility (%) |
|---|---|---|---|---|---|---|---|---|
| Control | 1 | - | - | $22.2 \pm 0.2^{Ba*}$ | $5.68 \pm 0.08^{Aa*}$ | $1.72 \pm 0.06^{Ac}$ | $3.74 \pm 0.09^{Ac*}$ | $78 \pm 3^{Ab*}$ |
| | | H | | $21.3 \pm 0.4^{Ba*}$ | $4.16 \pm 0.12^{Aa*}$ | $1.70 \pm 0.06^{Ac}$ | $4.16 \pm 0.11^{Ac*}$ | $70 \pm 2^{Aa*}$ |
| | 2 | - | - | $23.2 \pm 0.3^{Ac*}$ | $4.87 \pm 0.11^{Ba*}$ | $1.46 \pm 0.07^{Bc}$ | $2.96 \pm 0.15^{Bc*}$ | $78 \pm 3^{Aa*}$ |
| | | H | | $22.4 \pm 0.6^{Ab*}$ | $3.67 \pm 0.04^{Ba*}$ | $1.44 \pm 0.11^{Bc}$ | $3.65 \pm 0.11^{Bc*}$ | $69 \pm 4^{Aa*}$ |
| | 4 | - | - | $23.4 \pm 0.2^{Ab*}$ | $4.39 \pm 0.03^{Ca*}$ | $1.37 \pm 0.05^{Bc*}$ | $2.95 \pm 0.08^{Bc*}$ | $80 \pm 2^{Aa*}$ |
| | | H | | $21.9 \pm 0.5^{ABc*}$ | $3.50 \pm 0.08^{Ca*}$ | $1.30 \pm 0.05^{Cc*}$ | $3.48 \pm 0.19^{Bc*}$ | $70 \pm 1^{Aa*}$ |
| Papain | 1 | - | $4.3 \pm 0.2^{Ca}$ | $19.2 \pm 0.3^{Bc*}$ | $4.19 \pm 0.12^{Ac*}$ | $2.58 \pm 0.08^{Ba}$ | $5.77 \pm 0.17^{Ba*}$ | $79 \pm 3^{Aab*}$ |
| | | H | | $18.4 \pm 0.3^{Bc*}$ | $2.82 \pm 0.05^{Ac*}$ | $2.62 \pm 0.18^{Aa}$ | $6.32 \pm 0.16^{Ba*}$ | $66 \pm 3^{Ba*}$ |
| | 2 | - | $7.3 \pm 0.2^{Ba}$ | $24.3 \pm 0.4^{Ab*}$ | $3.61 \pm 0.07^{Bc*}$ | $2.65 \pm 0.16^{Ba}$ | $6.03 \pm 0.12^{Ba}$ | $76 \pm 4^{ABa*}$ |
| | | H | | $23.1 \pm 0.3^{Ab*}$ | $2.62 \pm 0.13^{Bc*}$ | $2.68 \pm 0.07^{Aa}$ | $6.23 \pm 0.20^{Ba}$ | $70 \pm 2^{Aa*}$ |
| | 4 | - | $9.1 \pm 0.2^{Aa}$ | $23.7 \pm 0.6^{Ab*}$ | $3.59 \pm 0.12^{Bc*}$ | $2.87 \pm 0.12^{Aa*}$ | $6.40 \pm 0.21^{Aa*}$ | $71 \pm 3^{Bb}$ |
| | | H | | $22.9 \pm 0.3^{Ab*}$ | $2.56 \pm 0.09^{Bc*}$ | $2.71 \pm 0.12^{Aa*}$ | $6.88 \pm 0.18^{Aa*}$ | $70 \pm 1^{Aa}$ |
| Bromelain | 1 | - | $2.2 \pm 0.1^{Cb}$ | $20.3 \pm 0.3^{Bb*}$ | $4.81 \pm 0.13^{Ab*}$ | $2.20 \pm 0.12^{Ab}$ | $4.72 \pm 0.12^{Bb*}$ | $83 \pm 3^{Aa*}$ |
| | | H | | $19.6 \pm 0.3^{Bb*}$ | $3.64 \pm 0.14^{Ab*}$ | $2.12 \pm 0.10^{Bb}$ | $4.97 \pm 0.23^{Bb*}$ | $62 \pm 3^{Bb*}$ |
| | 2 | - | $4.2 \pm 0.2^{Bb}$ | $26.2 \pm 0.6^{Aa}$ | $4.60 \pm 0.08^{Bb*}$ | $2.12 \pm 0.09^{Ab}$ | $4.61 \pm 0.14^{Bb*}$ | $77 \pm 4^{Ba*}$ |
| | | H | | $25.5 \pm 1.1^{Aa}$ | $3.44 \pm 0.16^{Bb*}$ | $2.20 \pm 0.07^{Bb}$ | $5.18 \pm 0.24^{Bb*}$ | $67 \pm 2^{Aa*}$ |
| | 4 | - | $8.8 \pm 0.3^{Ab}$ | $25.7 \pm 0.7^{Aa*}$ | $3.96 \pm 0.12^{Cb*}$ | $2.28 \pm 0.07^{Ab}$ | $6.02 \pm 0.10^{Ab*}$ | $75 \pm 5^{Bab*}$ |
| | | H | | $24.5 \pm 0.7^{Aa*}$ | $2.83 \pm 0.07^{Cb*}$ | $2.33 \pm 0.10^{Ab}$ | $6.23 \pm 0.10^{Ab*}$ | $64 \pm 3^{AB*}$ |

Capital letters indicate significant differences between the time of soaking within the same group of the type of soaking and with or without hull, lowercase letters indicate significant differences between the type of soaking within the same group of the time of soaking and with or without hull, and "*" indicates significant differences between with hull or without hull within the same group of the type and time of soaking ($p < 0.05$).

Thus, enzymatic hydrolysis during soaking resulted in hydrolysed flours with a higher content of phytic acid, phenolic compounds and tannins than control flours, which is an undesirable effect. To try to remove these antinutritional factors, the broad beans could be washed after soaking, as antinutritional factors may have remained on the legume surface, or cooked in water rather than steam, so that the antinutritional factors released during hydrolysis could leach into the cooking water.

### 3.4. Effect of the soaking-hydrolysis and dehulling on the *in vitro* protein digestibility of broad bean flours

The *in vitro* protein digestibility of the control and hydrolysed flours was determined to assess how it was affected by the soaking-hydrolysis and dehulling processes. The results, expressed as the DH obtained after the GID, are presented in Table 1. The DH for the control flours varied from 69.4 % to 79.6 %, whereas the DH for the hydrolysed flours ranged from 66 % to 79.4 % in those samples treated with papain and between 62 % and 82.8 % for those treated with bromelain. The hydrolysis of broad bean proteins during soaking did not seem to greatly affect the protein digestibility of the flours as the differences between control and enzyme-treated flours were, in general, not significant. Goertzen et al. (2021) also observed that *in vitro* protein digestibility of chickpea protein isolate was not changed after hydrolysis with pepsin, trypsin and papain. Nevertheless, dehulling broad beans significantly affected the protein digestibility of the flours. The flours with hull showed DH values between 62.0 and 70.4 %, while the flours without hull showed significantly higher DH, ranging from 71.3 to 82.8 %. Removing the hull significantly improved the digestibility of the samples, probably due to the elimination of antinutritional factors, such as tannins, mainly concentrated in the hull (Alonso et al., 2000). It has been reported that these factors can inhibit protein digestion by up to 28 % through the formation of tannin-protein complexes, direct inhibition of digestive proteases, or prevention of growth of gut microbiota (Ohanenye et al., 2020). Moreover, the protein digestibility decreased in the flours without hull as soaking time and DH increased, but

increased or remained constant in the flours with hull. The differences observed between flours could be due to the presence of antinutritional factors released by hydrolysis, but remaining in the flours. As previously mentioned, hydrolysed flours had a higher content of phytic acid, phenolic compounds, and tannins, as well as a lower content of trypsin inhibitors than the control flours. These antinutritional factors can interact with proteins and digestive enzymes during GID and lead to a reduction in their digestibility (Gu et al., 2023).

## 4. CONCLUSIONS

This study showed that the application of vacuum impregnation during soaking increased the hydration rate of broad beans. The incorporation of bromelain or papain enzymes in the soaking medium did not alter the hydration kinetics of the broad beans, but facilitated protein hydrolysis, reaching the maximum degree of hydrolysis after 4 h of soaking. The hydrolysis and dehulling process significantly affected the levels of antinutritional factors and protein digestibility in hydrolysed broad bean flours. So, protein hydrolysis led to increased contents of phytic acid, total phenolics, and tannins, while decreasing trypsin inhibitor levels. Additionally, dehulling increased the levels of phytic acid and trypsin inhibitor but reduced the tannin content, which would improve the protein digestibility. Thus, the application of vacuum enzyme impregnation during soaking opened the possibility of taking advantage of this processing stage for both legume hydration and protein hydrolysis, modifying the nutritional properties of the hydrolysed broad bean flours.

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**Author contributions: CRediT**

Milagros Arnal: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. Marta Gallego: Writing – review & editing, Supervision, Conceptualization. Leticia Mora: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. Pau Talens: Writing – review & editing, Visualization, Project administration, Funding acquisition, Conceptualization.

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# Discusión general

Las legumbres son una fuente excelente de proteínas que pueden convertirse en péptidos bioactivos tras un proceso de hidrólisis, como el que tiene lugar durante la DGI. Este proceso de hidrólisis dependerá de los tratamientos a los que hayan sido sometidas previamente las legumbres, ya que estos afectan a la estructura de las proteínas y, en consecuencia, a la liberación de péptidos. Además, su comercialización en forma de harinas puede ayudar a aumentar su consumo, ya que se podrán incorporar más fácilmente en la elaboración de distintos productos.

El principal objetivo de la presente tesis doctoral ha sido evaluar el impacto del proceso de cocción y de la DGI simulada sobre las propiedades bioactivas de diferentes legumbres y desarrollar harinas de legumbres con un perfil nutricional mejorado. Para alcanzar este objetivo, se han definido tres objetivos específicos, correspondientes a cada uno de los capítulos de la tesis.

El primer capítulo de la tesis se ha centrado en estudiar cómo afectan distintos tratamientos térmicos y la DGI simulada a la actividad antidiabética y al perfil peptídico de diferentes legumbres. En particular, se ha investigado la capacidad de algunas legumbres de inhibir las enzimas α-amilasa y α-glucosidasa, responsables de hidrolizar los carbohidratos complejos en azúcares simples durante la DGI. Para ello, se seleccionaron la soja, la alubia, el garbanzo y el guisante, ya que son las legumbres más producidas a nivel mundial, y se sometieron a distintos tipos de tratamientos térmicos, tales como cocción convencional, cocción a presión y cocción en microondas, que son los métodos más utilizados en el ámbito doméstico como paso previo a su consumo. En este sentido, la cocción puede afectar a la composición, la estructura de algunos compuestos y, como consecuencia, a las propiedades bioactivas de las legumbres (Gallego et al., 2021; Gu et al., 2023). Además, se simuló la DGI por fases (gástrica e intestinal) para estudiar en detalle la hidrólisis de proteínas y la generación de péptidos con actividad inhibidora de las enzimas α-amilasa y de la α-glucosidasa, así como evaluar el perfil peptídico tras cada etapa.

Los resultados mostraron un aumento de la bioactividad tras la DGI de las distintas legumbres sometidas a los tres tipos de cocción, alcanzando valores de inhibición de la α-amilasa entre el 50 y el 75 % tras la fase intestinal, salvo en el caso de la soja, que no mostró actividad, y entre el 22 y el 57 % de inhibición de α-glucosidasa. Este aumento podría deberse a la liberación de péptidos bioactivos tras la hidrólisis de las proteínas por acción de las enzimas digestivas (Gallego et al., 2021; Wang et al., 2019). Cabe señalar que la actividad inhibidora observada en algunas legumbres antes de ser digeridas podría atribuirse a la presencia de compuestos fenólicos resistentes al tratamiento térmico (Ganesan y Xu, 2017), ya que los péptidos bioactivos requieren de una hidrólisis para ser liberados de las proteínas (Acquah et al., 2022). Sin embargo, la ausencia de actividad inhibidora observada en la mayoría de los casos tras la fase gástrica podría deberse a la alta especificidad de la enzima pepsina, que generaría péptidos de elevado peso molecular y menor capacidad inhibidora.

Las muestras de legumbres obtenidas tras la fase intestinal de la DGI, se fraccionaron para posteriormente llevar a cabo la identificación de las secuencias peptídicas. En el estudio de la actividad inhibidora de la α-amilasa, el fraccionamiento se llevó a cabo por tamaño molecular. La fracción peptídica menor a 3 kDa presentó la mayor actividad en las tres legumbres (alubia, garbanzo y guisante) tras los diferentes tratamientos térmicos, mientras que, en el caso de los guisantes cocidos en microondas, la fracción con un peso molecular mayor a 10 kDa también mostró una actividad inhibidora significativa ($p < 0,05$). Estos resultados sugieren que péptidos con distinto tamaño molecular podrían ser capaces de ejercer actividad inhibidora de la α-amilasa (Castañeda-Pérez et al., 2021; González-Montoya et al., 2018; Ngoh y Gan, 2016; Oseguera-Toledo et al., 2015). Respecto a la actividad inhibidora de la α-glucosidasa, las muestras se fraccionaron por cromatografía líquida de alta resolución en fase reversa (RP-HPLC), la cual permite la separación de los péptidos según su polaridad. En todas las muestras, el perfil peptídico mostró 6 picos diferenciados y la mayor actividad inhibidora se concentró en la

fracción 2 (correspondiente a los minutos 3 y 4), conteniendo péptidos más hidrofílicos.

Los tratamientos térmicos utilizados para cocer las legumbres influyeron de forma significativa en la generación de péptidos bioactivos tras la DGI, pero sin un patrón definido. En general, las legumbres cocidas en microondas mostraron una actividad inhibidora de la α-amilasa significativamente ($p < 0,05$) mayor, tanto en el caso del extracto (75-85 %) como de sus fracciones (52-72 %). Sin embargo, la mayor actividad inhibidora de la α-glucosidasa se observó en las legumbres cocidas a presión (26-57 %) y, tras fraccionar, en las legumbres cocidas de forma convencional (44-63 %), aunque en ocasiones sin diferencias significativas ($p > 0,05$) con respecto a los otros tratamientos. Las diferencias observadas entre las muestras cocidas podrían atribuirse a los efectos de los diferentes tratamientos térmicos sobre las proteínas y/o la estructura de la matriz alimentaria, lo que va a determinar la hidrólisis de las proteínas durante la GID y la generación de péptidos bioactivos (Gallego et al., 2021).

Las fracciones que presentaron mayor actividad inhibidora se analizaron por espectrometría de masas en tándem (LC-MS/MS) para identificar las secuencias peptídicas. En el estudio de la actividad inhibidora de la α-amilasa se identificaron un total de 205 péptidos, mientras que en el estudio de la actividad inhibidora de la α-glucosidasa se identificaron 48 péptidos. El análisis *in silico* mostró que ninguno de los péptidos identificados había sido previamente descrito en la base de datos BIOPEP como inhibidor de la α-amilasa o de la α-glucosidasa, aunque algunos presentaban características determinadas para ello, como la presencia de dipéptidos formados por los aminoácidos prolina, cisteína, leucina y glicina en los péptidos previamente descritos como inhibidores de la α-amilasa (Ngoh y Gan, 2016), o la presencia de serina y lisina en el extremo N-terminal, o prolina cerca del extremo C-terminal en los péptidos con actividad inhibidora de la α-glucosidasa (Di Stefano et al., 2018; Ibrahim et al., 2018). Así, estos estudios demuestran el potencial de las legumbres como fuente de péptidos antidiabéticos, aunque

sería necesario sintetizar las secuencias peptídicas para poder confirmar su actividad biológica tanto *in vitro* como *in vivo*.

El segundo y tercer capítulo de la tesis se han centrado en obtener harinas de legumbres mejoradas nutricionalmente aprovechando la etapa de remojo para introducir compuestos hidrosolubles, como hierro (capítulo 2) o enzimas (capítulo 3), mediante la tecnología de impregnación a vacío. Esta tecnología consiste en sumergir un producto sólido en una solución, bajar la presión haciendo vacío (despresurización) y, tras un periodo de tiempo determinado, restablecer la presión atmosférica (Zanella-Díaz et al., 2014). De este modo, el aire ocluido dentro del alimento sale durante la despresurización, y al restablecerse la presión atmosférica, la solución penetra hacia el interior del producto debido al gradiente de presión generado, acelerando el proceso de hidratación. Para que esta tecnología sea efectiva, el producto debe tener una estructura porosa (Durán-Castañeda et al., 2024), por lo que se utilizó el método del picnómetro con tolueno para determinar la porosidad de varias legumbres (Figura 1). De entre ellas, se seleccionó el haba ya que presentó la mayor porosidad, probablemente debido a que su morfología y mayor tamaño hacen que los espacios entre la piel y el cotiledón, así como entre ambos cotiledones, sean más amplios.

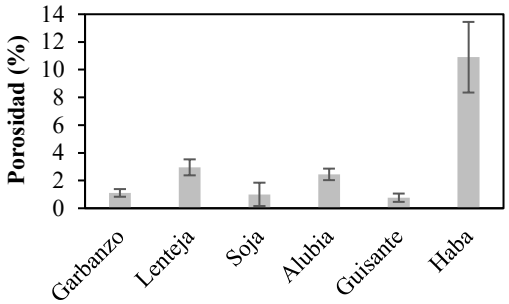


**Figura 1.** Porosidad de las legumbres determinada mediante el método del picnómetro con tolueno.

En ambos capítulos se evaluó la cinética de hidratación de las habas, con y sin la aplicación de la impregnación a vacío, a temperatura ambiente

(capítulo 2) y a la temperatura de incubación de las enzimas, 55 °C (capítulo 3). Los resultados mostraron que, al aplicar la impregnación a vacío, el tiempo de remojo se acortó aproximadamente un 75 %, lo que permitía reducir significativamente el tiempo de procesado de las habas. Además, el hecho de introducir compuestos hidrosolubles como el hierro o las enzimas en el medio de remojo, no modificó la cinética de hidratación de las habas, lo cual es un aspecto importante para poder aprovechar dicha etapa.

En el capítulo 2, las habas enriquecidas en hierro mediante remojo con impregnación a vacío se sometieron a una cocción en autoclave y/o un descascarillado como posibles alternativas del proceso previo a la obtención de las harinas, y se evaluó su impacto sobre distintos FAN, el contenido y la bioaccesibilidad del hierro, las propiedades fisicoquímicas y tecno-funcionales y la estabilidad durante el almacenamiento y la cocción. En total, se obtuvieron ocho harinas: harinas de habas remojadas con y sin piel, harinas de habas remojadas y cocidas con y sin piel, harinas de habas enriquecidas en hierro con y sin piel, y harinas de habas enriquecidas en hierro y cocidas con y sin piel.

Tras enriquecer las habas mediante impregnación a vacío, se obtuvieron harinas con un contenido en hierro significativamente ($p < 0,05$) superior, en concreto, 4,3 mg (sin piel) y 8,2 mg (con piel) más de hierro por 100 g de harina que las harinas no enriquecidas. Sin embargo, debido a la presencia de FAN quelantes en las habas (Man et al., 2021; Shubham et al., 2020), no todo el hierro añadido resultó bioaccesible, es decir, no todo el hierro añadido quedó disponible para su absorción tras la DGI. No obstante, las harinas enriquecidas presentaron un contenido de hierro bioaccesible significativamente ($p < 0,05$) superior, con 1,4 mg (sin piel) y 0,64 mg (con piel) más de hierro por 100 g de harina que las no enriquecidas.

La cocción de las habas previa a la obtención de las harinas afectó al contenido en taninos, al contenido en hierro y su bioaccesibilidad, así como a las propiedades fisicoquímicas y tecno-funcionales (Figura 2). En concreto, el contenido en taninos y hierro disminuyó en las harinas obtenidas de habas

con piel, y aumentó en las harinas de habas sin piel. Sin embargo, la fracción de hierro bioaccesible presentó una tendencia inversa. Al eliminar la piel de las habas tras la cocción, los taninos que se encuentran mayoritariamente en la piel y el hierro añadido que se había quedado en la piel, podrían haber difundido hacia el interior del cotiledón y/o hacia el vapor que condensa sobre las habas durante la cocción, resultando en un mayor contenido de estos compuestos en las harinas de habas cocidas sin piel. Sin embargo, las harinas de habas con piel presentaron un menor contenido de taninos y hierro que las harinas de habas sólo remojadas, ya que parte de estos compuestos podrían haber difundido hacia el vapor condensado. Se observó el efecto contrario en la fracción de hierro bioaccesible, probablemente porque los taninos forman complejos estables con el hierro y dificultan su absorción (Luo et al., 2010; Multari et al., 2015). Además, la cocción afectó de forma significativa a la mayoría de las propiedades fisicoquímicas y tecno-funcionales evaluadas en las harinas. Así, aumentó la capacidad de retención de agua y el índice de absorción, la capacidad de hinchamiento, la densidad aparente y el tamaño de partícula, mientras que disminuyó el índice de solubilidad en agua, el índice de blancura, la capacidad y estabilidad emulsionante, y la capacidad gelificante, debido probablemente a cambios de conformación resultantes de la gelatinización del almidón y la desnaturalización de las proteínas producidas durante el tratamiento térmico (Lin y Fernández-Fraguas, 2020).

El descascarillado previo al secado y la molienda de las habas provocó una reducción del contenido en taninos y hierro, aunque aumentó su bioaccesibilidad, ya que los taninos son compuestos quelantes que se ubican principalmente en la piel (Alonso et al., 2000; Luo y Xie, 2013). Sin embargo, en general, este proceso no afectó a las propiedades fisicoquímicas y tecno-funcionales de las harinas (Figura 2).

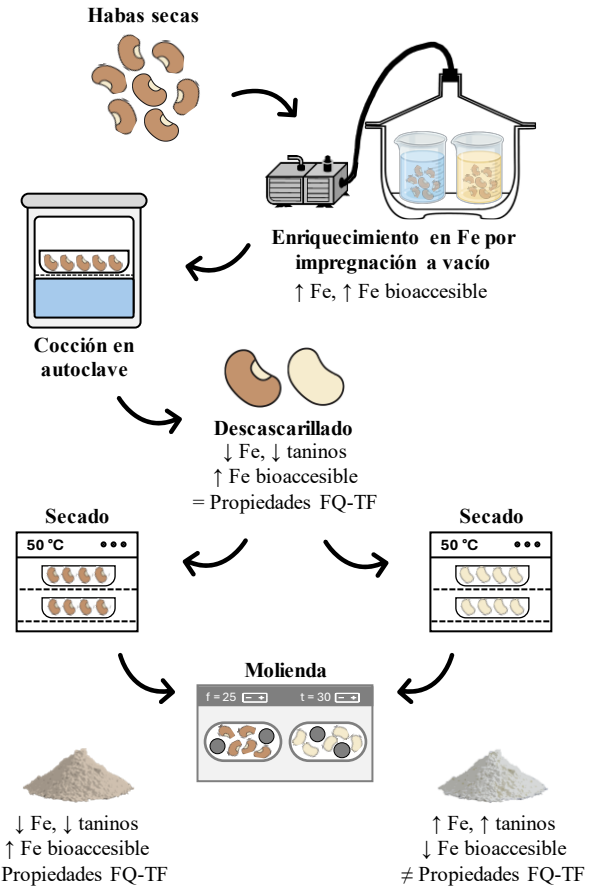


**Figura 2.** Proceso de obtención de las harinas de haba enriquecidas en hierro y efecto de cada una de las variables del proceso sobre el contenido y bioaccesibilidad del hierro, contenido de taninos, y propiedades fisicoquímicas y tecno-funcionales (FQ-TF).

Para evaluar la estabilidad de la harina enriquecida en hierro por impregnación a vacío, se determinó la oxidación lipídica y proteica durante el almacenamiento, y se compararon los resultados con los de una harina enriquecida por adición y una sin enriquecer. Además, se elaboró una pasta fresca con cada una de las distintas harinas para evaluar cómo se comportaba el

hierro añadido durante la cocción y la DGI. En este caso, se seleccionó la harina enriquecida por impregnación a vacío obtenida a partir de habas remojadas y cocinadas sin piel, ya que presentó un mayor contenido en hierro bioaccesible y mejores características para hacer pasta, tales como una mayor capacidad de retención de agua, un mayor índice de absorción de agua, una mayor capacidad de hinchamiento, y un menor índice de solubilidad en agua.

Los resultados mostraron una mayor oxidación lipídica y proteica en las harinas enriquecidas por impregnación a vacío que en las enriquecidas por el método tradicional, probablemente porque se cocieron cuando el hierro ya había sido incorporado al haba, y ambos factores, tanto el calor como el hierro, favorecen la oxidación (Barden y Decker, 2016). No obstante, la oxidación se mantuvo estable durante las 7 semanas de almacenamiento.

Durante la cocción de las pastas, la cantidad de hierro añadido de forma tradicional se redujo un 17 %, mientras que el incorporado por impregnación a vacío se mantuvo estable, probablemente porque este se habría incorporado a la matriz del haba (Durán-Castañeda et al., 2024), dificultando su difusión hacia el agua de cocción. Tras la DGI simulada de las pastas, se observó una mayor bioaccesibilidad del hierro en las harinas enriquecidas, independientemente del método de adición utilizado.

Así, estos estudios muestran que la impregnación a vacío es una tecnología útil durante el remojo para obtener harinas de haba enriquecidas en hierro. Sin embargo, será necesario realizar un estudio más profundo de la estabilidad de las harinas para confirmar que el nivel de oxidación se mantiene estable a lo largo de la vida útil del producto, así como una evaluación sensorial para comprobar que no se producen problemas organolépticos indeseables por la adición de hierro.

En el capítulo 3, la impregnación a vacío durante el remojo se utilizó para obtener harinas de habas hidrolizadas. Para ello, se añadieron en el agua de remojo las enzimas papaína y bromelina, que son endopeptidasas de origen vegetal (Gurumallesh et al., 2019; Mazorra-Manzano et al., 2018), y tras

aplicar la impregnación a vacío y dejar en reposo durante 6 h a 55 °C, se determinó la hidrólisis a distintos tiempos. El grado de hidrólisis máximo se alcanzó a las 4 h de remojo, con un valor de 9,1 % con papaína y de un 8,8 % con bromelina. Estos valores se consideran un grado de hidrólisis bajo-medio (<10 %) y se han relacionado con una mejora de las propiedades nutricionales de las legumbres (Gouseti et al., 2023; Mookerjee y Tanaka, 2023), mientras que niveles superiores se han relacionado con problemas organolépticos (Gu et al., 2023) o de agregación y precipitación de las proteínas (Mookerjee y Tanaka, 2023).

Para evaluar cómo afectaba el grado de hidrólisis y el descascarillado a los FAN y a la digestibilidad proteica, se elaboraron harinas con habas remojadas durante 1, 2 y 4 h, con y sin piel (Figura 3). Entre los distintos FAN se determinaron el ácido fítico, los compuestos fenólicos totales, los taninos y los inhibidores de tripsina, ya que estos pueden interferir y dificultar la digestibilidad de las proteínas (Bessada et al., 2019). Los resultados mostraron que la hidrólisis proteica provocó un aumento del contenido de ácido fítico, compuestos fenólicos totales y taninos, lo que podría atribuirse a la rotura de los complejos que estos compuestos forman con las proteínas (Ribéreau et al., 2018). Sin embargo, disminuyó los niveles de inhibidores de tripsina, lo cual podría deberse a que son hidrolizados por la acción enzimática o al efecto del calor, ya que tras la hidrólisis quedarían más expuestos y serían susceptibles a la desnaturalización (Goertzen et al., 2021; Konieczny et al., 2020; Ribéreau et al., 2018). No obstante, en general, no se observaron cambios significativos ($p < 0.05$) en la digestibilidad proteica, probablemente porque el efecto positivo de reducir el contenido en inhibidores de tripsina se vio contrarrestado por el aumento del resto de FAN.

El descascarillado previo al secado y molienda de las habas aumentó los niveles de ácido fítico e inhibidores de tripsina, pero redujo el contenido de taninos (Figura 3), probablemente debido a su ubicación en la legumbre, ya que el ácido fítico y los inhibidores se encuentran mayoritariamente en el cotiledón (Avilés-Gaxiola et al., 2018; Luo y Xie, 2013) y los taninos en la

piel (Singh et al., 2017). Sin embargo, en este caso, sí que se observó una digestibilidad proteica significativamente ($p < 0,05$) superior, posiblemente debida a la reducción del contenido en taninos, los cuales son FAN que forman complejos con las proteínas durante la digestión o inhiben a las enzimas digestivas (Ohanenye et al., 2020).

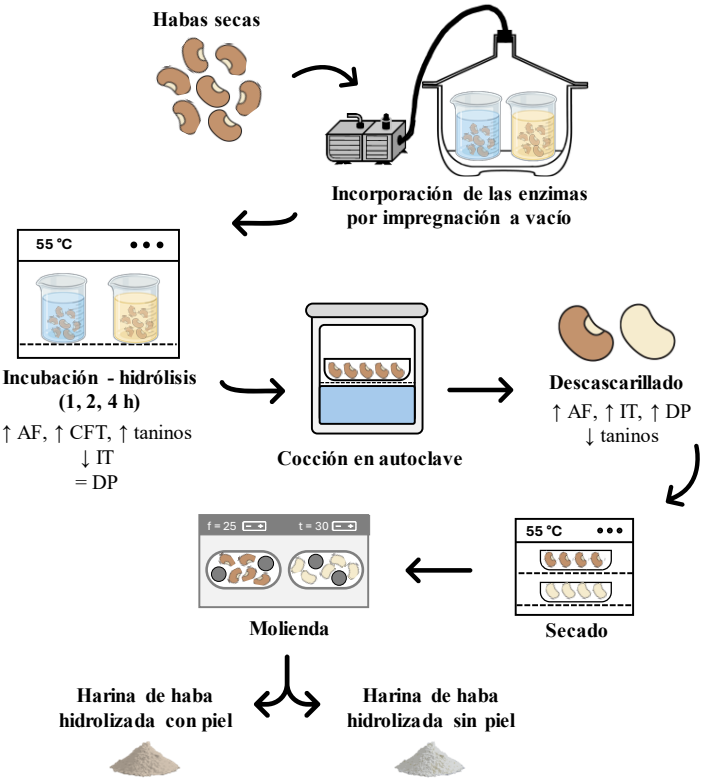


**Figura 3.** Proceso de obtención de las harinas de habas hidrolizadas y efecto de cada una de las variables del proceso sobre el contenido en ácido fítico (AF), compuestos fenólicos totales (CFT), taninos, inhibidores de tripsina (IT) y digestibilidad proteica (DP).

Este estudio demuestra que la impregnación a vacío puede utilizarse para hidrolizar las proteínas de las habas durante el remojo y con ello liberar algunos FAN, resultando una opción interesante para mejorar la digestibilidad

proteica de las harinas. Sin embargo, será necesario estudiar la combinación del remojo con enzimas con otro tipo de tratamiento que permita eliminar todos los FAN liberados durante la hidrólisis.

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# Conclusiones

Las conclusiones extraídas de los diferentes estudios realizados durante la presente tesis doctoral fueron las siguientes:

- Las alubias, la soja, los garbanzos y los guisantes cocidos de forma convencional, a presión o en microondas mostraron una potencial actividad antidiabética tras la digestión gastrointestinal, identificándose péptidos potencialmente bioactivos con herramientas *in silico*.

- El uso de la impregnación a vacío para incorporar hierro a las habas durante el remojo resultó útil para obtener harinas enriquecidas, cuyo contenido en hierro y bioaccesibilidad, contenido en factores antinutricionales y propiedades fisicoquímicas y tecno-funcionales, cambiaron en función del procesado de las habas previo a la obtención de la harina. Además, el hierro añadido aumentó ligeramente la oxidación de las harinas, aunque se mantuvo estable durante el almacenamiento, y no se produjeron pérdidas durante la cocción cuanto se utilizaron para la elaboración de pasta fresca.

- El uso de la impregnación a vacío durante el remojo para incorporar enzimas a las habas permitió obtener harinas de haba hidrolizadas y provocó un aumento del contenido en ácido fítico, compuestos fenólicos totales y taninos, y una disminución de los niveles de inhibidores de tripsina. A su vez, el descascarillado aumentó los niveles de ácido fítico e inhibidores de tripsina, redujo el contenido de taninos y mejoró la digestibilidad proteica.

- Los resultados de esta tesis doctoral demuestran cómo el procesado y el uso de la impregnación a vacío pueden utilizarse para potenciar el valor nutricional de las legumbres, promoviendo su consumo y su uso como harinas en la formulación de nuevos productos.